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Relationships between rapid changes in local aromatase activity and estradiol concentrations in male and female quail brain

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Abstract

Estradiol-17 β (E₂) synthesized in the brain plays a critical role in the activation of sexual behavior in many vertebrate species. Because E₂ concentrations depend on aromatization of testosterone, changes in aromatase enzymatic activity (AA) are often utilized as a proxy to describe E₂ concentrations. Utilizing two types of stimuli (sexual interactions and acute restraint stress) that have been demonstrated to reliably alter AA within minutes in opposite directions (sexual interactions= decrease, stress= increase), we tested in Japanese quail whether rapid changes in AA are paralleled by changes in E₂ concentrations in discrete brain areas. In males, E₂ in the pooled medial preoptic nucleus/medial portion of the bed nucleus of the stria terminalis (POM/BST) positively correlated with AA following sexual interactions. However, following acute stress, E₂ decreased significantly (approximately 2-fold) in the male POM/BST despite a significant increase in AA. In females, AA positively correlated with E₂ in both the POM/BST and mediobasal hypothalamus supporting a role for local, as opposed to ovarian, production regulating brain E₂ concentrations. In addition, correlations of individual E₂ in POM/BST and measurements of female sexual behavior suggested a role for local E₂ synthesis in female receptivity. These data demonstrate that local E₂ in the male brain changes in response to stimuli on a time course suggestive of potential non-genomic effects on brain and behavior. Overall, this study highlights the complex mechanisms regulating local E₂ concentrations including rapid stimulus-driven changes in production and stress-induced changes in catabolism.

Keywords

estrogens; aromatase; acute stress; reproductive behavior; non-genomic actions

INTRODUCTION

While testosterone mediates various behavioral and physiological effects, critical to the actions of this steroid in the brain is its conversion into estrogens via aromatization. Estradiol-17 β (E₂) synthesized in the brain has been shown to have a critical, often limiting, role in the activation of sexual behavior (e.g. Balthazart et al., 2009a), parental behavior (Trainor and Marler, 2002), aggression (e.g. Trainor et al., 2006), nociception (Evrard and

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Balthazart, 2004), cognition (Garcia-Segura, 2008) and neuroprotection (Garcia-Segura, 2008). Often the actions of locally synthesized estrogens are mediated by their genomic effects: they bind to specific nuclear receptors that then act as transcription factors (McEwen and Alves, 1999). However, evidence for faster non-genomic action is growing.

As described in Cornil et al. (2006), three main criteria should be identified to demonstrate a non-genomic behavioral action of E₂: 1) a behavioral effect should occur rapidly (within minutes, rather than hours or days), 2) a specific receptor and/or signaling pathway should be present to mediate such rapid effects of E₂, and 3) a rapid endogenous regulation of E₂ availability in the brain should be identified.

As an example of satisfying the first requirement, estrogens acutely affect a variety of physiological and behavioral endpoints including sexual behavior (for reviews, see Cornil et al., 2012; Luine and Frankfurt, 2012; Micevych and Christensen, 2012). For example, sexual behavior in male Japanese quail is rapidly regulated by local changes in brain estrogen concentrations (as reviewed in Balthazart et al., 2009b; Serendynski et al., 2013). For the second requirement, a large number of studies have identified and continue to describe cellular actions of E₂ mediated by membrane receptors for E₂ (e.g. Huang and Woolley, 2012; Srivastava et al., 2011; Woolley, 2007). Not surprisingly, such receptors have been implicated in the control of physiological and behavioral processes such as sexual behavior (Dewing et al., 2007; Kow and Pfaff, 2004; Serendynski et al., 2013) and auditory processing (Remage-Healey, 2012).

The third requirement, the existence of active mechanisms of regulation of local E₂ concentration, is thought to be fulfilled by the discovery of rapid changes of brain aromatase kinetics in response to conditions that mimic fluctuations of neuronal activity (Cornil and Charlier, 2010). Indeed, it has been shown that aromatase activity (AA) can be altered *in vitro* via protein phosphorylations such that the enzyme's capacity to generate estrogens is changed rapidly and reversibly (Balthazart and Ball, 2006; Charlier et al., 2011a). In addition, different external stimuli, such as sexual interactions or exposure to an acute restraint stress, elicit *in vivo* rapid changes in AA in a way that is nuclei-, sex- and context-specific. These changes are also reversible (de Bournonville et al., 2013; Dickens et al., 2012). Such acute variations in enzymatic activity would thus provide the endogenous source of steroid as well as the anatomical specificity (localized aromatase expression) and temporal resolution (rapid changes in enzymatic activity) enabling the best efficiency for the former two requirements (Cornil et al., 2006; Saldanha et al., 2011).

Experiments aiming to describe rapid fluctuations in local E₂ concentrations often rely on measurements of AA levels as a proxy due to technical limitations. Experiments utilizing microdialysis have begun to directly measure fluctuations in E₂ concentrations in response to environmental stimuli (e.g. Remage-Healey et al., 2012; Remage-Healey et al., 2008). However, so far this technique has only allowed the quantification of E₂ in samples collected over a period of 30 minutes and thus provides integrated measures of fluctuations over a relatively long period rather than a point sample. In addition, with microdialysis it is impossible to also identify the mechanisms mediating changes in E₂ concentrations (increased synthesis or decreased catabolism) although there is good evidence that such changes are aromatase driven (Remage-Healey et al., 2008). In this study, we sought to assess how AA levels and E₂ concentrations relate to each other at a specific time point in a specific brain region in a shorter time frame.

Two types of stimuli (sexual interactions and acute restraint stress) that have been studied in our laboratory reliably alter AA within minutes in a manner that is brain nuclei and sex specific (Dickens et al., 2012). Interestingly, the directionality of these changes in AA is

counterintuitive. For example, aromatization of testosterone to E₂ in the medial preoptic nucleus (POM) is known to be required for the activation of male sexual behavior (Balthazart et al., 2009a); however, males allowed to sexually interact with a female show decreases in AA in the POM (de Bournonville et al., 2013). In contrast, despite suggestion that acute stress suppresses sexual behavior (Wingfield et al., 1998), male Japanese quail that are exposed to acute stress show an increase in AA in the POM (Dickens et al., 2011). Finally, although females show similar patterns of AA changes in the POM, it is not clear why females would utilize the local aromatization pathway to control their brain E₂ concentrations when E₂ concentration is relatively high in the circulation.

In this experiment, we tested whether rapid changes in AA are reflected by parallel local changes in E₂ concentrations. To test this directly, we used tissue dissected from specific brain regions in specific individuals to measure *both* AA and E₂ within the same sample. We hypothesized that a direct relationship between AA and E₂ concentrations should be present such that AA increases (as expected for the POM/BST of stressed individuals) would correlate with increases in E₂ concentration whereas AA decreases (as expected for the POM/BST of individuals exposed to a sexual partner) would be associated to decreases in E₂ concentrations.

MATERIALS AND METHODS

Birds

A total of 62 quail (*Coturnix japonica*) were used (females, n=32 and males, n=30). Birds were randomly divided between three groups: control, copulation, and stress. *Control birds* (n=10–11 for each sex) were sampled directly after removal from their home cage. *Copulation group* birds (n=10 for each sex) were allowed to interact sexually for 5 min before being sampled (see below for details). Finally, *stress* group birds (n=10–11 for each sex) were restrained for 15 minutes prior to sampling. All experiments were conducted on 10 weeks-old quail raised from eggs obtained from our laboratory breeding colony. Birds were maintained on a long day photoperiod (16L:8D) and both males and females were kept gonadally intact. Birds were provided with food and water *ad libitum*. Experiments complied with the Belgian laws on “Protection and Welfare of Animals” and on the “Protection of experimental animals”. Animal use protocol (#1027) was approved by the Ethics Committee for the Use of Animals at the University of Liège.

Experimental procedures

All experiments were run between 0830–1200 across three experimental days. Individuals assigned to each group were tested in an alternating manner such that timing within the test day and across days was evenly distributed for all treatment groups and sex. Because we were mainly interested in testing how changes in AA directly correspond to local E₂ concentrations, the design of our experiment did not create groups for direct comparison but rather reproduced scenarios that have been thoroughly tested independently (de Bournonville et al., 2013; Dickens et al., 2012; Dickens et al., 2011) and have demonstrated to produce the most consistent changes in AA.

Control group—Birds were removed from their home cage and immediately sacrificed by decapitation. Animals were not anaesthetized to minimize potential neurochemical changes and decrease the exposure time to additional stressors. Trunk blood was collected for steroid analysis and brains were rapidly dissected from the skull and snap frozen on dry ice (average time from removal from home cage to ice < 90s). Blood was collected, without heparin, in Eppendorf® tubes and immediately placed at 4°C. After allowing the blood to clot

overnight, the blood was centrifuged for 10 minutes at 9,300 g to separate the serum. Serum was then stored at -80°C until assayed.

Copulation group—Males were pre-tested seven times in order to give them sexual experience prior to testing. During these tests, males were paired with females also assigned to the copulation group; pairings were different on each day such that males did not see the same female twice, including on the experimental test day. We pre-tested males and females in this group but not the other groups because our prior studies had shown consistent results (decrease in POM AA) for pre-tested individuals (e.g. de Bournonville et al., 2011). In contrast, prior studies with stressed individuals (increase in POM AA), utilized birds that had not been pre-tested (e.g. Dickens et al., 2011). Since we wanted to limit the number of animals, we chose to prioritize testing the direction of the changes induced in AA compared to E2, effectively choosing consistency with previous studies rather than consistency between groups.

All males achieved at least one full copulation (cloacal contact movement or CCM) prior to the testing day. During the pre-tests and on the experimental day, pairs of males and females were allowed to sexually interact in an arena ($60 \times 40 \times 50$ cm; length $\times$ width $\times$ height) for five minutes during which time behaviors were recorded (as described below). In previous studies (Cornil et al., 2005; de Bournonville et al., 2013), this time frame has given the most consistent decrease in AA in the medial preoptic nucleus (POM) and medial portion of the bed nucleus of the stria terminalis (mBST). These two nuclei were pooled here as the POM/BST. At the end of the behavior test on the testing day, birds were removed from the arena and immediately sacrificed. Sample collection and processing continued as described for control birds.

Stress group—Birds were restrained for 15 min in a metal cylinder (10 cm diameter $\times$ 16 cm length) that had been used in previous experiment (see Dickens et al., 2011). This time frame produced the most consistent increase in POM/BST AA for males and females. At the end of the restraint stress period, birds were removed from the metal cylinder and immediately sacrificed. Sample collection and processing continued as described for control birds.

Behavioral analysis

Males—All behavioral interactions were recorded with a video camera and we reviewed the videotape at a later point to score the male behavior. We recorded both the latency and the frequency of four well-documented male behaviors that are part of the full complement of the typical copulatory sequence in quail. These behaviors have been thoroughly described elsewhere (Adkins and Adler, 1972; Hutchison, 1978). Specifically, we measured neck grabs (NG), mount attempts (MA), mounts (M), and cloacal contact movements (CCM). We also made note of the time elapsed from the beginning of the test before each behavior was displayed (latency), the last behavior performed and the elapsed time between the last behavior and the end of the testing period.

Females—All behavioral interactions were recorded with a video camera and we reviewed the videotape at a later point to score the female behavior. The frequency of each of the following behavior patterns were measured (see Delville and Balthazart, 1987 for detailed descriptions): squat, crouch, stand up, short avoid, long avoid. Squat and crouch behaviors are both considered to indicate positive receptivity and were combined for analysis due to the small number of squats viewed. Stand up, short avoid, and long avoid all indicate negative receptivity of increasing degree such that stand up (simply standing upright with extended legs to discourage male mount attempts) is the least negative while long avoid

(female runs away and continues to run for 4s at least) is considered the most negative. For each pair, we also calculated the relative percentages of each behavioral display by the female relative to the total number of times the male approached her during the 5 min test.

Micropunches

Our method of isolating brain nuclei to assess region-specific AA followed the previously described protocol (Cornil et al., 2011; Dickens et al., 2011), which utilizes a modified Palkovits punch technique (Palkovits, 1973) validated for use in quail (Schumacher and Balthazart, 1987). Since only six nuclei are known to contain high densities of aromatase-positive neurons and only four of these nuclei demonstrate changes in AA following either acute restraint stress (Dickens et al., 2011) or sexual interactions (de Bournonville et al., 2013), we only targeted these specific areas. In the rostral forebrain, we punched and pooled under the name POM/BST the medial preoptic nucleus (POM) and the medial portion of the bed nucleus of the stria terminalis (mBST). In the mediobasal hypothalamus (MBH), we pooled the ventromedial and tuberal hypothalamus (VMN and Tub, respectively). We pooled tissue in this specific experiment due to the detection limits of the assays and since the pooled nuclei represent an anatomically continuous population of aromatase cells (Foidart et al., 1995) that shows similar changes in AA following stress and sexual interaction. We knew from previous experiments that punches from individual nuclei would allow us to measure aromatase activity but this was doubtful for the E₂ assay. Since we also did not know how low E₂ concentrations would be after some of the treatments, we erred on the side of caution. It would have been necessary to run almost the entire experiment to have an answer to these questions and ensure that sensitivity of the assays would be sufficient for measures in individual punches. We believe in retrospect that pooling punches from POM and BST on the one hand and of VMN and Tub on another hand was a wise decision. All experimental values for E₂ concentrations fell on the curves but some were on the low end of the curve and separating POM from BST and VMN from Tub would have produced lower values probably below detection.

Micropunches were obtained from 200 μ m coronal sections collected through the preoptic area and hypothalamus beginning at the caudal end of the tractus septopallio-mesencephalicus (TSM). Position of punches was determined using the quail atlas as presented in (Bayle et al., 1974). All 200 μ m sections for each animal were kept frozen until punched. Micropunches were obtained using a specialized 1.25 mm diameter brain punch apparatus (Leica Biosystems #39443001RM). Micropunches were kept frozen throughout and stored at -80°C until processing. Prior to assaying, micropunches were homogenized in 80 μ L of ice cold buffer (150 mM KCl, 1 mM Na-EDTA, 10 mM Tris-HCl, pH 7.2) using a glass pestle specific for a 1.5 mL Eppendorf tube. Immediately after homogenizing, samples were divided into aliquots to be used for AA analysis (20 μ L) and E₂ analysis (50 μ L).

E₂ extraction and assay in brain and serum

E₂ concentrations were measured in both brain tissue and serum using similar protocols for solid phase extraction and radioimmunoassay. The description presented below primarily concerns brain assays and the limited differences for serum assays are usually mentioned in parentheses.

Solid phase extraction—The 50 μ L fraction from brain tissue isolated for E₂ quantification was immediately added to a tube containing 250 μ L of HPLC-grade methanol, while 40 μ L aliquot of serum were added to a tube containing 2 mL of distilled water. Samples were extracted using the solid phase extraction method utilized previously (Newman et al., 2008) and validated for E₂ in songbirds (Charlier et al., 2011c). Following this method, brain samples were first sonicated for 5 min, placed on a shaker for 60 min at

1500 rpm, and then incubated at 4°C overnight. The following day, brain samples were placed on the shaker again for 60 minutes. The remaining of extraction procedure was strictly similar for brain tissue and serum except that smaller columns were used for serum (1 cc, 100 mg sorbent instead of 6 cc, 500 mg sorbent). As a consequence, the volumes mentioned below refer to the processing of brain tissue, the volumes used for the extraction from serum are presented in parentheses and can be deduced by dividing the volumes used for brain tissue by five. For the extraction, we used uncapped C18 columns (Sep-Pak C18, 6 cc, 500 mg sorbent, Waters; 1cc for serum) primed with 5 mL of HPLC-grade ethanol (1 mL for serum). For the priming step, the ethanol was allowed to drip through the column passively, but for the remaining steps, the liquid was manually forced through the column with an adapted syringe with rubber tubing attached to create a seal since a vacuum manifold was not available. Any effects of this technique over the technique previously used by Charlier et al. (2011c) who used a vacuum manifold to force liquids through the columns was accounted for when values were corrected for recovery percentage (see below). Immediately following the ethanol, columns were equilibrated with 10 mL of deionized water (2×5 mL; 2×1 mL for serum), followed by sample loading (samples were added to 10 mL of deionized water prior to loading; 2 mL for serum). Following sample loading, the column was washed with another 10 mL of deionized water (2×5 mL; 2×1 mL for serum) and then steroids were eluted with 5 mL of 90% HPLC-grade methanol (1 mL for serum). Eluates were dried under a steady stream of nitrogen at 37°C and dried samples were stored at -20°C until assayed. Recovery was tested independently prior to running experimental samples and during extraction of experimental samples by adding a known amount of tritiated E₂ (10000 cpm) when the sample was mixed with methanol and measuring its recovery. The independent recovery pre-tests showed an average recovery of 57.7% with a standard deviation of 3.9%. For recovery during experimental sample extraction, ten samples, across six extraction days and ranging from 1:3 to 1:25 dilutions were used for these tests and the average recovery of E₂ was 60.2% with a standard deviation of 3.9%. This value was then used to calculate the final concentrations for all experimental brain tissue samples.

For the serum samples, recovery was tested independently prior to running experimental samples and during extraction of experimental samples by adding a known amount of tritiated E₂ (30000 cpm) when the sample was mixed with methanol. Three extraction days were used with an average recovery of 41.5% and standard deviation of 3.7%. This value was used to calculate the final concentrations for all experimental serum samples.

A day prior to assaying, samples were reconstituted by adding HPLC-grade ethanol (0.7% of final volume, therefore, 2.5 µL or 4.5 µL for brain and serum samples respectively) directly to the dried sample, vortexing, and bringing the remaining sample to 325 µL (brain tissue) or 650 µL (serum) in phosphate buffered saline containing 0.1% gelatin.

Estradiol Radioimmunoassay—Samples were analyzed using a commercially available ¹²⁵I-E₂ RadioImmunoassay (DSL-4800, UltraSensitive Estradiol RIA, Beckman Coulter). Brain samples were run in singlet in order to have measurable amounts of E₂, while serum samples were run in duplicate. We used the protocol as optimized in (Charlier et al., 2011c) for measuring E₂ in brain tissue. For each individual assay, experimental blanks as well as low and high concentration controls that had been previously validated, were included.

For the assay, 100 µL of anti-estradiol antiserum (diluted 1:3.5) was added to 300 µL of sample. All tubes were vortexed and incubated at room temperature (4h) prior to the addition of 100 µL of ¹²⁵I-E₂ (diluted 1:3). Tubes were then vortexed and incubated at 4°C for 24 hrs. Following this incubation, 500 µL of precipitating reagent was added, tubes were

mixed and incubated at room temperature for 20 min. Tubes were then centrifuged at 1500 g for 20 min at room temperature. Supernatant was decanted and samples were counted. Concentrations per sample were calculated by comparing gamma counts to a standard curve of known E₂ quantities. Brain tissue samples were corrected for protein content by analyzing total protein in the original homogenized sample in the aliquots separated for AA analysis (as described below); therefore samples are expressed as pg × mg protein⁻¹. Serum samples are expressed as pg × mL⁻¹. All samples were corrected for recovery.

We validated the assay kit for quail by measuring a known quantity of radioinert E₂ (5 pg per brain sample and 1pg for serum sample) that had been added to homogenized punches from the nucleus taeniae of the amygdala (brain) or to serum from non-experimental birds prior to extraction. After correcting for recovery, spiked samples yielded the following values: 5.25 ± 0.69 for 5 pg spiked (brain) and 0.83 pg ± 0.18 for 1pg spiked (serum). We also ran serial dilutions using pooled brain samples (4 dilutions from 1:1 to 1:16; n = 2) and individual serum samples of males (4 dilutions from 1:1 to 1:16; n = 3) to assess parallelism in order to confirm that the solid phase extraction efficiently removed interfering substances from the samples. Serially diluted samples showed curves paralleling the standard curve for both sample types. The slopes of these dilution curves (lines when transformed in % B/B0 vs. Log of concentration) were very similar and not significantly different (Brain: p = 0.566, 95 % confidence interval of slope for brain curve: -0.389 to -0.261, for pool 1: -0.371 to -0.173, for pool 2: -0.366 to -0.238; Serum: p = 0.113, 95 % confidence interval of slope for serum curve: -0.404 to -0.286, for serum Sample 1: -0.534 to -0.356, for serum Sample 2: -0.566 to -0.159, for serum Sample 3: -0.620 to -0.257).

AA assay

We determined aromatase activity by measuring the production of tritiated water during the conversion of [1β-³H]-androstenedione into estrone (Baillien and Balthazart, 1997; Roselli and Resko, 1991). This assay has been thoroughly described and validated in published studies and adapted for micropunches (Cornil et al., 2011). Briefly, samples (20μl of a total of 80 μl, as described in the micropunch section) were run in duplicates (2 × 10μl) that were incubated at 37°C for 15 min in the presence of saturating concentrations of substrate (25 nM [1β-³H]-androstenedione) and cofactor (1.2 mM NADPH). After the reaction had been stopped, produced ³H-water was separated from steroids and tissue remains and counted with a beta counter. All samples were divided randomly between two assays. The intra-assay coefficient of variation was less than 6% while the inter-assay coefficient of variation was less than 5%.

AA results were corrected for the amount of tissue obtained for each micropunch sample by analyzing protein concentrations with a commercially available Coomassie Plus Protein Assay reagent (Pierce, Rockford, IL). For this assay, we used 10 μL of the micropunch homogenates (duplicates of 5 μL). With this correction, we are expressing AA as a measure of pmol of aromatization product × h⁻¹ × mg protein⁻¹.

Corticosterone Enzyme Immunoassay

CORT was assayed using Enzyme Immunoassay (EIA) kits (Cayman Chemical Company, Ann Arbor, MI) validated for quail (Dickens et al., 2011). We first extracted CORT from the protein component of the serum using dichloromethane and dried samples at 37°C under a constant stream of nitrogen gas. Samples were reconstituted to a 1:20 (10 μL serum) dilution using 200 μL EIA buffer supplied with the kit. Samples were run in duplicate and randomly assigned to one of two plates, that were both run in a single assay. For each plate, both the intra-assay coefficient of variation was less than 10% and inter-assay coefficient of variation was less than 15%. Sensitivity of the EIA, determined from the lowest reliable value

detectable when samples were diluted at a 1:20 dilution factor, was greater than 0.015 ng/tube.

Testosterone Enzyme Immunoassay

T was assayed using Enzyme Immunoassay (EIA) kits (Cayman Chemical Company, Ann Arbor, MI) validated for quail (Dickens et al., 2011). We extracted T from the protein component of the serum using dichloromethane and dried samples at 37°C under a constant stream of nitrogen gas. Samples from females were reconstituted to a 1:10 dilution (100 µL serum in 1000 µL of EIA buffer) and samples from males were reconstituted to a 1:40 dilution (25 µL serum in 1000 µL of EIA buffer). Samples were run in duplicate and randomly assigned to one of two plates, that were both run in the same assay. For each plate, both the intra-assay coefficient of variation was less than 4.45% and inter-assay coefficient of variation was less than 6.5%. Sensitivity of the EIA, determined from the lowest reliable value detectable when samples were diluted at a 1:10 dilution factor, was greater than 0.29 pg/tube.

Statistical Analysis

Data were analyzed with Prism, version 4.0b (2004; GraphPad Software, Inc., San Diego, CA) or Statistica 10 (Statsoft). AA and E₂ data were analyzed using two-way ANOVAs with the experimental group and sex as independent variables. When appropriate, additional paired comparisons were carried out using a Fisher Protected Least Significant Difference (PLSD) test. Effect size estimates were calculated using eta squared (η^2) for ANOVAs (Prism software) or Cohen's d for pairwise comparisons (<http://www.cognitiveflexibility.org/eta2/>). Individual values for E₂, AA and behavior for males and females were compared by linear regression analyses.

Data points were removed if technical issues were noted for the assay (e.g. samples within undetectable range for RIA, very low protein content, or if results could be considered as outliers - more than two standard deviations away from the mean for each group). Less than 2.5% of data points were removed for these reasons and such removals were evenly distributed between experimental groups and sexes. Such removal of data points has been thoroughly described elsewhere (Dickens et al., 2012). All data are presented by their mean $\pm$ SEM.

RESULTS

Estradiol concentrations

POM/BST—E₂ concentrations in the POM/BST were significantly affected by the experimental treatments ($F_{2,52}=4.39$; $p=0.017$; $\eta^2=10.34\%$) and sex ($F_{1,52}=16.55$; $p=0.0002$; $\eta^2=19.49\%$ with a significant sex*group interaction ($F_{2,52}=3.19$; $p=0.0493$; $\eta^2=7.52\%$; Figure 1A). Post hoc test revealed that the tissue concentration in E₂ of females regardless of treatments were significantly lower than the concentrations in control ($p<0.001$) and mated males ($p<0.0306$). Moreover stress reduced local E₂ concentrations in the male POM/BST by nearly half within 15 min ($p<0.0005$). Finally, local E₂ concentrations following 5 min sexual interaction were half way between concentrations measured at rest ($p=0.0629$) and after 15 min of restraint stress ($p=0.0708$) but these differences were not significant.

MBH—There were no significant differences of E₂ concentrations in the MBH between experimental groups ($F_{2,52}=0.74$; $p=0.480$; $\eta^2=2.71\%$) or sex ($F_{1,52}=0.95$; $p=0.333$; $\eta^2=1.74\%$) with no group*sex interaction ($F_{2,52}=0.26$; $p=0.771$; $\eta^2=0.77\%$; Figure 1C). This lack of a difference between males and females in the MBH E₂ concentration is in

contrast with the differences seen in the POM/BST. As a result, these data highlight the region- and sex- specificity of the local E₂ concentrations.

AA levels

POM/BST—As predicted from prior experiments, AA levels in the POM/BST were significantly affected by the experimental treatments ($F_{2,54}=9.13$; $p=0.0004$; $\eta^2=7.83\%$) and sex ($F_{1,54}=159.71$; $p<0.0001$; $\eta^2=68.45\%$, males > females; Figure 1B). There was no significant group*sex interaction ($F_{2,52}=2.37$; $p=0.103$; $\eta^2=2.03\%$). Post hoc analysis of the treatment effect revealed that this difference arises from the highest enzymatic activity measured in stressed birds compared to the other two groups ($p < 0.013$).

MBH—AA levels in the MBH were significantly affected by the experimental condition ($F_{2,53}=5.91$; $p=0.0048$; $\eta^2=9.50\%$) and sex ($F_{1,53}=49.29$; $p<0.0001$; $\eta^2=36.61\%$), with a significant group*sex interaction ($F_{2,53}=6.49$; $p=0.003$; $\eta^2=10.43\%$; Figure 1D). Post-hoc tests reveal that these effects were driven by the highest enzymatic activity displayed by stressed males compared to the two other groups ($p<0.001$) and the fact that all males groups were different from all female groups ($p < 0.02$) with the exception of mated males compared to control females. The significant effects in AA contrast with the lack of change in E₂ concentrations, suggesting that, in this region, E₂ concentrations may not solely depend on rapid fluctuations in AA.

E₂ versus AA

Males—As illustrated in figure 1, graphical comparison of the AA levels and E₂ concentrations in the POM/BST of males suggests a trend for a slight (non significant) decrease of both variables after sexual interactions but in contrast, AA and E₂ concentrations changed in opposite directions after exposure to acute stress. These data, therefore, imply that E₂ concentration in the POM/BST may not be controlled only by brain AA. We thus chose to compare these measures with three separate linear regressions, one for each experimental group. E₂ concentrations and AA for control (Fig. 2A) and stressed (Fig. 2C) males did not correlate significantly ($R^2 = 0.09$; $F_{1,8} = 0.17$; $p = 0.4$); however, in the copulation group a positive correlation between E₂ concentrations and AA was detected ($R^2 = 0.47$; $F_{1,8}=7.03$; $p=0.029$; Fig. 2B). In the MBH, there were no significant correlations for any of the three groups ($R^2<0.22$; $F_{1,7}<2.0$; $p>0.2$; Fig. 2D–F).

Females—Because the groups were not significantly different for E₂ concentrations nor AA, data from all individuals were analyzed in the same regression. In both the POM/BST and the MBH, AA and E₂ were positively correlated ($R^2=0.23$; $F_{1,28}=8.48$; $p=0.007$ and $R^2=0.16$; $F_{1,28}=5.47$; $p=0.027$, respectively; Figure 3). These data from females suggest that despite the lack of experimentally induced changes in E₂ and AA, minor individual fluctuations in AA in these brain nuclei represent an important component in the regulation of local concentrations of E₂ in the brain despite the presence of ovarian E₂ in the circulation.

E₂ versus sexual behavior

In both males and females, individual fluctuations in AA and E₂ were thus correlated for the birds that were allowed to sexually interact. Therefore, we wondered how these individual differences may be driven by or underlie sexual behavior. For males, we ran linear regression analyses to compare the number of mount attempts (MA) and cloacal contact movements (CCM) versus E₂ concentrations in the POM/BST and MBH. None of these regressions were associated with significant correlations ($R^2 = 0.02$; $F_{1,8} = 1.7$; $p = 0.3$; data not shown). In females, we considered the percentage of crouches, stand-ups, and avoidance

behaviors (long and short combined) performed in response to male approaches in relation with brain E₂ concentrations. E₂ in the POM/BST and percentage of stand-ups showed a significant negative linear relationship ($R^2=0.36$; $F_{1,8}=7.17$; $p=0.028$) suggesting that females with higher E₂ concentrations in the POM/BST may be more receptive (Figure 4A). There were no other significant correlations between E₂ and female behaviors in either nuclei ($R^2=0.36$; $F_{1,8}=1.5$; $p=0.25$).

In addition to these behavioral analyses, we considered whether or not females showed foam in their cloaca after sexual interactions. Male quail produce a meringue-like foam that is transferred to the female cloaca and aids fertilization (Seiwert and Adkins-Regan, 1998). Therefore, presence of this foam in the female cloaca indicates that the male was able to successfully complete the copulation sequence. We ran t-tests to compare the E₂ concentrations in females that did (yes: $n=4$) and did not (no: $n=5$) show foam (one female was not included in the analysis because presence of foam was not checked). For these comparisons, we found that E₂ concentrations in the MBH of females that did show foam in the cloaca were significantly higher than in those that did not ($t=3.55$; $df=7$; $p=0.009$; Cohen's $d=2.4$; Figure 4B). It is important to note that two of the five females that did not have visible foam, received at least one CCM from the male and these females were not outliers in the data set. In contrast, E₂ concentrations in the POM/BST of females were not significantly different between females showing foam versus not ($t=0.92$; $df=7$; $p=0.389$; Cohen's $d=0.61$).

To determine whether these effects are linked with behavior exhibited by the male, we ran a linear regression for male behavior versus female E₂ concentrations. E₂ concentrations in the POM/BST showed a significant positive linear correlation with the number of CCMs observed ($R^2=0.44$; $F_{1,10}=6.36$; $p=0.036$, Figure 4C). This correlation further suggests a potential role for E₂ in the female POM/BST in the control of receptivity since males were more likely to complete the full copulatory sequence with females showing higher concentrations of E₂ in this brain region. Comparing the number of MAs, there was a significant negative linear relationship with E₂ concentrations in the female MBH ($R^2=0.46$; $F_{1,10}=6.69$; $p=0.032$; Figure 4D). We have observed that females harassed by unsuccessful males (attempted repeated mount without achieving CCM) tend to become less receptive as the time in the presence of the male passes. These data thus suggest a role for E₂ concentrations in the MBH for receptivity in the females. However, there was not a significant linear relationship for either number of MA versus E₂ concentrations in the female POM/BST or number of CCM versus E₂ concentrations in the female MBH ($R^2=0.08$; $F_{1,10}=0.68$; $p=0.432$). Together with the absence of treatment effect on brain E₂ concentrations, these results suggest that there might be subtle region-dependent variations in brain E₂ concentrations in response to the social context in females.

AA levels versus sexual behavior

For the behaviors that we found correlated with E₂, we ran statistical analyses to determine if these behaviors also corresponded to differences in AA levels in the same samples. For percentage of stand-ups, there was no significant relationship to AA in the female POM/BST ($R^2=0.26$; $F_{1,8}=2.87$; $p=0.129$). For foam observed in the cloaca, there was similarly no significant difference for the AA in the MBH between the two subgroups ($t=0.40$; $df=6$; $p=0.70$; Cohen's $d=0.30$).

Serum E₂ and T concentrations

E₂ concentrations were significantly affected by sex ($F_{1,43}=51.38$; $p<0.00001$; $\eta^2=52.20\%$) but there were no significant effects of group ($F_{2,43}=0.49$; $p=0.62$; $\eta^2=1.26\%$) or sex*group interaction ($F_{2,43}=0.09$; $p=0.91$; $\eta^2=0.22\%$). For females, means

of serum E₂ were as follows: controls = 214 ± 16 pg/mL; copulation = 211 ± 14 pg/mL; and stress = 239 ± 39 pg/mL. For males, means of serum E₂ were as follows: controls = 68 ± 11 pg/mL; copulation = 85 ± 17 pg/mL; and stress = 95 ± 15 pg/mL. There was no significant linear relationship between serum E₂ concentrations and E₂ concentrations in either the female POM/BST ($R^2 < 0.01$; $F_{1,28} = 0.01$; $p = 0.96$) or the MBH ($R^2 = 0.02$; $F_{1,28} = 0.46$; $p = 0.50$). In males, since the copulation group was the only group that showed a linear relationship between AA and E₂ concentrations, we tested the linear relationship between serum E₂ and POM/BST E₂ concentrations and found no significant relationship ($R^2 = 0.01$; $F_{1,8} = 0.10$; $p = 0.76$).

T concentrations were significantly affected by sex ($F_{1,56} = 163.27$; $p < 0.00001$; $\eta^2 = 73.64\%$) but there were no significant effects of group ($F_{2,56} = 1.13$; $p = 0.33$; $\eta^2 = 1.03\%$) or sex*group interaction ($F_{2,56} = 0.25$; $p = 0.78$; $\eta^2 = 0.23\%$). For females, means of serum T were as follows: controls = 0.81 ± 0.09 ng/mL; copulation = 0.66 ± 0.05 ng/mL; and stress = 0.66 ± 0.15 ng/mL. For males, means of serum T were as follows: controls = 3.3 ± 0.32 ng/mL; copulation = 2.97 ± 0.17 ng/mL; and stress = 2.84 ± 0.38 ng/mL.

As expected, the circulating concentrations in testosterone and E₂ were different between males and females. Interestingly, however, these concentrations did not appear to be affected by sexual behavior and stress further highlighting the link existing between brain-derived estrogens and these processes.

E₂ and corticosterone concentrations

As illustrated in figure 5, CORT concentrations were significantly affected by the experimental treatments ($F_{2,55} = 9.56$; $p = 0.003$; $\eta^2 = 25.74\%$) but there were no significant effects of sex ($F_{1,55} = 0.19$; $p = 0.6751$; $\eta^2 = 0.21\%$) or sex*group interaction ($F_{2,55} = 0.81$; $p = 0.4478$; $\eta^2 = 2.12\%$). Post hoc analysis of the treatment effect revealed that stressed subjects regardless of their sex presented a significantly elevated circulating CORT compared to control ($p < 0.001$) or mated subjects as observed in our previous study (Dickens et al., 2012). There was no significant effect comparing the control group to the copulation group ($p > 0.05$).

Data from our past studies suggested that AA does not correlate to circulating CORT concentrations (Dickens et al., 2012) and since AA did not correlate to E₂ concentrations in the stressed males, we wanted to determine if individual CORT concentrations could be driving E₂ despite lack of correlations with AA. However, data from the present experiment also failed to identify correlations between circulating CORT concentrations in stressed individuals and E₂ concentrations in the male POM/BST or MBH ($R^2 < 0.03$; $F_{1,9} < 0.6$; $p > 0.49$), as well as in the female POM/BST or MBH ($R^2 < 0.05$; $F_{1,10} < 0.40$; $p > 0.55$).

DISCUSSION

The data from this study identify a number of new observations concerning the dynamic relationships between brain AA and local changes in neural E₂ concentrations. First, the levels of AA and E₂ measured in control condition in POM/BST and MBH present the expected sex difference based on previous reports of the distribution of the aromatase protein (Balthazart et al., 1996) and its enzymatic activity (Cornil et al., 2011; Schumacher and Balthazart, 1987). Secondly, these data demonstrate that, in response to environmental stimuli, AA and E₂ concentrations change with a time course compatible with non-genomic effects of locally synthesized estrogens, while circulating testosterone and E₂ concentrations remain unaffected. Thirdly, they show that these effects are localized to discrete brain regions in the males, while in females, E₂ concentrations and AA remain relatively stable regardless of stimuli. Fourthly, they show that, at the individual level, a higher AA in the

female brain correlates with a higher concentration of E_2 suggesting that the brain enzymatic activity might play a substantial role in the control of local concentrations of the steroid even in females where circulating E_2 concentration are clearly higher than in males (Balthazart et al., 1986; Schumacher et al., 1988). Fifthly, they demonstrate that in females and in males exposed to sexual interactions, individual differences in AA correlate with individual differences in local but not serum E_2 concentrations an observation that also suggests a role of the enzyme in controlling local concentrations of the steroid. Finally, these data indicate that although AA can often be used as a proxy for local E_2 concentration in specific conditions, major discrepancies can be observed in the short-term variations of these two measures. Specifically, AA increased markedly in stressed males but in contrast we observed a major decrease in local E_2 concentrations indicating that these concentrations either are modulated with a different time-course or are affected by additional, currently unidentified, factors. We will discuss these new results separately for males and females since, in several cases, different results were observed in the two sexes.

Control males

Further building on the decades of research emphasizing the important role for neurally synthesized estrogens in male behavior (Balthazart et al., 2009b), these data show that in the POM/BST, the brain region most critical to male sexual behavior, E_2 concentrations are nearly twice higher in males than in females despite females having nearly 3-fold higher E_2 concentrations in the circulation. In control males, individual E_2 concentrations were not directly related to AA levels. However, we saw anatomical relationships between these two variables: higher AA concentrations in the POM/BST were reflected in higher E_2 concentrations as compared to lower AA and E_2 concentrations in the MBH. Therefore, these data are consistent with the notion that aromatase in the POM/BST plays a role in the *maintenance* of high E_2 concentrations in this region of the male brain. The specific role of short-term fluctuations of AA on E_2 concentrations following external stimulation (here: stress and sexual interaction), however, appears to be more complex.

Males exposed to sexual interactions

In males exposed to a female and allowed to interact with her, a small non-significant decrease in both AA and E_2 concentrations was observed in the POM/BST. We had identified in similar conditions a significant decrease of AA in the POM or in the BST following sexual interactions but significant effects were not observed after the same latency. The failure to reach statistical significance here might relate to the fact that pooled samples were analyzed (dilution effect) or to a difference in females behavior (variability of the stimulus). Interestingly however, in the POM/BST of males that were allowed here to interact sexually with a female there was a positive linear relationship between AA and E_2 concentrations. These data thus suggest that effects of sexual interaction on E_2 concentrations in this region potentially result from changes in AA. However, male sexual behavior did not correlate with the two neural measurements (AA and E_2) possibly due to differences in the rate at which behavior and brain measures can change and be measured but also possibly due to the role that female behavior and receptivity plays in controlling the male ability to copulate. Nevertheless, recent evidence suggests that local E_2 administration can rapidly (within 15–30 min) stimulate behavior (Serendynski et al., 2013). Since AA and E_2 were measured at a single time point here, after 5 min of sexual interactions, it is possible that changes in AA and in the resulting E_2 concentration did not last long enough to affect in a reliable manner the expression of sexual behavior. It should also be noted that in the Serendynski et al. (2013) study, E_2 rapidly affected measures of appetitive sexual behavior that were not collected here and did not affect, at least when tests were performed in a small arena as done here, the copulatory performance. Multiple reasons can thus explain the lack of correlation between brain measures of AA and E_2 and copulatory behavior. Obviously

unresolved questions remain concerning the timing of enzymatic and E₂ concentration changes and the timing of the acute regulation of behavior by estrogens.

Males exposed to acute stress

In acutely stressed males, AA levels increased significantly as previously reported (Dickens et al., 2012; Dickens et al., 2011). In stark contrast, E₂ concentrations in this region significantly decreased to nearly half of baseline concentrations. Charlier et al. (2011b) emphasizes that environmental stimulation (aggression) can result in rapid changes in tissue concentrations of E₂ that may not be reflected in alterations of AA levels. However, the results from the study by Charlier and colleagues show little to no change in AA despite significant decreases in E₂ in several nuclei investigated. We are the first to show a significant increase in AA with a significant decrease in E₂.

Despite this discrepancy between changes in E₂ concentrations and AA in acutely stressed birds, the direction of the change in E₂ concentration is in agreement with what would be expected of the effects of acute stress on sexual behavior. Stress is often assumed to have a suppressive effect on reproduction and reproductive behavior (Sapolsky et al., 2000; Wingfield et al., 1998), but a potential mechanism underlying stress-induced inhibition of behavior that would allow for both rapid and reversible effects has never been identified (as explained in Dickens et al., 2011). This being said, it has to be noted that contrary to this prediction, acute stress exposure does not seem to interfere with sexual behavior in Japanese quail (Dickens et al., 2012). The stress induced increase in AA was then interpreted as a mechanism of compensation to maintain local estrogen levels. The present data obviously do not support this hypothesis. An alternative hypothesis arose in the light of the recent finding that brain-derived estrogens acutely modulate sexual motivation rather than performance (Serendynski et al., 2013). Acute stress could thus alter the motivation to copulate rather than the ability to perform the copulatory sequence. Such a reduced motivation should in theory result in a parallel change in copulatory frequency. However, Serendynski et al. (2013) showed that when tested in a small arena, male copulatory behavior is not affected by short-term changes in estrogen bioavailability in the brain while sexual motivation, as assessed by appetitive sexual responses, is markedly decreased. The effects of the decreased motivation on sexual performance only becomes apparent when males are tested in a much larger arena where they have to actively search and pursue the female in order to achieve copulation. Effects of stress on copulation were never assessed in these latter conditions and thus whether stress does or does not affect sexual behavior in male quail thus remains to be tested.

Decreases in E₂ concentrations could theoretically arise from a change in the bioavailability of the aromatase substrate coming from the periphery (i.e., testosterone) that has been shown to decrease after at least 15 min of restraint stress (Deviche et al., 2010; Dickens et al., 2011). However, since the E₂ decrease is possibly occurring sooner (Dickens et al., 2011) and appears to be restricted to the POM/BST (present study), this potential mechanism is unlikely. Accordingly, we show that plasma T concentrations were not affected by the experimental treatments in the present experiment so that changes in substrate for the aromatase enzyme cannot explain the changes in brain E₂ concentration. Another possible mechanism underlying the decrease in E₂ following stress may relate to changes in the activity of other steroidogenic enzymes upstream from aromatase in the synthetic pathway going from cholesterol to E₂, such as 3 β -HSD or CYP17 (Pradhan et al., 2008). These modified enzymatic activities would limit the substrate of the aromatase enzyme leading to a decreased E₂ production *in vivo* while this effect would no longer be observed *in vitro* when AA is measured with saturating concentrations of substrate.

An alternative view that deserves further exploration is the potential for stress-induced clearance of E_2 by catabolism. Rapid clearance may represent a fast way to inactivate E_2 produced at the synapse (Cornil, 2009) and, as a consequence, rapidly terminate sexual behavior activation due to hormonal action. A disconnect between changes induced by stress in AA & E_2 concentrations might relate to a differential catabolism of E_2 in control vs. stressful condition, therefore revealing a previously unidentified clearance mechanism to rapidly modulate local E_2 concentrations following stressor exposure. In this framework, increased AA might be interpreted as a compensatory mechanism to rapidly return stress-induced depleted E_2 concentrations back to normal levels (as discussed in Dickens et al., 2011).

While additional research will be necessary to best describe the catabolic pathway(s) that is (are) potentially activated in the POM/BST following acute stress, one potential mechanism has already been described and could directly relate to the rapid increase in AA occurring in this brain region after acute stress. Despite its well-described role in estrogen synthesis, aromatase has also been shown to act as an estrogen 2-hydroxylase (Osawa et al., 1993). An increase in enzymatic activity (as seen with acute stress) could thus also result in an increased *catabolism* of available E_2 via transformation into 2-hydroxy-estradiol. Evidence of aromatase switching from its commonly accepted role as estrogen synthase to an estrogen hydroxylase has been described in the placenta (Osawa et al., 1993) and suggested in the brain (Cornil et al., 2006). In addition, estrogen hydroxylase activity has been demonstrated in the quail brain regions analyzed in this experiment (Balthazart et al., 1994). Since the switch of aromatase from estrogen synthase to hydroxylase is pH dependent (Osawa et al., 1993), our *in vitro* enzymatic assay of post-mortem tissue in standardized conditions of pH, substrate and cofactor concentration would not be able to distinguish between increased AA representing increased aromatase activity or hydroxylase activity. In contrast, *in vivo*, the enzyme could catalyze one or the other reaction depending on local conditions and this possibility should be experimentally investigated. Since blocking E_2 action at neuronal membrane has been demonstrated to rapidly decrease sexual motivation in male quail (Serendynski et al., 2013), a stress-induced clearance of E_2 at the synapse should be explored further as a potential mechanism linking the actions of stress pathways and sexual behavior.

Finally, the discordance between changes in E_2 and in AA following stress exposure might simply be an undesired consequence of our experimental procedures. While E_2 concentrations were assayed directly on frozen brain tissue, AA was evaluated *ex vivo* in optimized conditions of enzymatic substrate and cofactor. As shown by Pradhan et al. (2010) artificially saturating cofactor concentrations in *in vitro* assays may mask enzymatic changes occurring *in vivo*. One could thus imagine that *in vivo* aromatase was actually inhibited due to the acute stress exposure but this inhibition was artificially masked by the optimal conditions used to measure aromatase activity *in vitro*.

Females

Although females in this study did not show any clear average changes in AA levels and E_2 concentrations after exposure to sexual interactions or restraint stress, individual differences in the two measurements did positively correlate in both brain regions studied. In addition, individual measures of receptivity in the females allowed to sexually interact with a male correlated with the local E_2 concentration in the POM/BST but not in MBH suggesting that locally synthesized E_2 potentially regulates female behavior. Significant correlations (in opposite directions) were also observed between E_2 concentrations in POM/BST and MBH and the behavior displayed by the male. Although nuclei-specific action of estrogens on female sexual behavior has been documented in rodents (Blaustein, 2009; Pfaff et al., 1994)

and birds (Gibson and Cheng, 1979), the source of these estrogens has always been assumed to be gonadal.

However, if availability of E₂ in the brain tissue were solely derived from circulating E₂ originating from the ovaries, we would predict no correlation between region-specific AA and E₂ concentrations in the female. The significant correlations between individual differences in AA and E₂ concentrations in both POM/BST and MBH of females challenge this prediction. In addition, there were no significant correlations between individual serum E₂ concentrations and E₂ concentrations in either brain region.

It has been shown that local changes in E₂, either injected or derived from local synthesis, can have rapid effects on the neuron firing frequency in the female NCM, a nucleus associated with auditory processing in zebra finches, *Taeniopygia guttata* (Tremere et al., 2009; Tremere and Pinaud, 2011). In addition, rapid and anatomically specific increases in E₂ concentrations in this nucleus in the female brain are observed in direct response to male song, and linked to neuronal responsiveness (Remage-Healey et al., 2012). A potential role for neural aromatase in female behavior has also been suggested in the hamster (Hsu, 1990) and musk shrew (Veney and Rissman, 2000). Similar to what was recently shown in male quail (Serendynski et al., 2013), it would thus be interesting to determine whether rapid changes in local synthesis of E₂ in the female brain directly relate to changes in receptivity as suggested by the present correlative data.

Conclusions

Together, these results confirm that acute changes in the environmental context are rapidly reflected in highly dynamic changes in aromatase activity, that are region-, sex- and stimulus-specific. They also provide evidence for a control of local estrogen concentrations in the female brain that would occur independently from ovarian secretion. They finally demonstrate that in stable environmental conditions measures of AA in specific brain nuclei represent a good proxy for local brain E₂ concentrations. We found indeed that sex differences in AA were reflected in sex differences in E₂ concentrations (males > females), that the higher AA in POM/BST as compared to the MBH corresponds to a higher E₂ concentration and that in females where experimental treatments had no impact on either AA or E₂ concentrations, the individual values of these two variables are nicely correlated. The situation, however, becomes more complex in dynamic conditions when AA is affected by experimental treatments and discrepancies between these two measures were identified. Such mismatches could arise from (1) changes in the availability of peripheral testosterone (unlikely here), (2) changes in local (brain) testosterone synthesis, (3) differences in the time course of changes in AA and E₂ concentrations, (4) a region-specific catabolism possibly involving aromatase itself or finally (5) from differences between AA as it is expressed *in vivo* and the activity of the same enzyme as measured in the optimized *in vitro* conditions. Overall, these results highlight the complexity of the regulatory mechanisms involved in local neurosteroidogenesis and the resulting behavioral effects.

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References

- Adkins EK, Adler NT. Hormonal control of behavior in the Japanese quail. *Journal of Comparative and Physiological Psychology*. 1972; 81:27–36. [PubMed: 5074305]
- Baillien M, Balthazart J. A direct dopaminergic control of aromatase activity in the quail preoptic area. *J Steroid Biochem Mol Biol*. 1997; 63:99–113. [PubMed: 9449211]
- Balthazart J, Ball GF. Is brain estradiol a hormone or a neurotransmitter? *Trends Neurosci*. 2006; 29:241–249. [PubMed: 16580076]
- Balthazart J, Cornil CA, Charlier TD, Taziaux M, Ball GF. Estradiol, a key endocrine signal in the sexual differentiation and activation of reproductive behavior in Quail. *Journal of Experimental Zoology Part A: Ecological Genetics and Physiology*. 2009a; 311:323–345.
- Balthazart J, Delville Y, Sulon JC, Hendrick JC. Plasma levels of luteinizing hormone and of five steroids in photostimulated, castrated and testosterone-treated male and female Japanese quail (*Coturnix coturnix japonica*). *General Endocrinology*. 1986; 5:31–36.
- Balthazart J, Stoop R, Foidart A, Granneman JCM, Lambert JGD. Distribution and regulation of estrogen-2-hydroxylase in the quail brain. *Brain Res Bull*. 1994; 35:339–345. [PubMed: 7850484]
- Balthazart J, Taziaux M, Holloway K, Ball GF, Cornil CA. Behavioral effects of brain-derived estrogens in birds. *Ann N Y Acad Sci*. 2009b; 31–48. [PubMed: 19456326]
- Balthazart J, Tlemcani O, Harada N. Localization of testosterone- sensitive and sexually dimorphic aromatase-immunoreactive cells in the quail preoptic area. *J Chem Neuroanat*. 1996; 11:147–171. [PubMed: 8906458]
- Bayle JD, Ramade F, Oliver J. Stereotaxic topography of the brain of the quail (*Coturnix coturnix japonica*). *Journal de Physiologie*. 1974; 68:219–241. [PubMed: 4607102]
- Blaustein, JD. Feminine Reproductive Behavior and Physiology in Rodents: Integration of Hormonal, Behavioral, and Environmental Influences. In: Pfaff, DW.; Arnold, AP.; Etgen, AM.; Fahrbach, SE.; Rubin, RT., editors. *Hormones, Brain and Behavior*. Academic Press; San Diego, CA: 2009. p. 68-1007.
- Charlier TD, Harada N, Balthazart J, Cornil CA. Human and quail aromatase activity is rapidly and reversibly inhibited by phosphorylating conditions. *Endocrinology*. 2011a; 152:4199–4210. [PubMed: 21914772]
- Charlier TD, Newman AEM, Heimovics SA, Po KWL, Saldanha CJ, Soma KK. Rapid Effects of Aggressive Interactions on Aromatase Activity and Oestradiol in Discrete Brain Regions of Wild Male White-Crowned Sparrows. *J Neuroendocrinol*. 2011b; 23:742–753. [PubMed: 21623961]
- Charlier TD, Po KWL, Newman AEM, Shah AH, Saldanha CJ, Soma KK. 17 β -Estradiol levels in male zebra finch brain: Combining Palkovits punch and an ultrasensitive radioimmunoassay. *Gen Comp Endocrinol*. 2011c; 167:18–26. [PubMed: 20144613]
- Cornil CA. Rapid regulation of brain oestrogen synthesis: The behavioural roles of oestrogens and their fates. *J Neuroendocrinol*. 2009; 21:217–226. [PubMed: 19207825]
- Cornil CA, Ball GF, Balthazart J. Functional significance of the rapid regulation of brain estrogen action: Where do the estrogens come from? *Brain Res*. 2006; 1126:2–26. [PubMed: 16978590]
- Cornil CA, Ball GF, Balthazart J. Rapid control of male typical behaviors by brain-derived estrogens. *Frontiers in Neuroendocrinology*. 2012; 33:425–446. [PubMed: 22983088]
- Cornil CA, Ball GF, Balthazart J, Charlier TD. Organizing Effects of Sex Steroids on Brain Aromatase Activity in Quail. *PLoS ONE*. 2011; 6:e19196. [PubMed: 21559434]
- Cornil CA, Charlier TD. Rapid Behavioural Effects of Oestrogens and Fast Regulation of Their Local Synthesis by Brain Aromatase. *J Neuroendocrinol*. 2010; 22:664–673. [PubMed: 20456609]
- Cornil CA, Dalla C, Papadopoulou-Daifoti Z, Baillien M, Dejace C, Ball GF, Balthazart J. Rapid decreases in preoptic aromatase activity and brain monoamine concentrations after engaging in male sexual behavior. *Endocrinology*. 2005; 146:3809–3820. [PubMed: 15932925]
- de Bournonville, C.; Ball, GF.; Balthazart, J.; Cornil, CA. Rapid changes of aromatase activity in discrete brain regions following social interactions. In: Panzica, G.; Gotti, S., editors. *Steroids and Nervous System. Trabajos del Instituto Cajal*; Torino, Italy: 2011. p. 147(Abtract)

- de Bournonville C, Dickens MJ, Ball GF, Balthazart J, Cornil CA. Dynamic changes in brain aromatase activity following sexual interactions in males: where when and why? *Psychoneuroendocrinology*. 2013
- Delville Y, Balthazart J. Hormonal control of female sexual behavior in the Japanese quail. *Horm Behav*. 1987; 21:288–309. [PubMed: 3666684]
- Deviche PJ, Hurley LL, Fokidis HB, Lerbour B, Silverin B, Sabo J, Sharp PJ. Acute stress rapidly decreases plasma testosterone in a free-ranging male songbird: Potential site of action and mechanism. *Gen Comp Endocrinol*. 2010; 169:82–90. [PubMed: 20691650]
- Dewing P, Boulware MI, Sinchak K, Christensen A, Mermelstein PG, Micevych P. Membrane Estrogen Receptor- α Interactions with Metabotropic Glutamate Receptor 1a Modulate Female Sexual Receptivity in Rats. *The Journal of Neuroscience*. 2007; 27:9294–9300. [PubMed: 17728443]
- Dickens MJ, Balthazart J, Cornil CA. Brain aromatase and circulating corticosterone are rapidly regulated by combined acute stress and sexual interaction in a sex specific manner. *J Neuroendocrinol*. 2012; 24:1322–1334. [PubMed: 22612582]
- Dickens MJ, Cornil CA, Balthazart J. Acute stress differentially affects aromatase activity in specific brain nuclei of adult male and female quail. *Endocrinology*. 2011
- Evrard HC, Balthazart J. Aromatization of androgens into estrogens reduces response latency to a noxious thermal stimulus in male quail. *Horm Behav*. 2004; 45:181–189. [PubMed: 15047013]
- Foidart A, Reid J, Absil P, Yoshimura N, Harada N, Balthazart J. Critical re-examination of the distribution of aromatase-immunoreactive cells in the quail forebrain using antibodies raised against human placental aromatase and against the recombinant quail, mouse or human enzyme. *J Chem Neuroanat*. 1995; 8:267–282. [PubMed: 7669272]
- Garcia-Segura LM. Aromatase in the brain: Not just for reproduction anymore. *J Neuroendocrinol*. 2008; 20:705–712. [PubMed: 18601693]
- Gibson MJ, Cheng MF. Neural mediation of estrogen-dependent courtship behavior in female ring doves. *J Comp Physiol Psychol*. 1979; 93:855–867.
- Hsu CH. Blockade of lordosis by androst-1,4,6-triene-3,17-dione (ATD) and tamoxifen in female hamsters primed with testosterone propionate. *Horm Behav*. 1990; 24:14–19. [PubMed: 2328967]
- Huang GZ, Woolley CS. Estradiol Acutely Suppresses Inhibition in the Hippocampus through a Sex-Specific Endocannabinoid and mGluR-Dependent Mechanism. *Neuron*. 2012; 74:801–808. [PubMed: 22681685]
- Hutchison RE. Hormonal differentiation of sexual-behavior in Japanese quail. *Horm Behav*. 1978; 11:363–387. [PubMed: 753700]
- Kow LM, Pfaff DW. The membrane actions of estrogens can potentiate their lordosis behavior-facilitating genomic actions. *Proceedings of the National Academy of Sciences of the United States of America*. 2004; 101:12354–12357. [PubMed: 15302933]
- Luine VN, Frankfurt M. Estrogens facilitate memory processing through membrane mediated mechanisms and alterations in spine density. *Frontiers in Neuroendocrinology*. 2012; 33:388–402. [PubMed: 22981654]
- McEwen BS, Alves SE. Estrogen actions in the central nervous system. *Endocr Rev*. 1999; 20:279–307. [PubMed: 10368772]
- Micevych P, Christensen A. Membrane-initiated estradiol actions mediate structural plasticity and reproduction. *Frontiers in Neuroendocrinology*. 2012; 33:331–341. [PubMed: 22828999]
- Newman AEM, Chin EH, Schmidt KL, Bond L, Wynne-Edwards KE, Soma KK. Analysis of steroids in songbird plasma and brain by coupling solid phase extraction to radioimmunoassay. *Gen Comp Endocrinol*. 2008; 155:503–510. [PubMed: 17949721]
- Osawa Y, Higashiyama T, Shimizu Y, Yarborough C. Multiple functions of aromatase and the active-site structure - aromatase is the placental estrogen 2- hydroxylase. *J Steroid Biochem Mol Biol*. 1993; 44:469–480. [PubMed: 8476762]
- Palkovits M. Isolated removal of hypothalamic or other brain nuclei of the rat. *Brain Res*. 1973; 59:449–450. [PubMed: 4747772]

- Pfaff, DW.; Schwartz-Giblin, S.; McCarthy, MM.; Kow, LM. Cellular and molecular mechanisms of female reproductive behaviors. In: Knobil, E.; Neill, JD., editors. *The physiology of reproduction*. 2. Raven Press; New York: 1994. p. 107-220.
- Pradhan DS, Newman AEM, Wacker DW, Wingfield JC, Schlinger BA, Soma KK. Aggressive interactions rapidly increase androgen synthesis in the brain during the non-breeding season. *Horm Behav*. 2010; 57:381–389. [PubMed: 20116379]
- Pradhan DS, Yu Y, Soma KK. Rapid estrogen regulation of DHEA metabolism in the male and female songbird brain. *J Neurochem*. 2008; 104:244–253. [PubMed: 17949414]
- Remage-Healey L. Brain estrogen signaling effects acute modulation of acoustic communication behaviors: A working hypothesis. *Bioessays*. 2012; 34:1009–1016. [PubMed: 23065844]
- Remage-Healey L, Dong SM, Chao A, Schlinger BA. Sex-specific, rapid neuroestrogen fluctuations and neurophysiological actions in the songbird auditory forebrain. *J Neurophysiol*. 2012; 107:1621–1631. [PubMed: 22190616]
- Remage-Healey L, Maidment NT, Schlinger BA. Forebrain steroid levels fluctuate rapidly during social interactions. *Nat Neurosci*. 2008; 11:1327–1334. [PubMed: 18820691]
- Roselli, CE.; Resko, JA. In vitro assay of aromatase activity in the central nervous system. In: Greenstein, B., editor. *Neuroendocrine Research Methods*. Harwood Academic Publishers; Chur, Switzerland: 1991. p. 937-951.
- Saldanha CJ, Remage-Healey L, Schlinger BA. Synaptocrine Signaling: Steroid Synthesis and Action at the Synapse. *Endocr Rev*. 2011; 32:532–549. [PubMed: 21622487]
- Sapolsky RM, Romero LM, Munck AU. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. *Endocr Rev*. 2000; 21:55–89. [PubMed: 10696570]
- Schumacher M, Balthazart J. Neuroanatomical distribution of testosterone-metabolizing enzymes in the Japanese quail. *Brain Res*. 1987; 422:137–148. [PubMed: 3676776]
- Schumacher M, Sulon J, Balthazart J. Changes in serum concentrations of steroids during embryonic and post-hatching development of male and female Japanese quail (*Coturnix-coturnix-japonica*). *J Endocrinol*. 1988; 118:127–134. [PubMed: 3411279]
- Seiwert CM, Adkins-Regan E. The foam production system of the male Japanese quail: Characterization of structure and function. *Brain Behavior and Evolution*. 1998; 52:61–80.
- Serendynski AL, Balthazart J, Christophe VJ, Ball GF, Cornil CA. Neuroestrogens Rapidly Regulate Sexual Motivation But Not Performance. *J Neurosci*. 2013; 33:164–174. [PubMed: 23283331]
- Srivastava DP, Waters EM, Mermelstein PG, Kramar EA, Shors TJ, Liu F. Rapid Estrogen Signaling in the Brain: Implications for the Fine-Tuning of Neuronal Circuitry. *J Neurosci*. 2011; 31:16056–16063. [PubMed: 22072656]
- Trainor BC, Kyomen HH, Marler CA. Estrogenic encounters: How interactions between aromatase and the environment modulate aggression. *Frontiers in Neuroendocrinology*. 2006; 27:170–179. [PubMed: 16376420]
- Trainor BC, Marler CA. Testosterone promotes paternal behaviour in a monogamous mammal via conversion to oestrogen. *Proc R Soc Lond Ser B-Biol Sci*. 2002; 269:823–829.
- Tremere LA, Jeong JK, Pinaud R. Estradiol Shapes Auditory Processing in the Adult Brain by Regulating Inhibitory Transmission and Plasticity-Associated Gene Expression. *J Neurosci*. 2009; 29:5949–5963. [PubMed: 19420261]
- Tremere LA, Pinaud R. Brain-Generated Estradiol Drives Long-Term Optimization of Auditory Coding to Enhance the Discrimination of Communication Signals. *J Neurosci*. 2011; 31:3271–3289. [PubMed: 21368039]
- Veney SL, Rissman EF. Steroid implants in the medial preoptic area or ventromedial nucleus of the hypothalamus activate female sexual behaviour in the musk shrew. *J Neuroendocrinol*. 2000; 12:1124–1132. [PubMed: 11069128]
- Wingfield JC, Maney DL, Breuner CW, Jacobs JD, Lynn S, Ramenofsky M, Richardson RD. Ecological bases of hormone-behavior interactions: The emergency life history stage. *Am Zool*. 1998; 38:191–206.
- Woolley, CS. *Annu Rev Pharmacol Toxicol*. Annual Reviews; Palo Alto: 2007. Acute effects of estrogen on neuronal physiology; p. 657-680.

HIGHLIGHTS

- Comparison of aromatase activity and localized estradiol concentrations
- Post-copulation, aromatase activity correlates with estradiol in male preoptic area
- Post-stress, aromatase activity increases, estradiol decreases in male preoptic area
- Measurements of female receptivity correlate with localized, neural estradiol
- Rapid, stimuli-specific, local changes in estradiol suggest non-genomic effects

FIGURE 1.

Effects of sexual interactions or acute stress on local estradiol concentrations (E₂ in pg/mg protein; A, C) and aromatase activity (AA in pmol/h/mg protein, B, D) in the medial preoptic nucleus/medial part of the bed nucleus of the stria terminalis (POM/BST, A–B) and the mediobasal hypothalamus (MBH, C–D) of males and females. Control animals did not receive any manipulation prior to sample collection. N.S., *, **, *** indicate the degree of statistical significance (non-significant, or < 0.05, 0.01 or 0.001 respectively) for the main effect of sex and treatment as well as the interaction. Results from post-hoc tests are represented by asterisks (*) above the bars to identify significant differences in analysis of the main effect of treatment or by letters to identify statistical differences in the post-hoc analysis of the interaction; bars that do not share a same letter are statistically different.

FIGURE 2. Linear regressions of E₂ concentrations (pg/mg protein) versus AA levels (pmol/hour/mg protein) in males

The three experimental groups were split to compare individual values in the medial preoptic nucleus and medial part of the bed nucleus of the stria terminalis (POM/BST; A–C) and mediobasal hypothalamus (MBH; D–F). A positive linear relationship, as indicated by a black line, is observed only in the POM/BST of males in the copulation group ($R^2=0.47$, $p=0.03$). No significant linear relationship ($R^2<0.22$, $p>0.2$) was observed in all other cases (light grey line).

FIGURE 3. Linear regressions of E₂ concentrations (pg/mg protein) versus AA levels (pmol/hour/mg protein) in females

Data from the three experimental groups were pooled to compare individual values in A) the medial preoptic nucleus and medial part of the bed nucleus of the stria terminalis (POM/BST) and B) mediobasal hypothalamus (MBH). A positive linear relationship is observed in both the POM/BST ($R^2=0.23$, $p=0.007$) and the MBH ($R^2=0.16$, $p=0.027$).

FIGURE 4. Measurements of female receptivity and E₂ concentrations

A) Linear regression of E₂ concentrations in the female POM/BST versus the percentage of stand-ups (a negative measurement of receptivity) relative to other behaviors exhibited towards male in arena ($R^2=0.36$, $p=0.028$). B) Comparison of females exhibiting foam (yes) or not exhibiting foam (no) transferred from the male cloaca during copulation. E₂ concentrations were significantly different in these two subgroups of females ($p=0.009$, significance indicated by *). Sample sizes are indicated within the columns. C) Linear regression of E₂ concentrations in the female POM/BST versus the number of cloacal contact movements (CCMs, indicative of male achieving full copulatory sequence; $R^2=0.443$; $p=0.036$). D) Linear regression of E₂ concentrations in the female MBH versus the number of mount attempts (MAs, indicative of male's continuous attempt to mate; $R^2=0.455$; $p=0.032$).

FIGURE 5. Measurements of corticosterone (CORT) concentrations in males and females following sexual interactions or exposure to acute stress

Control animals did not receive any manipulation prior to sample collection. In the legend, ** and N.S. reflect the degree of statistical significance (<0.01 or non-significant, respectively) for the main effect of sex and treatment as well as the interaction. The asterisk above the columns identifies the significant differences in the post-hoc analysis of the main effect of treatment.