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Dynamic changes in brain aromatase activity following sexual interactions in males: where, when and why?

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Summary

It is increasingly recognized that estrogens produce rapid and transient effects at many neural sites ultimately impacting physiological and behavioral endpoints. The ability of estrogens to acutely regulate cellular processes implies that their concentration should also be rapidly fine-tuned. Accordingly, rapid changes in the catalytic activity of aromatase, the limiting enzyme for estrogen synthesis, have been identified that could serve as a regulatory mechanism of local estrogen concentrations. However, the precise anatomical localization, time-course, triggering stimuli and functional significance of these enzymatic changes *in vivo* are not well understood. To address these issues as to where, when and why aromatase activity (AA) rapidly changes after sexual interactions, AA was assayed in six populations of aromatase-expressing cells microdissected from the brain of male quail that experienced varying durations of visual exposure to or copulation with a female. Sexual interactions resulted in a rapid AA inhibition. This inhibition occurred in specific brain regions (including the medial preoptic nucleus), in a context-dependent fashion and time-scale suggestive of post-translational modifications of the enzyme. Interestingly, the enzymatic fluctuations occurring in the preoptic area followed rather than preceded copulation and were tied specifically to the female's presence. This pattern of enzymatic changes suggests that rapid estrogen effects are important during the motivational phase of the behavior to trigger physiological events essential to activate mate search and copulation.

Keywords

aromatase; estrogen synthesis; non-genomic action of estrogens; copulatory behavior; sexual motivation

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Contributors

CdB and CAC performed the behavioral experiments and aromatase assays and did all statistical analyses. MJD did the testosterone assays. The research was designed by CAC, GFB and JB. All authors contributed to the preparation of the manuscript.

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1. Introduction

Estrogens profoundly affect a multitude of physiological and behavioral responses such as social behaviors, nociception and neuroprotection (Evrard and Balthazart, 2004; Trainor et al., 2006; Garcia-Segura, 2008; Ball and Balthazart, 2009; Hull and Rodriguez-Manzo, 2009). Estrogen effects are classically attributed to the transcriptional activity of their liganded nuclear receptors (Tsai and O'Malley, 1994), usually arise slowly (hours-days) and coincide with the slow fluctuations in circulating concentrations of gonadal steroids associated with specific physiological states (e.g. estrus cycle, breeding cycle; Ball and Balthazart, 2009). Estrogens also rapidly regulate neuronal activity through membrane-initiated events (Maggi et al., 2004; Vasudevan and Pfaff, 2007) that translate into fast (within minutes) and transient modulations of physiological and behavioral responses (Cornil and Charlier, 2010; Roepke et al., 2011). Estrogen's ability to trigger rapid and reversible responses implies that mechanisms exist to regulate their concentrations on a comparable time-scale.

Estrogens are synthesized from androgens by aromatase, an enzyme present in various tissues (gonads, muscles, adipose tissue, ...) but also in discrete brain regions (Foidart et al., 1995; Ball and Balthazart, 2009). Previous research on brain aromatase largely focused on the regulation of aromatase concentration in relation to different reproductive states. It was recently demonstrated that aromatase activity (AA) can also be rapidly and reversibly modulated by calcium-dependent phosphorylations resulting from neuronal depolarization or by glutamate release (Balthazart et al., 2003; Balthazart et al., 2006; Remage-Healey et al., 2008; Charlier et al., 2011; Remage-Healey et al., 2011). Moreover, rapid AA fluctuations occur *in vivo* in response to social interactions (Cornil et al., 2005; Remage-Healey et al., 2008; Remage-Healey et al., 2009) or acute stress (Dickens et al., 2011). This acute control of AA preferentially occurs at the synapse, and could thus serve as a rapid and spatially restricted control mechanism of local estrogen concentrations (Remage-Healey et al., 2011; Cornil et al., 2012). However, the precise anatomical localization, time-course, triggering stimuli and functional significance of these rapid enzymatic changes *in vivo* are not well understood.

To address these issues as to where, when and why AA rapidly changes after sexual interactions, AA was quantified in aromatase-rich regions that were microdissected from the brains of male Japanese quail exposed to varying durations of visual exposure to or copulation with a female. Since this species expresses much higher AA than rodents and is the system most consistently demonstrating the fastest fluctuations in AA (Cornil et al., 2005), it constitutes an exquisite model to study these questions. Changes in enzyme kinetics were analyzed in parallel with the behavior exhibited by males in different social conditions. Results identified rapid, region- and context-specific changes in AA, occurring on a time-scale suggestive of post-translational modifications of the enzyme. The careful analysis of where and when these enzymatic changes occur with respect to the timing of sexual behavior suggests that rapid changes in brain estrogens mediate fast changes in sexual motivation but not in copulatory performance, which would be affected only by slower genomic effects of steroids.

2. Materials and methods

2.1. Subjects

198 male Japanese quail (*Coturnix japonica*) were used as subjects. Animals were obtained from a local breeder in Belgium or derived from our breeding colony at the University of Liège. All animals were adult (> 8 weeks), individually housed, maintained on a long day photoperiod (16 h light and 8 h dark), provided with food and water *ad libitum* and kept

gonadally intact. Experiments complied with the Belgian laws on the “Protection of experimental animals” and were approved by the Ethics Committee for the Use of Animals at the University of Liège (Protocol #1235).

2.2 Experimental procedures

Four separate experiments were performed to determine the timing and neuroanatomical specificity of female-induced changes in AA. Prior to each experiment, subjects were pre-tested for copulatory behavior until they had gained sufficient experience (see below) and then assigned to different experimental groups matched for their cloacal gland size and behavioral performance during the pre-tests. During the experimental tests, subjects were exposed for a given duration (see below) to a sexually experienced female that they had never been paired with. In all experiments, control (CTL) birds were killed immediately after being removed from their home cage without exposure to a female. Immediately after these manipulations, birds were killed by rapid decapitation. Trunk blood was collected and stored at 4°C, while the brain was rapidly (< 2 min) collected, frozen on dry ice and stored at -80°C. On the next day, blood samples were centrifuged (9 min at 9000 g), the plasma was collected and stored at -80°C.

In Experiment 1, 65 males were allowed to copulate with a female during 2 (n=14), 5 (n=15), 10 (n=15) or 15 (n=6) min before brain collection or left in their home cage as a control (n=15). Experiment 2 tested whether the rapid changes in AA observed in Experiment 1 directly result from the intense sexual activity occurring during the first 2 min of interaction. Males (n=57) were allowed to copulate with a female for 2 min, and left in the empty experimental arena for 0 (n=11), 3 (n=11), 8 (n=11) or 13 (n=12) min before brain collection or left in their home cage as a control (n=12). Experiment 3 investigated whether the visual interaction in the absence of physical contacts with a female produces changes in AA similar to those observed after copulation. Males (n=49) were allowed to view a female for 2 (n=10), 5 (n=9), 10 (n=10) or 15 (n=10) min before brain collection or left in their home cage as a control (n=10). Finally, Experiment 4 assessed whether rapid changes in AA are reversible in a time-scale compatible with changes in the enzyme's concentration or enzyme catalytic activity. Males (n=28) were allowed to copulate for 5 min with a female. Brains were collected either immediately after the sexual interaction (n=10) or after 115 additional min in their home cage (n=8) or in birds left in their home cage as a control (n=8).

2.3. Behavioral tests

Appetitive sexual behavior was assessed by measuring the frequency of rhythmic cloacal sphincter movements (RCSM). The procedure was described previously (Taziaux et al., 2004). Briefly, tests were performed in an aquarium (40×20×25 cm) placed above a mirror at a 45° angle, which provides an unobstructed view of the male's cloacal area. The aquarium was divided into two chambers by a glass partition. The experimental male was placed in one chamber and the stimulus egg-laying female in the other chamber. The male had thus visual access to the stimulus female, but birds could not physically interact. The frequency of the cloacal contractions displayed by males in response to visual exposure to a female provides a measure of sexual motivation (Balthazart et al., 1998; Seiwert and Adkins-Regan, 1998).

Consummatory sexual behavior was measured in a test arena (60×40×50 cm) where a male was allowed to freely interact with a receptive female. The latency and frequency of the stereotyped male sexual behaviors (neck grab [NG], mount attempt [MA], mount [M], and cloacal contact movements [CCM] (see (Adkins and Adler, 1972; Hutchison, 1978)) for a detailed description) were recorded. Because frequencies and latencies of NG and MA, on

the one hand, and of M and CCM, on another hand, were nearly identical, results for MA and CCM only are presented to avoid redundancy.

During pre-tests, males were allowed to interact with a female for 5 min during which their behavioral frequencies and latencies were recorded. Depending on the experiment, 5 to 7 pre-test sessions were needed to reach the criterion for sexual performance (at least one CCM with a latency shorter than 10 sec). In the experimental tests of experiments 1 and 3, the behavior frequencies were recorded minute by minute to allow a fine analysis of their evolution over time.

2.4. Brain dissections

In experiments 1-3, AA was quantified in specific brain nuclei microdissected using the Palkovits punch method (Palkovits, 1973) adapted for quail (Cornil et al., 2011). Briefly, brains were cryosectioned in 200 μ m-thick coronal sections. Punches were dissected by an 18 or 21G needle (respectively 0.838 or 0.514 mm inner diameter and 1.270 or 0.8192 mm outer diameter) attached to a cold air-filled syringe, expelled in a frozen 1.5 ml tube, kept frozen, and stored at -80°C. Six brain nuclei containing aromatase-expressing cell populations (Foidart et al., 1995) were collected: the medial preoptic nucleus (POM), the medial portion of the bed nucleus of the stria terminalis (BST), the nucleus taeniae of the amygdala (TnA), the ventromedial (VMN) and tuberal (Tub; presumably homologous to the mammalian arcuate nucleus) hypothalamus, and the mesencephalic periaqueductal gray (PAG). The small size of the dissection needles allowed to confine dissection largely within the aromatase-expressing cell groups and erred on the side of collecting only the nucleus of interest rather than collecting all of it (See Cornil et al., 2011 for a fuller description).

Although these nuclei belong to different brain structures as defined by Nissl staining, the ARO-ir cells of (1) the POM and BST and (2) the VMN and the Tub form two distinct continuous cell populations (Foidart et al., 1995). For this reason and to allow a comparison with Experiment 4 (see below), AA was analyzed separately in each of these nuclei and also as the total AA measured in the microdissected tissue collected from these two contiguous cell populations i.e., the POM+BST and VMN+Tub re-labeled mediobasal hypothalamus or MBH.

In Experiment 4, larger pieces of tissue were collected in an attempt (unfortunately unsuccessful) to obtain samples that would be large enough to measure the aromatase protein concentration by Western blot along with its enzymatic activity. A home-made U-shaped tool was used to microdissect a squared piece of tissue (2×2 mm) from successive 200 μ m-thick brain slices. Two samples were collected per brain. The first sample containing the POM and BST was collected from the section where the tractus septopallio-mesencephalicus (TSM) reaches 50% of its maximal extension to the section following the full extension of the commissura anterior (4 sections). The second sample covering the VMN and Tuber was collected from the section containing the decussatio supraoptica dorsalis to the last section containing the full median eminence (12 sections). For all dissections, the vertical axis of the dissecting tool was aimed at the midline, its bottom targeting the most ventral portion of the brain.

2.5. Aromatase activity assay

Micropunches or microdissections were respectively homogenized in 120 μ l or in 240 μ l of ice-cold TEK buffer (150 mM KCl, 1 mM Na-EDTA, and 10 mM Tris-HCl; pH 7.2) and stored at -80°C until assayed. AA was quantified by measuring the production of tritiated water associated with the conversion of [1! -³H]-androstenedione into estrone (Baillien and Balthazart, 1997; Roselli, 2001) as described previously (Cornil et al., 2011). Briefly,

samples were incubated in the presence of TEK buffer, ^3H -androstenedione (final concentration 25 nM, specific activity 23.6 Ci/mmol; Perkin-Elmer), and NADPH (4.8 mM) at 37°C for 15 min. The reaction was stopped by adding 2% activated charcoal in 10% trichloroacetic acid. Samples were then centrifuged to collect the supernatant (H_2O and $^3\text{H}_2\text{O}$) that was filtered through Dowex cation exchange columns. ^3H -water was quantified by adding Optiphase “Highsafe” 3 (Perkin Elmer) and counting for 3 min on a Wallac Winspectral 1414 Liquid Scintillation Counter. Inter- and intra-assay variation coefficients were below 10.2 and 4.3 respectively.

The protein content of each sample was assayed using the commercial Coomassie Plus Protein Assay Reagent (Pierce, Rockford, IL) and used to normalize the enzymatic activity of micropunched samples that was then expressed in pmol/h.mg protein after correction of the counts for quenching, recovery, blank values and percentage of tritium in ! -position in the substrate (see Baillien and Balthazart, 1997). Total AA in POM+BST and VMN+Tub was calculated by adding the AA expressed in pmol/h on the one hand and protein content of the two nuclei on the other hand and then dividing the summed AA by the summed protein mass. Total AA in the microdissected brain areas during Experiment 4 was not corrected for protein content because the size of the dissection exceeded the surface area of distribution of aromatase, so that the whole population of aromatase expressing cells had been collected. A correction by the total amount of protein would thus obscure any experimental effect on AA since the correction would use a denominator that does not relate in any way to the enzymatic measure.

2.6. Testosterone Enzyme Immuno assays (EIA)

Circulating concentrations of testosterone were assayed using an Enzyme Immunoassay (EIA) kit (Cayman Chemical Company, Ann Arbor, MI) which has been validated previously for use in quail (Dickens et al., 2011). The cross-reactivity of the testosterone antiserum as reported by the company is 140% with 19-Nortestosterone, 100% for testosterone, 27% for 5! -dihydrotestosterone, 19% for 5! -dihydrotestosterone and <5% for all other steroids. Testosterone was extracted from the protein component of the plasma using dichloromethane and dried at 37°C under a stream of nitrogen gas. Samples were reconstituted to a 1:20 dilution using EIA buffer supplied with the kit. Samples were assayed in duplicate and randomly assigned to one of six plates, which were all run in the same assay. For each plate, the intra-assay coefficient of variability was less than 3% and the coefficient of variation between plates was less than 10%. No samples had concentrations below the limit of detection that was below 0.15 ng/mL with samples diluted 1:20.

2.7. Statistical analysis

All data were analyzed with Statistica 10.0 (Statsoft, Inc.). The AA data were analyzed for each nucleus separately using one-way ANOVAs with AA level as the dependent variable and the experimental conditions as a categorical factor. When significant, the ANOVA was followed by Fisher protected least-significant difference post-hoc tests. A small number of data points were removed from the analysis when histological analyses revealed wrong anatomical location of punches, technical errors were noted during the enzymatic or protein assays or values were more than two standard deviations away from the mean for each group.

Behavioral frequencies recorded every minute were analyzed by repeated measures ANOVAs. By definition, the four experimental groups were subjected to different durations of stimulation, rendering a global analysis with all individuals impossible. Therefore, data were analyzed by four separate ANOVAs comprising a decreasing number of subjects as the stimulation duration increased (see Fig.1).

Spearman's correlation coefficients were used to analyze relationships between AA and latency of brain collection. Differences were considered significant for $p < 0.05$. All data are presented by their mean $\pm$ SEM.

3. Results

3.1. Experiment 1: Rapid changes in AA after copulation with a female

With the exception of controls that remained in their home cage and were not allowed to meet a female, all males copulated except two in the “2 min” group and 2 others in the “10 min” group. The analysis of copulatory frequencies over time indicates that these males, probably because they had gained extensive sexual experience during the pretests, were immediately active when placed in the presence of a female, exhibiting their highest copulatory frequency during the first minute. This frequency then rapidly decreased so that less than one behavior occurrence per min was observed after one (for CCM) or three minutes (for MA; Fig. 1A-B) of interaction. Statistical analysis by one-way ANOVAs of changes in behavioral frequencies over time revealed significant time effects (MA: 1-2 min: $F_{1,49} = 50.67$, $p < 0.001$; 1-5 min: $F_{4,140} = 33.87$, $p < 0.001$; 1-10 min: $F_{9,180} = 20.74$, $p < 0.001$; 1-15 min: $F_{14,70} = 4.94$, $p < 0.001$; CCM: 1-2 min: $F_{1,49} = 40.28$, $p < 0.001$; 1-5: $F_{4,140} = 24.82$, $p < 0.001$; $F_{9,180} = 16.39$, $p < 0.001$; 1-15 min: $F_{14,70} = 7.99$, $p < 0.001$). Such a decrease in male behavioral frequencies replicates a pattern found in prior studies (Hutchison, 1978).

Although plasma testosterone concentrations appeared to decrease after copulatory interactions, this trend was not significant ($F_{4,59} = 1.41$, $p = 0.241$; Fig. 2A).

Interactions with the female significantly reduced AA in the POM ($F_{4,60} = 2.75$, $p = 0.036$) and Tub ($F_{4,60} = 2.83$, $p = 0.032$), but not in BST and VMN (Fig. 3) nor in the two other nuclei (TnA and PAG) expressing lower enzymatic activity (AA lower than 4 pmol/h.mg prot; data not shown; all $F_{4,58} < 0.83$; $p > 0.509$). Post-hoc analyses indicated that in the POM this effect resulted from an enzymatic decrease of about 28% after 5 min compared to 0 and 2 min (Fig. 3A) that reached a maximal inhibition of 38% after 15 min. In the Tub, a marked decrease (-26%) was already detected after 2 min and persisted up to 15 min when it attained -42% of controls (Fig. 3E).

The analysis of total AA in POM+BST and VMN+Tub (MBH) considered as a single cluster of aromatase-expressing cells revealed significant enzymatic changes in the POM+BST ($F_{4,59} = 2.90$, $p = 0.029$; Fig. 3C). In the MBH, although a general decrease in AA was observed, this trend did not reach statistical significance ($F_{4,59} = 1.84$, $p = 0.134$; Fig. 3F). Post-hoc analyses performed on POM+BST revealed that animals that interacted with a female for more than 5 min had a lower AA (up to -20%) than those that did not or did but for a shorter duration.

No correlation was found between the time elapsed between the bird was killed and his brain was frozen and AA in the POM ($r = -0.162$; $n = 50$; $p = 0.260$), BST ($r = 0.004$; $n = 50$; $p = 0.976$), TnA ($r = 0.042$; $n = 50$; $p = 0.776$), VMN ($r = 0.130$; $n = 50$; $p = 0.377$), Tub ($r = 0.126$; $n = 50$; $p = 0.383$) and PAG ($r = 0.119$; $n = 50$; $p = 0.410$). This suggested that the dissection procedure was not having a significant impact on the level of brain AA that supposedly reflected the enzymatic activity present *in vivo*.

To summarize, copulation induced rapid and region-specific changes in AA. This effect was detectable within 2 min in the Tub and appeared slightly delayed in the POM. In both nuclei, the effect was still present, even stronger, after 15 minutes despite the dramatic decrease in the mean sexual activity indicating that most males were no longer engaging in behavior.

Therefore, based on these data, it remains unclear whether the reduced enzymatic activity results from slow and long lasting effects of copulation or simply depends on contextual cues provided by the continuous presence of the female and/or the test arena in which they had previously copulated. These questions were addressed in the follow-up experiments.

3.2. Experiment 2: Effect of 2 min of copulation on AA measured after different latencies

To investigate whether the rapid changes in AA detected in the POM and Tub are a consequence of the sexual activity displayed during the first 2 min, four groups were allowed to copulate for 2 min after which their brain was immediately collected or they were left undisturbed for different durations in the test arena from which the female had been removed. This waiting time in the empty arena was determined to match the interaction durations of the previous experiment when added to the 2 min of copulation. With the exception of one male from the “3 min” group and another from the “8 min” group, all males copulated and no differences in behavioral frequencies were detected between groups ($F_{3,41} = 0.47$, $p = 0.7065$).

Plasma testosterone concentration significantly decreased with time after this short interaction with the female ($F_{4,51} = 3.74$, $p = 0.010$). Specifically, the two groups that waited longer than 8 min showed reduced testosterone levels compared to controls (Fig. 2B).

No treatment effect was observed on AA in any nucleus ($F = 1.98$; $F = 0.111$; Table 1) with the exception of an increase in AA in the TnA ($F_{4,51} = 2.69$; $p = 0.041$) resulting from a higher activity after 3 and 13 min than 8 min and controls. Therefore, even if most sexual activity was observed during the first 2 min of interaction during Experiment 1, copulating for 2 min does not seem sufficient to induce a significant enzymatic change in the POM and Tub suggesting that the effects observed in the previous experiment are not linked to sexual performance *per se* or exposure to the environmental context where copulation occurred previously. Instead, it is possible that the reduction in aromatization rate simply results from the female's presence.

3.3. Experiment 3: Rapid enzymatic changes after viewing a female for various durations

To test this idea, another set of males was allowed to see a female for various durations without being able to physically interact with her. The analysis of the frequency of rhythmic cloacal sphincter movements (RCSM) over time confirmed that males immediately respond to the view of a female (Cornil and Ball, 2010). As for copulatory activity, RCSM frequency was the highest during the first minute of interaction and rapidly decreased over the first five minutes. Then, it slowly continued to decrease but did not completely disappear (Fig. 1C). Accordingly, the analysis by one-way ANOVAs of changes in behavioral frequencies over time revealed significant time effects (1-2 min: $F_{1,39} = 21.03$, $p < 0.001$; 1-5 min: $F_{4,116} = 20.69$, $p < 0.001$; 1-10 min: $F_{9,171} = 19.33$, $p < 0.001$; 1-15 min: $F_{14,126} = 5.57$, $p < 0.001$). No significant change in plasma testosterone concentrations was detected ($F_{4,59} = 1.11$, $p = 0.366$; Fig. 2C).

Although there was a consistent numerical decrease in AA measured in the POM after visual exposure to a female (except at the 10 min point), this effect was not significant most likely due to the absence of change at 10 min ($F_{4,44} = 1.83$; $p = 0.141$; Fig. 4A). Similarly, in the Tub, no treatment effect was detected despite a noticeable drop in AA after 10 and 15 min ($F_{4,44} = 1.08$; $p = 0.379$; Fig. 4E). However, a significant treatment effect was detected in the BST ($F_{4,44} = 3.18$, $p = 0.022$; Fig. 4B). Post-hoc analyses revealed that this effect resulted from a significant enzymatic decrease after 2 and 15 min compared to controls. AA measured after 10 min did not significantly differ from controls and consequently differed from 2 and 15 min. No treatment effect was detected in VMN (Fig. 4D) nor in the two other

nuclei TnA and PAG displaying much lower enzymatic activity ($AA < 4.5$ pmol/h.mg prot; data not shown, $F_{1,22}$, $p = 0.318$). It is important to note that in POM and BST the 10 min group showed a mean activity close to controls. Although it cannot be ruled out that the aromatase of these individuals did not respond to the female as in the other groups, it is more likely that this absence of overall effect in the ANOVA reflects a pre-existing higher aromatase content in this group.

As expected based on the response pattern of individual nuclei, when considered globally, AA significantly differed between treatments in POM+BST ($F_{4,44} = 3.86$, $p = 0.009$; Fig. 4C). Post-hoc analysis revealed an overall enzymatic decrease following 2, 5 and 15 min long visual exposures but not after 10 min. No change was detected in the MBH ($F_{4,44} = 0.74$, $p = 0.569$; Fig. 4F). Together, these results suggest that seeing a female for a period induces rapid and sustained changes in AA in the rostral part of the HPOA.

3.4. Experiment 4: Recovery of enzymatic activity

The enzymatic changes observed in previous experiments were prolonged and lasted at least as long as the female was present. These enzymatic decreases are compatible with a mechanism based on the degradation as well as on conformational changes of the enzyme. To tease these two options apart, males were allowed to copulate with a female for 5 min and their brain was immediately collected or birds were returned to their home cage for 115 min before brain collection. The assumption was that a recovery of basal activity levels within 2 hours would more likely result from changes in the enzymatic efficiency due to post-translational modifications (such as phosphorylations) of the enzymatic molecule rather than from new protein synthesis following copulation-induced protein degradation.

AA in these brains displayed a numerical decrease in both microdissected POM/BST and MBH after 5 minutes of interaction and a recovery to control levels after 2 hours. Groups significantly differed in the MBH ($F_{2,23} = 3.91$, $p = 0.035$; Fig. 5B). Post-hoc analysis indicated that this effect resulted from a significant reduction in samples collected immediately after copulation compared to controls ($p = 0.042$) and a tendency to recover in samples collected 2 hours after copulation initiation compared to samples collected immediately after copulation ($p = 0.060$). Due presumably to the larger individual differences possibly related to the different dissection procedure or lower statistical power, the qualitative differences detected in POM/BST were, however, not statistically significant ($F_{2,23} = 1.42$, $p = 0.2596$; Fig. 5A).

Because some males (3 in each experimental groups) did not copulate during the test session, we checked whether AA was affected by copulation. A hierarchical two-way ANOVA with AA as the dependent variable and the groups as the categorical predictor with copulation as dichotomous nested factor showed no significant effect of copulation in both nuclei (POM+BST, $F_{2,21} = 0.55$, $p = 0.587$; MBH, $F_{2,21} = 0.40$, $p = 0.673$). However, this hierarchical ANOVA confirmed the main effect of the experimental groups in the MBH microdissection but not in the "POM/BST" sample (POM/BST, $F_{2,21} = 1.37$, $p = 0.275$; MBH, $F_{2,21} = 3.70$, $p = 0.042$). Together, these data are thus consistent with the idea that AA is rapidly inhibited in the POM/BST and MBH after sexual interactions and that the enzymatic activity returns to control levels at least 2 hours after the female has been removed.

4. Discussion

This study was designed to determine the spatial and temporal pattern of changes in brain AA following sexual interactions, the nature of the stimuli driving these changes and their functional significance. The data confirm that sexual interactions induce a rapid AA

inhibition (Cornil et al., 2005) and demonstrate that these enzymatic changes occur in specific brain regions in a context-dependent fashion. In particular, preoptic enzymatic fluctuations are tied to the female's presence rather than to copulatory behavior *per se*, providing important insights into the function of this rapid enzymatic decrease. We will discuss the questions of “where”, “when” and “why” AA is changing in order.

“Where”

We demonstrate here that rapid AA changes occur in specific neuronal populations. As expected, enzymatic changes of larger amplitude than those measured in entire HPOA blocks (Cornil et al., 2005) were detected in microdissected nuclei after sexual interactions. The specific neuronal populations affected depend on the context to which subjects were exposed: copulation induces changes in the POM and Tub, while just seeing the female induces changes in the BST and possibly in POM. The POA and BST are key sites for the steroid-dependent activation of sexual motivation and copulatory behavior (Balthazart et al., 2009; Hull and Rodriguez-Manzo, 2009). Preoptic aromatization is critical for the control of copulatory behavior (Balthazart et al., 2004) but the precise functional significance of other aromatase cell groups is unknown. Although little is known about the role of aromatase in the BST, it was recently shown that BST aromatase-immunoreactive neurons are differentially activated following expression of both appetitive and consummatory sexual behavior (Xie et al., 2011). This corroborates our observation that, AA in BST varies only in response to the view of the female.

“When”

One component to answer this question relates to when AA changes occur with respect to the stimuli encountered and the behaviors displayed. Brain-derived estrogens are required for the short- and long-term activation of male sexual behavior (Cross and Roselli, 1999; Cornil et al., 2006a; Cornil et al., 2006b; Taziaux et al., 2007; Seredynski et al., 2012). It was thus hypothesized that rapid changes in preoptic AA would be associated with behavior initiation. However, the comparison of behavioral and enzymatic activities over time reveals that sexual activity ceased before preoptic AA declined, suggesting that the rapid enzymatic changes are the consequence rather than the cause of behavior. Yet, AA did not change in samples collected at different latencies after a brief period of interactions during which most of the copulatory activity occurs. This implies that enzymatic changes are not linked to sexual performance *per se*, nor to the environmental context, but require the female's presence for a longer duration. Moreover, copulating or simply seeing a female induces very similar enzymatic responses suggesting that enzymatic changes are not tied to behavior display but to the female's presence.

Another dimension of the answer concerns temporal resolution. AA changes initiated *in vivo* exhibit a time-course similar to the activity-dependent inhibition reported *in vitro* (Balthazart et al., 2006). Here, we show that AA changes even faster than previously observed (Cornil et al., 2005). The activity remains reduced as long as the interaction lasts and returns to pre-copulatory levels within 2 hours after the interaction ceased. Comparing Experiments 1 and 2 suggests that AA might return to baseline even faster, likely within minutes after female removal. Thus, rapid and localized changes in estrogen production occur in a time-scale compatible with the temporal resolution associated with their non-genomic actions (Cornil and Charlier, 2010; Roepke et al., 2011).

“Why”

This question refers to both the mechanisms regulating AA and their functional significance. We shall first consider the mechanisms and how they can generate changes on this time-scale. The rapid enzymatic inhibition following sexual encounters could reflect a diminution

of aromatase concentration or of its catalytic ability. Here, we show that inhibited AA returns to baseline within 2 hours after female removal. Protein synthesis is a relatively slow process. In castrates, preoptic AA only increases 20% within eight hours of testosterone treatment and 48 hours are needed to reach the maximal enzymatic activity (Balthazart et al., 1990). Enzymatic recovery in less than 2 hours (possibly even less, see above) is thus inconsistent with new protein synthesis. *In vitro*, AA is rapidly and reversibly down-regulated independently from protein degradation by phosphorylations induced by neuronal activity (Balthazart et al., 2003; Charlier et al., 2011). The rapidly reversible changes observed *in vivo* are thus likely mediated by the mechanisms described *in vitro* that are independent from enzyme degradation.

Rapid and reversible AA inhibitions by glutamate have been observed *in vitro* (Balthazart et al., 2006) and suggested *in vivo* (Remage-Healey et al., 2008). In male rats, sexual interactions are accompanied by glutamate release in the POA (Dominguez et al., 2006). Preoptic neurons, including aromatase neurons, are sensitive to glutamate (Karlsson et al., 1997; Cornil et al., 2004). AA down-regulation could thus arise from increased preoptic glutamate concentration following copulation and this mechanism should now be tested.

Regarding functional significance, one obvious hypothesis is that the fall in AA leads to a post-copulatory refractory period. However, the present data demonstrate that this decrease also occurs after visual exposure to a female, does not result from engaging in copulation *per se*, and follows the decline in copulatory activity. One can speculate that rapid actions of estrogens modulate on a short-term basis the expression of sexual motivation but that sexual performance is controlled by genomic actions of estrogens that adjust over the course of days and weeks the neurochemical circuit underlying this aspect of sexual behavior. Moment to moment changes in the expression of copulatory behavior *sensu stricto* would then be controlled by neurotransmitters such as dopamine.

This notion is consistent with a theory involving the interaction of sex steroids and neurotransmitter systems in the control of copulation in rodents (Hull and Dominguez, 2006). In this model, male sexual behavior depends on dopamine's action in the POA. Preoptic extracellular dopamine concentration increases in response to the view of a female (Bazzett et al., 1992; Hull et al., 1992). Importantly, this essential response for the activation of copulatory behavior depends on the recent presence of testosterone or its estrogenic metabolites (Hull et al., 1995; Putnam et al., 2003). These findings established that dopamine's action is downstream from the genomic action of estrogens in the cascade of physiological events regulating copulation. This is consistent with the observation that ER! knock-out mice do not copulate unless they are treated with the non-selective dopamine receptor agonist apomorphine (Wersinger and Rissman, 2000).

Recent studies established that, like in rats, dopamine is released in the POA of male quail exposed to a female (Kleitz-Nelson et al., 2010a; Kleitz-Nelson et al., 2010b). Importantly, this response only occurs in males that go on to copulate (Kleitz-Nelson et al., 2010a; Kleitz-Nelson et al., 2010b) and the extent of dopamine's rise is positively correlated with cloacal gland size which provides a sensitive measure of plasma testosterone concentrations and indirectly of its brain estrogenic metabolites. Dopamine action, itself under the genomic control of estrogens, thus seems to regulate copulatory performance in quail as it does in rat.

Together, these findings support the general scenario that short-term changes in motivation would be under the control of brain-derived estrogens but short-term changes in sexual performance would be under the control of transmitters such as dopamine. This would also fit in well with the observation that acute manipulations of estrogens action in the brain rapidly affect measures of sexual motivation but not or much less measures of sexual

performance (Cornil et al., 2006b; Seredynski et al., 2012). This theory is consistent with all available data but should obviously be tested experimentally.

In conclusion, this study demonstrates that fast changes in AA occur in specific brain regions, including the POM, after a sexual interaction with a conspecific partner. Strikingly, these changes are induced by the mere presence of a female rather than by the male's sexual performance. Together these data provide evidence that rapid and reversible changes in AA, presumably controlled by post-translational modifications of aromatase, occur *in vivo*. Importantly, such a control mechanism of brain estrogen provision offers (1) the spatial specificity required for the activation of neuronal circuits involved in behavioral control and (2) dynamic changes in a time-scale compatible with the non-genomic effects of estrogens. The pattern of AA changes observed is consistent with the view that rapid estrogen effects are important during appetitive sexual behavior to trigger physiological events that are essential for the activation of the mate search and copulation but that are no longer needed after the male actually starts engaging in behavior. Given the critical role played by aromatase in the control of steroid-dependent processes such as aggression and cognition, it is likely that this general observation extends to other behavioral systems.

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Figure 1.

MA (A), CCM (B) (Experiment 1) and RSCM (C) (Experiment 3) frequencies across time. Data of the four experimental groups within each experiment were pooled for statistical analysis based on the total duration of the test in each group (2, 5 10 or 15 min). Data from 4 groups are thus available for the first 2 min, from 3 groups for the first 5 min, from two groups for the first 10 min and from only one group for the total duration of 15 min, hence the decreasing numbers of subjects indicated at the top of the graphs. The probabilities above the graphs refer to the result of the one-way ANOVA of the behavioral frequencies with each of these different time bins (0-2, 0-5, 0-10 and 0-15 min).

Figure 2.

Concentrations of plasma testosterone (in ng/ml) measured (A) in control males and males allowed to copulate with a female for different durations (Experiment 1); (B) in control males and males allowed to copulate for 2 min before waiting in the absence of the female for different durations (Experiment 2); (C) in control males and males allowed to see a female for different durations (Experiment 3). * $p < 0.05$ vs. CTL.

Figure 3.

Effect of an interaction with a female during 0 (CTL), 2, 5, 10 or 15 minutes on male aromatase activity measured in (A) the medial preoptic nucleus (POM), (B) the medial part of bed nucleus of the stria terminalis (BST), (C) POM+BST, (D) the ventromedial nucleus of the hypothalamus (VMN), (E) the tuberal hypothalamus (Tub), and (F) VMN+Tub (MBH). Enzymatic activity expressed as percentage of the control group is indicated in each column. (*), * and ** $p < 0.1$, 0.05 and 0.01 respectively vs. CTL. (†), † and †† $p < 0.1$, 0.05, and 0.01 respectively vs. 2 min.

Figure 4.

Effect of viewing at a female during 0 (CTL), 2, 5, 10 or 15 minutes on male aromatase activity in (A) the POM, (B) the BST, (C) POM+BST, (D) VMN, (E) Tub, and (F) VMN/Tub (MBH). Enzymatic activity expressed as percentage of the control group is indicated in each columns. (*) and * $p < 0.1$ and 0.05 respectively vs. CTL. (+) and + $p < 0.1$ and 0.05 vs 10 min, respectively.

Figure 5.

Effect of a recovery time. AA in (A) the POM/BST and (B) the MBH following interaction with a female for 0 (CTL), 5min, and 5 minutes followed by 115 min of recovery. Note that these data were not corrected for protein content, which explains the difference in absolute values as compared to data in Figure 3 and 4. * $p < 0.05$ vs CTL. (†) $p < 0.1$ vs. recovery.

Table 1

Average aromatase activity ($\pm$ SEM; pmol/h.mg prot) measured in POM, BST, TnA, VMN, Tub and PAG as a function of the time between female removal and brain collection.

	CTL	0 min	3 min	8 min	13 min
POM	105.53 $\pm$ 15.61	109.07 $\pm$ 11.54	118.48 $\pm$ 19.82	113.69 $\pm$ 16.84	105.81 $\pm$ 13.75
BST	55.62 $\pm$ 6.74	65.58 $\pm$ 6.50	54.92 $\pm$ 7.06	48.39 $\pm$ 4.54	47.31 $\pm$ 3.42
TnA	6.43 $\pm$ 0.41 *	7.65 $\pm$ 0.48 (+)	8.28 $\pm$ 0.50	6.34 $\pm$ 0.38 *	7.88 $\pm$ 0.82
VMN	26.11 $\pm$ 1.56	23.03 $\pm$ 1.46	20.68 $\pm$ 1.47	22.11 $\pm$ 1.76	21.11 $\pm$ 1.53
Tub	25.77 $\pm$ 2.31	24.03 $\pm$ 1.91	25.63 $\pm$ 3.18	27.11 $\pm$ 3.67	23.14 $\pm$ 1.60
PAG	4.17 $\pm$ 0.50	4.57 $\pm$ 0.53	4.31 $\pm$ 0.67	3.63 $\pm$ 0.35	3.58 $\pm$ 0.45

* p < 0.05 vs. 3 min ; + and (+) p < 0.05 and p < 0.1 vs. 13 min.