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ORIGINAL ARTICLE

Rapid Effects of Aggressive Interactions on Aromatase Activity and Oestradiol in Discrete Brain Regions of Wild Male White-Crowned Sparrows

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Testosterone is critical for the activation of aggressive behaviours. In many vertebrate species, circulating testosterone levels rapidly increase after aggressive encounters during the early or mid-breeding season. During the late breeding season, circulating testosterone concentrations did not change in wild male white-crowned sparrows after an aggressive encounter and, in these animals, changes in local neural metabolism of testosterone might be more important than changes in systemic testosterone levels. Local neural aromatisation of testosterone into 17β-oestradiol (E_2) often mediates the actions of testosterone, and we hypothesised that, in the late breeding season, brain aromatase is rapidly modulated after aggressive interactions, leading to changes in local concentrations of E_2. In the present study, wild male white-crowned sparrows in the late breeding season were exposed to simulated territorial intrusion (STI) (song playback and live decoy) or control (CON) for 30 min. STI significantly increased aggressive behaviours. Using the Palkovits punch technique, 13 brain regions were collected. There was high aromatase activity in several nuclei, although enzymatic activity in the CON and STI groups did not differ in any region. E_2 concentrations were much higher in the brain than the plasma. STI did not affect circulating levels of E_2 but rapidly reduced E_2 concentrations in the hippocampus, ventromedial nucleus of the hypothalamus and bed nucleus of the stria terminalis. Unexpectedly, there were no correlations between aromatase activity and E_2 concentrations in the brain, nor were aromatase activity or brain E_2 correlated with aggressive behaviour or plasma hormone levels. This is one of the first studies to measure E_2 in microdissected brain regions, and the first study to do so in free-ranging animals. These data demonstrate that social interactions have rapid effects on local E_2 concentrations in specific brain regions.

Key words: estradiol, estrogens, songbird, aggression, hippocampus.

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Numerous behavioural actions of testosterone, including the regulation of aggressive and reproductive behaviour, require the aromatisation of testosterone into 17β-oestradiol (E_2) within the brain (1–4). This conversion of testosterone into E_2 is catalyzed by the enzyme cytochrome P450 aromatase (CYP19). Aromatase expression in the avian and mammalian brain is normally restricted to specific neuronal populations located mainly in the hypothalamic/preoptic and limbic system (5–7), as well as in the telencephalon in songbirds (8–10). Aromatase activity is modulated at the transcriptional level, a slow process (11–13). In addition, rapid modulation of aromatase activity has been demonstrated recently. *In vitro*, a rapid reduction of aromatase activity is observed in homogenate of the quail medial preoptic area incubated in calcium-rich and phosphorylating conditions (14, 15). Also, the activation of glutamatergic receptors in medial preoptic area explants rapidly reduces aromatase activity (16). *In vivo*, aromatase activity is rapidly reduced in specific brain regions after the expression of sexual behaviour in quail (17).

Rapid, local changes in aromatase activity are likely to modulate the concentrations of oestrogens within specific locations (18, 19). Although mostly studied for its actions at the genomic level (20), E_2 also affects brain and behaviour rapidly and independently of *de novo* transcription. For example, within seconds to minutes, E_2 modulates neuronal firing rate (21), a variety of intracellular signalling pathways (22) and reproductive behaviours (23–25). E_2 also rapidly influences agonistic behaviour. Indeed, aggressive behaviour in Beach mice and California mice rapidly (within 10 min) increases after E_2 treatment (26, 27). In zebra finches, acute treatment with E_2 increases the neuronal firing rate evoked by song playback within 5 min in the caudomedial nidopallium (NCM), a cortical-like auditory region involved in song recognition (28, 29).

Only one study has investigated local concentrations of E_2 in the brain immediately after behavioural interactions (30). The quantification of E_2 in discrete brain regions is technically challenging, and so most work has focused on aromatase activity and assumed that E_2 concentrations change in parallel with aromatase. To our knowledge, E_2 quantification at the level of a single nucleus in wild animals has not been performed, nor has concurrent measurement of brain aromatase activity and E_2 concentrations within individuals.

We recently developed a specific and sensitive assay to measure local E_2 concentrations (31). The present study aimed to: (i) measure the E_2 content in discrete brain regions in wild male white-crowned sparrows (*Zonotrichia leucophrys pugetensis*); (ii) correlate aromatase activity and E_2 concentration within individuals; and (iii) test whether exposure to a 30-min simulated territorial intrusion (STI) affects aromatase activity and/or E_2. We hypothesised that an aggressive interaction would rapidly affect aromatase activity, leading to a subsequent change in E_2 concentrations.

Materials and methods

All animal protocols were approved by the University of British Columbia Committee on Animal Care and followed the guidelines of the Canadian Council on Animal Care. All necessary permits were obtained.

Behavioural recording and blood sampling

Experiment 1

Wild adult male white-crowned sparrows from Vancouver, BC, Canada were studied in the late breeding season, just before moulting (4–27 July 2006 and 8–16 July 2007). Data on aggressive behaviour and plasma levels of testosterone, progesterone, corticosterone and corticosteroid binding globulin were previously presented (32) and, in the present study, we focus on a randomly selected subset (n = 20) of these subjects and provide important new data on aromatase activity and E_2 levels in 13 brain regions (approximately 520 data points).

Briefly, behavioural tests and blood sampling were performed between 06.00 h and 12.00 h. A mist net was quickly set up, furled and placed near the ground within a subject's territory. Subjects were exposed for 30 min to either: (i) a simulated territorial intrusions using conspecific song playback and live decoy (STI; n = 11) or (ii) an empty cage without playback (CON; n = 9). During the challenge, we recorded the time it took for the subject to fly toward the decoy (latency), as well as the numbers of songs, flights, trills and wing waves. We also recorded the amount of time spent within 1 m and 5 m of the decoy. Songs and flights toward an intruder are recognised as aggressive in this context (33, 34) and trills and wing waves are also observed during territorial disputes (33, 35). A composite aggression score (PC1) was obtained from a principal components analysis and loaded heavily on the number of songs, number of flights and time spent near the decoy, and loaded only moderately on latency to approach (32). In the present study, the behavioural data for only the present subset of subjects was analysed.

After the CON or STI, the mist net was quickly unfurled and playback was used to capture subjects within 7 min (CON: 3.97 ± 0.69 min; STI: 3.33 ± 0.69 min, d.f. = 18, t = 0.63, P = 0.54). Blood from the jugular and brachial veins was collected, and subjects were immediately sacrificed by rapid decapitation (time of sacrifice after capture, CON: 3.58 ± 0.36 min, STI: 3.81 ± 0.27 min, d.f. = 18, t = 0.52, P = 0.61). The brain was quickly removed from the skull and frozen on powdered dry ice. The testes were also collected and frozen on powdered dry ice. The brains and testes were kept at −80 °C.

Several endocrine variables were measured in these plasma samples by techniques previously described in detail by Charlier *et al.* (32). Briefly, circulating levels of corticosteroid-binding globulin (CBG) were determined using a radioligand binding assay. Corticosterone levels in plasma were measured using a corticosterone radioimmunoassay (MP Biomedicals, Cat# 07-120103; Santa Ana, CA, USA) that was validated for use with songbird plasma. Testosterone was extracted from plasma with dichloromethane and measured using a testosterone radioimmunoassay (DSL-4100) that was modified to increase sensitivity. Progesterone was extracted from plasma with diethyl ether and measured using a progesterone radioimmunoassay (DSL-3400) that was modified to increase sensitivity. The endocrine data obtained from only the brachial vein for the present subset of subjects was analysed.

Experiment 2

To assess possible changes in systemic E_2 levels, another group of wild adult male white-crowned sparrows was studied again in the late breeding season, just before moulting (14–26 July 2010). Subjects were exposed to a simulated territorial intrusion using conspecific song playback and live decoy for 15 min (STI: 15 min, n = 7) or 30 min (STI: 30 min, n = 7). Controls were exposed to an empty cage without playback for 30 min (CON; n = 7). After the CON or STI, the mist net was quickly unfurled and playback was used to capture subjects within 7 min. Upon capture, a blood sample (approximately 150 μl) was immediately collected from the brachial vein.

Palkovits punch technique

The technique originally developed by Palkovits for rat brain (36) and validated for zebra finch brain (31, 37) was used with only minor modifications. Coronal sections (300 μm) were made on a cryostat (Microm HM505E; Thermo Fisher Scientific Inc., Waltham, MA, USA) at −12 °C and were collected starting from the caudal part of the brain. The plane of the sections was adjusted to match as closely as possible the plane of the zebra finch brain atlas (38). From these sections, individual nuclei were isolated from one to six adjacent sections, depending on the size of the region of interest, by punching them out with a stainless steel cannula (Brain Punch Set, #57401; Stoelting Co., Wood Dale, IL, USA). Punches (0.94 mm diameter) were obtained from Area X (X), medial magnocellular nucleus of anterior nidopallium (MMAN), medial preoptic area (mPOA), rostral hippocampus (Hp), ventromedial nucleus of the hypothalamus (VMN), bed nucleus of the stria terminalis (BST), HVC (used as a proper name), NCM, nucleus taeniae of the amygdala (TnA), nucleus robustus of the arcopallium (RA), mesencephalic central grey (GCt), optic lobes (OL) and cerebellum (Cb) (Fig. 1).

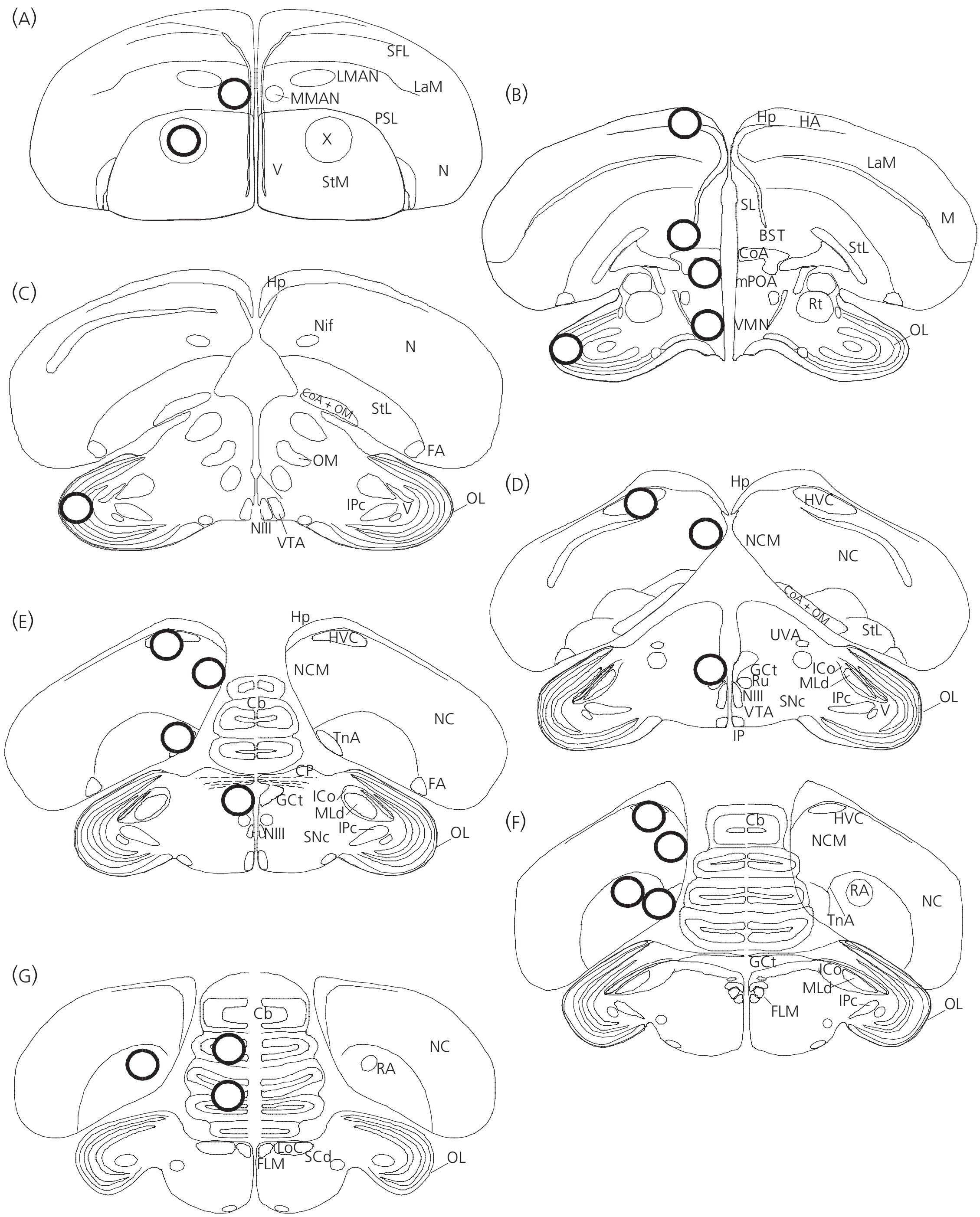

Fig. 1. Schematic representation of the location of the punches, as identified by circles in the transversal sections. (A–G) Sections arranged in a rostral to caudal order. Cb, cerebellum; CoA, anterior commissure; CP, posterior commissure; BST, bed nucleus of the stria terminalis, FA, fronto-arcopallial tract; FLM, fasciculus longitudinalis medialis (medial longitudinal bundle); GCt, mesencephalic central grey (periaqueductal grey); HA, accessory part of the hyperpallium; Hp, hippocampus; HVC, used as a proper name; ICo, intercollicular nucleus; IPc, parvocellular part of the isthmi nucleus; LaM, mesopallial lamina; LMAN, lateral magnocellular nucleus of the anterior nidopallium; LoC, locus ceruleus; M, mesopallium; MLd, lateral mesencephalic nucleus; MMAN, medial magnocellular nucleus of the anterior nidopallium; mPOA, median preoptic area; N, nidopallium; NC, caudal nidopallium; NCM, caudal medial nidopallium; NIf, nucleus interface of the nidopallium; NIII, oculomotor nerve; OL, optic lobe; OM, occipito-mesencephalic tract; PSL, pallial-subpallial lamina; RA, robust nucleus of arcopallium; Rt, nucleus rotundus; Ru, red nucleus; SCd, dorsal subceruleus nucleus; SFL, superior frontal lamina; SL, lateral septal nucleus; SNc, substantia nigra, pars compacta; StM, medial striatum; StL, lateral striatum; TnA, nucleus taeniae of the amygdala; UVA, nucleus uveaformis; V, ventricle; VMN, ventromedial nucleus of the hypothalamus; VTA, ventral tegmental area; X, Area X.

Punches from the left and right sides were collected into separate 'Pellet Pestle' 0.5 ml microcentrifuge tubes (Kimble/Kontes, Vineland, NJ, USA) and stored at −80 °C. The punches from the left side of the brain were used for the aromatase assay, whereas punches from the right side of the brain were used for E_2 quantification. For each region within a subject, the number of punches taken from the left and right sides were always identical (Table 1).

Because the very small amount of tissue could introduce an error in the weight measurement, tests were performed to estimate the weight of a single punch. Briefly, three pools of punches (each containing 75 punches) were obtained from three sparrow brains. The weight of each pool was recorded and used to calculate the weight of one punch. The average weight of one punch was calculated to be 0.1919 mg, and this value was used to estimate the wet weight of punches from the right side of the brain (Table 1). Note that some of the punches were not complete due to the proximity of ventricles (e.g. Hp, HVC and TnA). However, for each region analysed, no weight difference was observed between CON and STI groups (Student's t-test, P > 0.1).

Brain punches from the left side were homogenised in the microcentrifuge tubes with a specific pestle (CTFE/stainless steel 'Pellet Pestle,' #749516-0500; Kimble/Kontes) with 200 μl of TEK (10 mM Tris-HCl, pH 7.2, 1 mM Na-ethylenediaminetetraacetic acid, 150 mM KCl). Protein concentration was measured using the Bradford method (Bio-Rad, Hercules, CA, USA) in accordance with the manufacturer's instructions. The protein content was approximately 6% of the estimated wet weight, similar to studies with Japanese quail brain (39). Again, no difference in protein concentration was observed between CON and STI groups (Student's t-tests, P > 0.1). Moreover, the protein concentration (from the left side) was highly correlated with the estimated weight (from the right side) (r = 0.96, P < 0.0001), further supporting the validity of using estimated weight.

Table 1. Number, Estimated Weight and Protein Content of Punches from the 13 Different Brain Regions.

	Number of punches	Estimated weight (mg)	Protein content (mg)
High aromatase			
mPOA	4.85 ± 0.17	0.93 ± 0.03	0.055 ± 0.003
Hp	3.30 ± 0.21	0.63 ± 0.04	0.035 ± 0.003
VMN	1.50 ± 0.11	0.29 ± 0.02	0.019 ± 0.004
BST	1.45 ± 0.11	0.28 ± 0.02	0.011 ± 0.001
NCM	4.35 ± 0.23	0.83 ± 0.04	0.055 ± 0.004
TnA	2.45 ± 0.17	0.47 ± 0.03	0.026 ± 0.003
Low aromatase			
Area X	2.35 ± 0.19	0.45 ± 0.04	0.026 ± 0.003
MMAN	2.37 ± 0.16	0.45 ± 0.03	0.026 ± 0.003
HVC	2.80 ± 0.17	0.54 ± 0.03	0.039 ± 0.003
RA	1.94 ± 0.20	0.37 ± 0.04	0.027 ± 0.003
GCt	1.80 ± 0.12	0.34 ± 0.02	0.020 ± 0.002
OL	3.35 ± 0.18	0.64 ± 0.03	0.036 ± 0.003
Cb	3.30 ± 0.24	0.63 ± 0.05	0.041 ± 0.003

The values (mean ± SEM) correspond to punches obtained from one side of the brain only. The left side was used to determine aromatase activity and the right side for the 17β-oestradiol concentration. For each region within a subject, the number of punches collected from the right and left sides of the brain was always identical.

BST, bed nucleus of the stria terminalis; Cb, cerebellum; GCt, mesencephalic central grey (periaqueductal grey); Hp, hippocampus; HVC, used as a proper name; MMAN, medial magnocellular nucleus of the anterior nidopallium; mPOA, median preoptic area; NCM, caudal medial nidopallium; OL, optic lobe; RA, robust nucleus of arcopallium; TnA, nucleus taeniae of the amygdala; VMN, ventromedial nucleus of the hypothalamus.

Aromatase activity

Aromatase activity was quantified by measuring the production of tritiated water from [1β-^{3}H]-androstenedione as described by Thompson and Roselli (40, 41) and validated for songbird brains (42, 43). On an ice bath, 50 μl of TEK buffer, 50 μl of 100 nM [1β-^{3}H]-androstenedione, and 50 μl of 4.8 mM NADPH were added to aliquots (50 μl) of homogenate. The final volume of the reaction was 200 μl, the final concentration of [^{3}H]-AE was 25 nM, and the final concentration of NADPH was 1.2 mM. These concentrations were previously used for 1–8 mg of tissue per tube (42, 43), whereas our quantification was performed on 0.07–0.23 mg of tissue (Table 1). Therefore, we are confident that substrate was not limiting in our assays and was present at saturating concentrations. Samples were quantified in duplicate. Background values were obtained for each sample by processing brain samples in the presence of an excess (final concentration, 40 μM) of the potent and specific aromatase inhibitor, fadrozole (gift from Novartis, Camberley, UK). All these steps were conducted at 4 °C in 1.5-ml microcentrifuge tubes, which were then quickly capped and incubated for 20 min at 37 °C. The reaction was stopped by cooling the samples in an ice/water bath and adding 0.4 ml ice-cold 10% trichloroacetic acid containing 2% activated charcoal. After centrifugation at 1200 *g* for 15 min, supernatants were applied to small columns made of Pasteur pipettes plugged with glass beads and filled (3 cm high) with a Dowex cation exchange resin (AG 50W-X4, 100–200 mesh; Bio-Rad, Richmond, CA, USA). Distilled water (3 × 0.6 ml) was then added to the columns. Effluents were collected in scintillation vials, and 10 ml of scintillation liquid were added. Vials were counted for 3 min on a liquid scintillation counter (Beckman Coulter, LS6500, Fullerton, CA, USA). Enzyme activity was expressed as fmol/h/mg of protein, after correction of the d.p.m. for recovery (95%), background values and percentage of tritium in β-position in the substrate (76.8%).

Solid-phase extraction

To measure E_2 in brain samples (Experiment 1), tissue was homogenised in the microcentrifuge tubes with the Pellet Pestle with 50 μl of ice-cold deionised water, and then 250 μl of ice-cold high-performance liquid chromatography (HPLC)-grade methanol was quickly added. Samples were left overnight at 4 °C. Steroids were then extracted with solid-phase extraction (31, 44). Briefly, C18 columns (500 mg sorbent, 6 ml column volume; United Chemical Technologies, Bristol, PA, USA) were primed with HPLC-grade ethanol and equilibrated with deionised water. Then, 10 ml of deionised water was added to the brain samples before loading onto the C18 columns. After sample loading, the C18 columns were washed with deionised water (10 ml), and the steroids were eluted with 90% HPLC-grade methanol (5 ml). The eluates were dried (Speedvac; Thermo Fisher Scientific Inc.) before resuspension. We resuspended samples in 350 μl phosphate-buffered saline with 0.1% gelatin (PBSG) containing 0.7% absolute ethanol.

To measure E_2 in plasma samples (Experiment 2), we also used solid-phase extraction with C18 columns (Sep-Pak Vac C18 cartridge, 500 mg sorbent, 3 ml column volume; Waters Corp, Milford, MA, USA). For logistical reasons, the C18 columns used for plasma samples were obtained from a different manufacturer but, importantly, contain the same type of sorbent (C18) and the same amount of sorbent (500 mg). Columns were primed and equilibrated as above. After sample loading (29.2 μl of plasma + 10 ml of deionised water), the columns were washed with 40% HPLC-grade methanol (10 ml). Steroids were eluted with 90% HPLC-grade methanol (5 ml), and eluates were dried and resuspended as above. In preliminary studies with the Sep-Pak C18 columns, we measured E_2 in zebra finch plasma, and the results matched our previous results with C18 columns from United Chemical Technologies (30; Heimovics, Ma and Soma, unpublished results). Moreover, the C18 columns from both manufacturers gave similar results for standards containing known amounts of E_2, water blanks, and recovery (see below).

E_2 radioimmunoassay

Resuspended samples were then assayed as singletons (to maximise the number of detectable samples) with a double-antibody ^{125}I-E_2 radioimmunoassay (DSL-4800; Beckman Coulter Canada, Inc., Mississauga, ON, Canada) that we modified and validated for songbird brain samples (31). Briefly, 100 μl of diluted anti-oestradiol antiserum (dilution: 1 ml of stock antibody + 2.5 ml of PBSG) was added to 300 μl of sample, the tubes were quickly vortexed and incubated at room temperature for 4 h. Then, 100 μl of diluted ^{125}I-E_2 (dilution: 1 ml of stock tracer + 2 ml of PBSG) was added, and the tubes were vortexed and incubated for 24 h at 4 °C. Then, 500 μl of precipitating reagent was added, and tubes were vortexed and incubated for 20 min at room temperature. The tubes were centrifuged at 1500 *g* for 15 min at 4 °C, the supernatant was decanted, and tubes were counted.

The E_2 antibody has a low cross-reactivity with oestrone (2.4%), oestriol (0.64%), 17α-oestradiol (0.21%), 17β-oestradiol-3-glucuronide (2.56%), 17β-oestradiol-17-glucuronide (< 0.01%), oestradiol-3-sulphate (0.17%), testosterone (< 0.01%) and dehydroepiandrosterone (< 0.01%) in accordance with the manufacturer's instructions. The lowest point on the standard curve was 0.1875 pg per tube. E_2 values below the lowest point on the standard curve were set to zero. In the assays with brain samples, water blank values were 0.138 $\pm$ 0.057 pg/tube (n = 5) and, in the assay with plasma samples, water blanks were 0.055 $\pm$ 0.036 pg/tube (n = 2). For low (0.375 pg per tube) and high (1.125 pg per tube) standards, intra-assay variation was 0.92% and 4.37%, and inter-assay variation was 5.46% and 5.36%, respectively. The low intra-assay variation confirms the validity of using singletons to maximise the number of detectable samples. We also examined the recovery of a known quantity of radioinert E_2 (0.375 pg per tube) added to brain tissue (dorsomedial telencephalon containing NCM) or plasma before solid-phase extraction. We calculated recovery by comparing the quantity of E_2 in spiked (n = 5) and unspiked samples (n = 5). Data were corrected for recovery (brain samples: 75.95%, plasma samples: 84.00%), similar to our previous results (31).

Nissl staining

After the punch collection, sections were mounted on glass slides, dried overnight and Nissl-stained to confirm the location of the punches. Briefly, sections were brought to room temperature and postfixed in 4% paraformaldehyde for 15 min. The sections were rinsed three times in phosphate-buffered saline (0.1 M) and stained in 0.2% toluidine blue in Walpole solution (0.3 M sodium acetate, 0.12% acetic acid) for 2 min. The sections were rinsed in deionised water, destained in Walpole solution, and the stain was fixed in 0.04 M ammonium molybdate. Sections were dehydrated with increasing concentrations of ethanol, and incubated in acetone and then xylene before coverslipping (Permount; Fisher Scientific Co., Pittsburgh, PA, USA).

Statistical analysis

All data are presented as the mean $\pm$ SEM. Data analysis included Student's t-tests, Mann–Whitney tests, one-way ANOVAs, and mixed-design two-way ANOVAs, which were performed using STATVIEW, version 5.0.1 (Abacus Concept Inc., Berkeley, CA, USA). When appropriate, ANOVA tests were followed by post-hoc Tukey's honestly significant tests. $P \leq 0.05$ was considered statistically significant.

Results

Morphology

CON and STI groups did not differ with respect to body mass (CON: 24.00 $\pm$ 0.85 g, STI: 24.18 $\pm$ 0.78 g, d.f. = 18, t = 0.151, P = 0.88) and left tarsus length (CON: 20.36 $\pm$ 0.29 mm, STI: 20.65 $\pm$ 0.28 mm, d.f. = 18, t = 0.703, P = 0.49). The testes were starting to regress, and there was no difference between groups in total testes mass (CON: 361 $\pm$ 53 mg, STI: 379 $\pm$ 30 mg, d.f. = 18, t = 0.309, P = 0.76) or the length of the left testis (CON: 8.81 $\pm$ 0.61 mm, STI: 8.77 $\pm$ 0.31 mm, d.f. = 18, t = 0.059, P = 0.95).

Behaviour

The STI elicited a robust aggressive response (Table 2). STI significantly decreased the latency to approach the cage, increased the number of songs, increased the number of flights, and increased the time spent within 1 m and 5 m of the decoy (Table 2). Trills and wing waves were rarely observed and not significantly different between the groups (number of trills: CON: 0.00 $\pm$ 0.00, STI: 1.54 $\pm$ 1.36, Z = 0.684, P = 0.49; number of wing waves: CON: 0.00 $\pm$ 0.00, STI: 0.91 $\pm$ 0.73, Z = 0.684, P = 0.49). The aggression score (PC1) was significantly different between groups (CON: −2.15 $\pm$ 0.19, STI: 1.25 $\pm$ 0.21, d.f. = 18, t = 11.875, P < 0.0001).

Endocrine measurements

In Experiment 1, we examined endocrine measurements obtained from the brachial plasma (Table 2). Total plasma corticosterone levels were significantly elevated in the STI group, and there was a strong trend for plasma CBG levels to increase in the STI group. No changes in plasma progesterone or testosterone levels were observed in this subset of birds.

In a separate group of subjects (Experiment 2), exposure to STI for 15 or 30 min did not affect systemic E_2 levels in brachial plasma compared to the CON group ($F_{2,18}$ = 0.522, P = 0.602) (Fig. 2). In general, plasma E_2 levels were low but above the detection limit in all samples.

Brain aromatase activity

We analysed the effect of STI on aromatase activity by a mixed-design two-way ANOVA with a between-subjects factor (Treatment) and a within-subjects factor (Region). This analysis revealed no main effect of Treatment ($F_{1,13}$ = 2.510, P = 0.137), an effect of Region ($F_{1,12}$ = 13.924, P < 0.0001) and no interaction between the two factors ($F_{12,156}$ = 0.450, P = 0.940). As expected from previous reports, high aromatase activity was detected in the mPOA, Hp, VMN, BST, NCM and TnA (Fig. 3A). Low or nondetectable aromatase activity was detected in Area X, MMAN, HVC, RA, GCt, OL and Cb. It should be noted that separate t-tests were also performed for each brain region and, again, no significant differences were found between CON and STI groups (P > 0.15 in all cases).

Table 2. Aggressive Responses and Endocrine Measures in Control (CON) (n = 9) and Simulated Territorial Intrusion (STI) (n = 11) Subjects.

	CON	STI	t or Z	P
Behaviour				
Latency (s)	1010.44 ± 292.69	55.70 ± 25.12	Z = 2.531	***P = 0.01**
Number of songs	13.89 ± 7.14	110.82 ± 21.85	Z = 2.773	***P = 0.006**
Number of flights	0.78 ± 0.57	49.27 ± 7.02	Z = 3.761	***P = 0.0002**
Time in 1 m (s)	0.00 ± 0.00	862.73 ± 159.00	Z = 3.419	***P = 0.0006**
Time in 5 m (s)	3.33 ± 3.33	1647.64 ± 44.54	Z = 3.761	***P = 0.0002**
Endocrine measures				
Plasma CBG (nM)	141.30 ± 17.67	214.03 ± 30.94	Z = 1.937	(*)P = 0.053
Plasma corticosterone (nM)	27.14 ± 8.72	87.50 ± 18.16	Z = 2.849	***P = 0.004**
(ng/ml)	9.39 ± 3.02	40.25 ± 6.28		
Plasma progesterone (nM)	2.17 ± 0.55	2.18 ± 0.40	t = 0.003	P = 0.998
(ng/ml)	0.68 ± 0.17	0.69 ± 0.12		
Plasma testosterone (nM)	4.94 ± 3.17	2.92 ± 1.74	t = 0.584	P = 0.564
(ng/ml)	1.42 ± 0.87	0.84 ± 0.24		

Plasma corticosteroid-binding globulin (CBG) is expressed in nM, and steroid concentrations are shown as both nM and ng/ml. P in bold represent statistically significant differences. (*) represents close to significance.

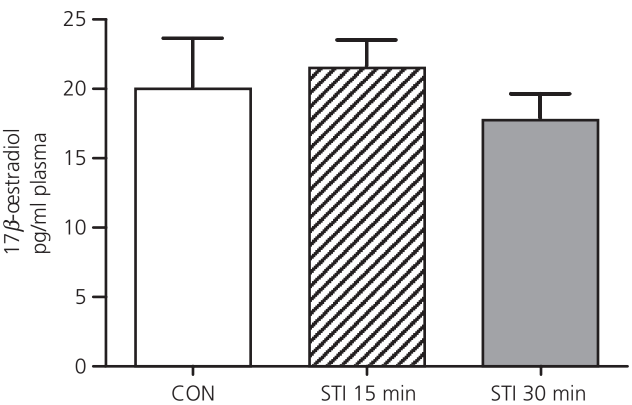

Fig. 2. Bar graphs representing plasma 17β-oestradiol concentrations in controls (CON, white) and subjects exposed to a simulated territorial intrusion (STI) for 15 min (dashed) or 30 min (grey). Subjects were wild adult male white-crowned sparrows, in the late breeding season.

Brain 17β-oestradiol concentrations

The use of singletons for E_2 quantification increased the number of detectable samples compared to that previously obtained in zebra finch (compare Table 3 and the results of Charlier *et al.* (31)]. A mixed-design two-way ANOVA of E_2 concentrations revealed no main effect of Treatment ($F_{1,12}$ = 0.056, P = 0.817; Fig. 3B), an effect of Region ($F_{1,12}$ = 3.818, P < 0.0001) and a strong trend for an interaction between the two factors ($F_{12,144}$ = 1.816, P = 0.0506). E_2 concentrations varied across brain regions, albeit less than aromatase activity did. Among the regions with high aromatase activity, higher levels of E_2 were found in the VMN and BST, whereas lower E_2 concentrations were observed in the mPOA and NCM.

Because we expected *a priori* stronger modulation by STI of E_2 concentrations in aromatase-rich regions, a mixed-design two-way ANOVA was also used to assess the effects of STI specifically in the six brain regions with high aromatase activity (mPOA, Hp, VMN, BST, NCM and TnA). This ANOVA revealed a trend for an effect of Treatment ($F_{1,16}$ = 3.054, P = 0.099), a strong effect of Region ($F_{1,5}$ = 9.758, P < 0.0001) and a significant interaction ($F_{5,80}$ = 2.465, P = 0.0396). Tukey's post-hoc tests revealed statistically significant reductions of E_2 in Hp (approximately 60%; P < 0.05), VMN (approximately 50%; P < 0.05) and BST (approximately 50%; P < 0.01) in the STI group (Fig. 3B). No significant change was observed in mPOA, NCM or TnA.

Importantly, E_2 concentrations in the brain punches (Fig. 3B) were much higher than those in the plasma (approximately 40-fold higher in mPOA and NCM, and up to approximately 200-fold higher in BST; compare the axes of Figs 2 and 3B). When comparing brain and plasma concentrations, note that 1 ml of plasma weighs almost 1 g (45, 46).

Correlations between brain aromatase activity and brain E_2

We found no correlations between aromatase activity and brain E_2 concentration (all data points: n = 245, r = 0.029, P = 0.647) (Fig. 4A). The correlational analyses were also performed separately for the CON and STI groups, as well as for individual brain regions. Again, no significant correlations were found (P > 0.1 in all cases). The analysis of the average aromatase activity and E_2 concentration from individual nuclei, in CON and STI groups, and separately in high aromatase regions (Fig. 4B) and low aromatase regions (Fig. 4C), did not reveal any correlations (P > 0.2).

Unexpectedly, our results suggest that brain E_2 concentration is not always directly linked to aromatase activity. Indeed, relatively low E_2 levels are observed in the aromatase-rich NCM, and E_2 con-

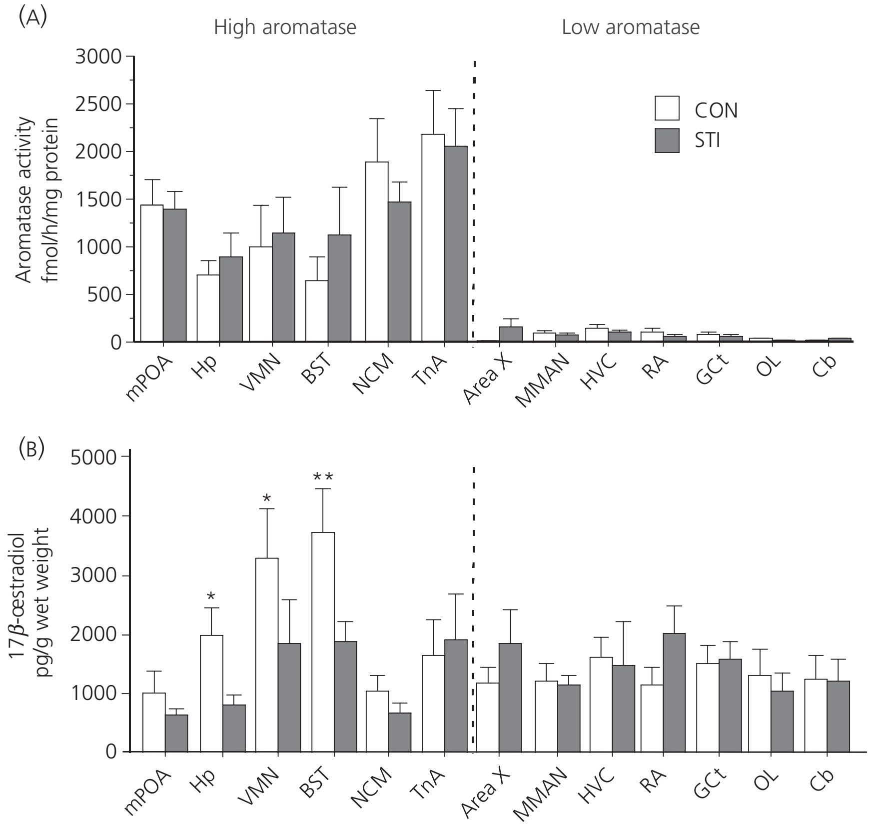

Fig. 3. Bar graphs representing aromatase activity (A) and 17β-oestradiol concentration (B) in 13 brain regions in the controls (CON, white) and subjects exposed to a simulated territorial intrusion (STI, grey) for 30 min. Subjects were wild adult male white-crowned sparrows, in the late breeding season. *$P < 0.05$; **$P < 0.01$. BST, bed nucleus of the stria terminalis; Cb, cerebellum; GCt, mesencephalic central grey (periaqueductal grey); Hp, hippocampus; HVC, used as a proper name; MMAN, medial magnocellular nucleus of the anterior nidopallium; mPOA, median preoptic area; NCM, caudal medial nidopallium; OL, optic lobe; RA, robust nucleus of arcopallium; TnA, nucleus taeniae of the amygdala; VMN, ventromedial nucleus of the hypothalamus.

centrations are similar between aromatase-rich TnA and low aromatase regions such as HVC, OL and Cb.

We also analysed the association of brain E_2 or aromatase activity from each individual brain regions with the endocrine (corticosterone, progesterone, testosterone and CBG) and behavioural (latency, flight, song, PC1) measures. Again, no significant correlations were found ($P > 0.1$ in all cases).

Discussion

The present study is one of the first to measure E_2 in microdissected brain regions (31), and the first to do so in free-ranging animals in their natural habitat. To our knowledge, the present study is also the first to examine the effects of aggressive interactions on E_2 levels in the brain. Moreover, we measured both brain aromatase activity and brain E_2 concentrations in the same subjects, which has not been carried out previously. Brain aromatase activity levels were similar in the control and STI groups, whereas brain E_2 concentrations were significantly reduced in Hp, BST and VMN in the STI group. Unexpectedly, brain E_2 concentrations were not correlated with aromatase activity. These data demonstrate that social interactions have rapid effects on local E_2 concentrations in specific brain regions.

Brain aromatase activity

Regional differences in aromatase activity

Very little information exists on regional differences in aromatase activity in the brain of wild songbirds. Aromatase activity has been studied using relatively large brain regions, such as the telencephalon or diencephalon (43, 47). In the closely-related Gambel's white-crowned sparrow caught in the nonbreeding season, aromatase activity was high in the telencephalon and relatively low in the diencephalon (48). However, aromatase-expressing cells are present in very discrete locations in the diencephalon and, thus, the use of whole diencephalon dilutes aromatase-expressing cells with the surrounding tissue.

The Palkovits punch technique allowed us to study aromatase activity in specific brain nuclei. Although this technique was previously used in two domesticated bird species, the Japanese quail and the zebra finch (37, 49), the present study is the first to investigate aromatase activity in microdissected brain regions from a wild bird species. We showed that two hypothalamic nuclei (mPOA and VMN), as well as several telencephalic regions (BST, Hp, NCM and TnA), have high levels of aromatase activity. Note that subjects were caught late in the breeding season, and aromatase activity in

Table 3. Percentage of Detectable Samples for Aromatase Activity and Oestradiol in the 13 Different Brain Regions for Control (CON) and Simulated Territorial Intrusion (STI) Groups.

	Aromatase activity		17β-Oestradiol	
	CON	STI	CON	STI
High aromatase				
mPOA	100	100	78	91
Hp	100	100	100	73
VMN	100	100	89	64
BST	67	100	100	91
NCM	100	100	78	91
TnA	100	100	78	82
Low aromatase				
Area X	57	80	71	80
MMAN	87	90	75	100
HVC	100	91	100	73
RA	100	90	71	80
GCt	75	60	87	80
OL	87	36	78	80
Cb	78	64	78	82

BST, bed nucleus of the stria terminalis; Cb, cerebellum; GCt, mesencephalic central grey (periaqueductal grey); Hp, hippocampus; HVC, used as a proper name; MMAN, medial magnocellular nucleus of the anterior nidopallium; mPOA, median preoptic area; NCM, caudal medial nidopallium; OL, optic lobe; RA, robust nucleus of arcopallium; TnA, nucleus taeniae of the amygdala; VMN, ventromedial nucleus of the hypothalamus.

some brain regions (e.g., mPOA) might be higher earlier in the breeding season (37, 48, 50).

Relationship between aromatase activity and aggressive behaviour

Aromatisation of testosterone is critical for the regulation of male aggression in birds and mammals (51, 52). In birds, seasonal changes in territorial aggression are associated with seasonal changes in aromatase in certain brain regions, such as the diencephalon and TnA (42, 47, 50, 53, 54). Furthermore, aromatase inhibitor treatments reduce aggression, and this effect can be rescued by E_2 replacement (3, 55, 56). In rodents, aromatase is also important in the control of aggressive behaviour. For example, aggressive behaviour is reduced in aromatase-deficient mice (57, 58) and in mice treated with an aromatase inhibitor (59). E_2 treatment increases aggressive behaviour in house mice (58, 60) and California mice housed under short photoperiods (26). Most of these studies have focused on the effects of long-term changes in aromatase activity, and little is known about rapid changes in aromatase activity associated with aggression (26, 27).

Effects of aggressive behaviour on aromatase activity

In the present study, aromatase activity was not different between the CON and STI groups in any region. Several reasons could explain the lack of a group difference. First, we might have missed a transient change in aromatase activity by analysing aromatase activity after 30 min of STI. It is possible that aromatase activity is modulated, for example, within 10 min and returns to baseline by 30 min. Indeed, aromatase activity in male quail hypothalamus is significantly reduced after 2–15 min of exposure to a female, with a recovery to baseline levels after 30 min (17). Second, the aromatase protein itself might not be rapidly modulated by aggressive encounters but, rather, its cofactor (NADPH) might be modulated. Our assay included a saturating level of cofactor and would have missed this difference. A similar scenario has been suggested for 3β-hydroxysteroid dehydrogenase (3β-HSD) and its cofactor (61). It should be emphasised that the endogenous substrate, testosterone (total from the plasma or free extrapolated from changes in CBG, progesterone and corticosterone), did not vary after STI (32). Third, perhaps the rapid initiation of territorial behaviour is independent of rapid synthesis of E_2. In this case, aromatase activity simply might not be rapidly modulated by aggressive interactions.

Plasma and brain E_2 levels

E_2 has numerous effects on behaviours associated with reproduction and aggression. In songbirds specifically, song production, song perception and the song control system are sensitive to sex steroids, such as E_2 (28, 62–65). The results of the present study show that E_2 is present at high concentrations within the male songbird brain, relative to plasma. Low plasma levels of E_2 were also detected in other adult male songbirds (66, 67). In wintering male Gambel's white-crowned sparrows, levels of plasma oestrogens (E_2 and oestrone combined) are approximately 250 pg/ml, which is also lower than the brain E_2 levels seen in the present study (68). The origin of plasma E_2 in male white-crowned sparrows is unclear. In male zebra finches, E_2 in the general circulation originates mainly from the brain, and not from the gonads or other organs (68). However, in other avian species, low aromatase activity was detected in the liver (43). In the brain, E_2 in brain regions with low to nondetectable aromatase activity might be the result of passive diffusion away from high aromatase regions or of sequestration (specific or nonspecific).

Region-specific changes in E_2

The data obtained in the present study demonstrate that E_2 concentrations are reduced following STI in three brain nuclei: VMN, BST and Hp. Besides its well-known role in the regulation of female sexual behaviour, the VMN also regulates other social behaviours (69). Immediate early gene induction is observed in VMN after agonistic encounters (69, 70). Furthermore, lesions, electrical stimulation or neuropeptide injection within VMN modulate aggression (71–73). The BST is involved in the control of social behaviours, including inter-male aggression, sexual behaviour, pair-bonding and parental behaviour (74, 75). The Hp plays a major role in spatial orientation (76–78), and a change in E_2 in the Hp might relate to remembering the location of the intruder within the territory. The Hp shows major structural and activity changes following social interaction and social stress (79) and is important for social

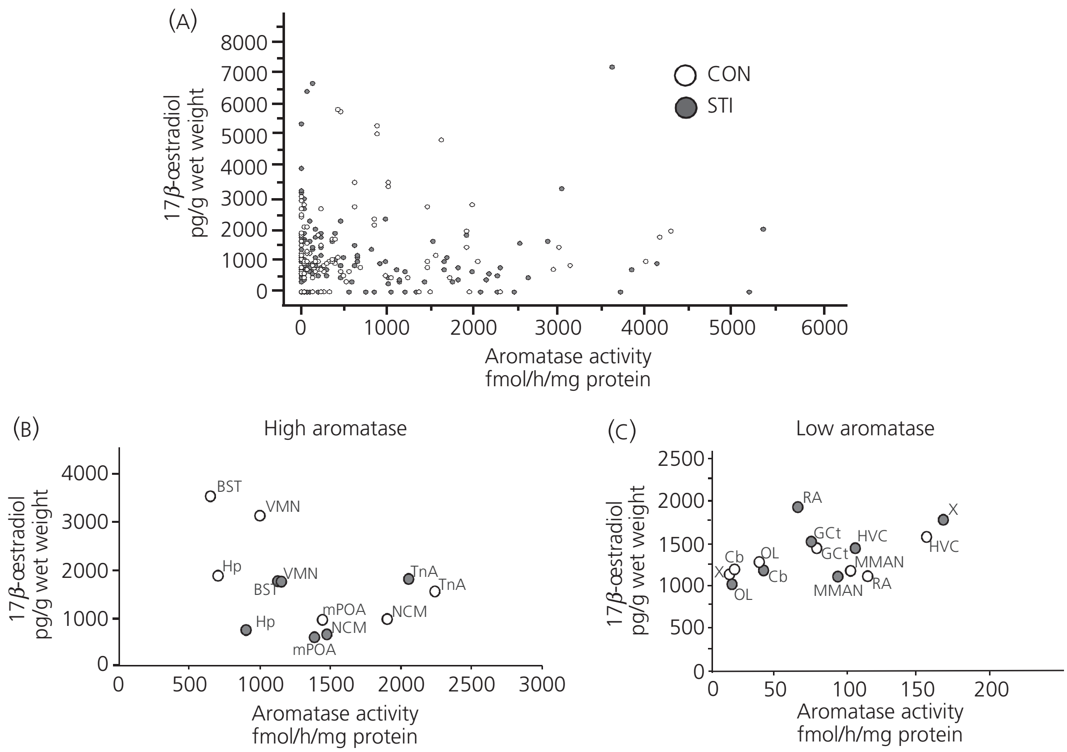

Fig. 4. Graphs showing the absence of correlations between brain aromatase activity (fmol/h/mg of protein) and brain 17β-oestradiol concentration (pg/g of wet weight) in control (CON, empty circles) and simulated territorial intrusion (STI, grey circles) animals. (A) Graph that includes all data from all subjects. (B, C) Aromatase activity (group average) as a function of oestradiol concentration (group average) for high aromatase and low aromatase regions in CON (empty circles) and STI (grey circles) subjects.

recognition in mammals (80). Both oestrogen receptor-α and β are present in these three regions (81–83).

It is unexpected that no group difference was observed in NCM E_2 levels. Using microdialysis, Remage-Healey *et al.* (30) showed an increase in E_2 content in the zebra finch NCM following playback exposure. The microdialysis samples were collected over 30 min, rendering direct comparison with our data difficult. In addition, in the control subjects, E_2 levels are low in the aromatase-rich NCM, which was also unexpected. Our previous data from zebra finches suggest that E_2 concentration was highest in the NCM (31). Note, however, that aromatase activity is similar in the POA and NCM of white-crowned sparrows (present study), whereas aromatase activity in the telencephalon far exceeds the activity in the preoptic area of zebra finches (43).

Decreases in brain E_2 concentrations: possible mechanisms

The decreases in E_2 levels in VMN, BST and Hp were unexpected, given that oestrogens typically stimulate aggressive behaviour (26). Also unexpected is the absence of correlations between brain aromatase activity and brain E_2 concentrations. There are several possible explanations. First, aromatase activity might have been transiently reduced during the start of the STI, leading to a subsequent decrease in E_2 content in specific brain regions. Second, decreases in local E_2 levels might be the result of decreases in other steroidogenic enzymes in the brain (e.g. 3β-HSD or CYP17) (61). Third, decreases in local E_2 concentrations could be a result of mechanisms designed to increase local androgen levels. In mice, androgens act via neural androgen receptors to mediate the 'winner effect,' whereby winners of aggressive encounters are more likely to win future aggressive encounters (84, 85). In the present study, in the late breeding season, no increase in circulating testosterone levels, either total or free, was detected in the STI group (present data; 32). Fourth, the reductions in E_2 concentrations could result from increased catabolism of E_2, subsequent to oestrogen signalling. Similar mechanisms have been shown for dopamine signalling. A reduction in extracellular dopamine, along with an increase in the dopamine metabolites homovanillic acid (HVA) or dihydroxyphenylacetic acid (DOPAC), results in an increased HVA/dopamine or DOPAC/dopamine ratio, indicating an increase in dopaminergic signalling (86–88). The catabolism of oestrogens occurs in the brain, as well as the liver, and is a key mechanism to control local oestrogen levels (19, 89).

Conclusions

There is considerable evidence in a wide variety of species suggesting that oestrogens play an important role in the regulation of social and aggressive behaviours. The causes and consequences of rapid variations in local steroid levels, however, remain largely unknown, and studies of wild animals in their natural environments should help elucidate these important issues. The lack of a simple correlation between brain E_2 concentrations and brain aromatase activity suggests that there are complex mechanisms for the production and metabolism of this brain steroid. The present data clearly demonstrate that social interactions have rapid effects on local E_2 concentrations in specific brain regions, independent of systemic E_2 concentrations.

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