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Steroid metabolism in the brain: From bird watching to molecular biology, a personal journey

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Abstract

Since Arnold Adolph Berthold established in 1849 the critical role of the testes in the activation of male sexual behavior, intensive research has identified many sophisticated neurochemical and molecular mechanisms mediating this action. Studies in Japanese quail demonstrated the critical role of testosterone action and of testosterone aromatization in the sexually dimorphic medial preoptic nucleus in the activation of male copulatory behavior. The development of an immunohistochemical visualization of brain aromatase in quail then allowed further refinement in the localization of the sites of neuroestrogens production. Testosterone aromatization is required for the activation of both appetitive and consummatory aspects of male sexual behavior. Brain aromatase activity is modulated by steroid-induced changes in the transcription of the corresponding gene but also more rapidly by phosphorylations processes. Sexual interactions with a female also rapidly regulate brain aromatase activity in an anatomically specific manner presumably via the release and action of endogenous glutamate. These rapid changes in estrogen production modulate sexual behavior and in particular its motivational component with latencies ranging between 15 and 30 minutes. Brain estrogens seem to act in a manner akin to a neurotransmitter or at least a neuromodulator. More recently, assays of brain estradiol concentrations in micropunched samples or in dialysis samples obtained from behaviorally active males suggested that aromatase activity measured *ex vivo* might not be an accurate proxy to the rapid changes in local neuroestrogens production and concentrations. Studies of brain testosterone metabolism are thus not over and will keep scientists busy for a little longer.

Keywords

Japanese quail; Aromatization; Testosterone metabolism; Sexual behavior; Preoptic area; Calcium-induced phosphorylations; Glutamate; Membrane-initiated steroid action

«For such a large number of problems there will be some animal of choice or a few such animals on which it can be most conveniently studied» (August Krogh 1929)

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(August Krogh 1929)

Introduction

My interest for birds started during my early childhood when I was trapping birds with my father during their autumn migration, an activity that was at the time very widespread in Belgium but became illegal (for good reasons) in the 1970's. I had at that time started studies in biology (then called zoology) and my interest for birds naturally transformed into a passion for bird watching and photography. At the end of my college years, I started a PhD project and quite naturally this research focused on birds. I was initially assigned a project attempting to determine the role of olfactory communication in the control of social behavior in ducks and I actually managed to collect experimental data suggesting the existence of pheromonal communication in ducks (Balthazart & Schoffeniels, 1979; Jacob *et al.*, 1979) but this topic became recognized and matured only several decades later (e.g., Hagelin *et al.*, 2003; Bonadonna & Nevitt, 2004; Hagelin, 2007; Balthazart & Taziaux, 2009)). During my first year of PhD, I faced however a problem that I had not anticipated, even if it was obvious: ducks are seasonal breeders and testing the contribution of pheromones to the control of sexual behavior was only feasible during a few months each year which left me unoccupied for long period of time. When the reproductive season ended, I therefore decided to try injecting testosterone to my male ducks to test whether this would activate sexual activity and thus allow me to analyze the effects of female odors on male behaviors. This experiment turned out to be very successful (Balthazart, 1974; Balthazart & Stevens, 1975) and over the years the endocrine controls of behavior occupied an increasing part of my scientific activity. Briefly summarized, this is how I became a behavioral endocrinologist...! even though this discipline was not represented in Belgium and only very scarcely in Europe.

Radioimmunoassays (RIA) for steroids were at that time beginning to generalize in biomedical laboratories and I took advantage of this new technology to analyze with a previously unmatched refinement the relationships between changes in plasma testosterone concentration and reproductive behaviors in captive male Rouen ducks (*Anas platyrhynchos*) (Balthazart, 1976; Balthazart & Hendrick, 1976; Balthazart *et al.*, 1977a). In collaboration with Dr. Jean Claude Hendrick (University of Liege) we also established at that time the first RIA for follicle-stimulating hormone (FSH) based on a heterologous antibody (Croix *et al.*, 1974) and combining this assay with the luteinizing hormone (LH) RIA developed a few years before by Brian Follett, Colin Scanes and Frank Cunningham (Follett *et al.*, 1972) we became capable for the first time to monitor in a longitudinal manner the changes in activity of the pituitary-gonadal axis across the annual or daily cycles and correlate them with behavior (Balthazart & Hendrick, 1976; Balthazart *et al.*, 1977a; Balthazart *et al.*, 1977b). Significant correlations between changes in time of hormones and behavior were identified (Balthazart & Hendrick, 1976; Balthazart *et al.*, 1977b) but to my surprise, I discovered on this occasion that individual differences in hormones, and in particular testosterone, concentrations did not relate to individual differences in behavior (Dessi-Fulgheri *et al.*, 1976; Balthazart *et al.*, 1977a; Ramenofsky, 1984)(Figure 1).

This absence of relationship had actually been suggested in an indirect manner before the advent of sensitive assays for steroid hormones. In a classical study, Grunt and Young had shown that individual differences in sexual behavior of guinea pigs (*Cavia porcellus*) disappeared following castration (sexual behavior progressively vanished) but were quickly re-established when castrated subjects were all treated with a standard dose of exogenous testosterone (Grunt & Young, 1953). This idea was confirmed in multiple subsequent studies and it was established that in general, individual concentrations of hormones correlate with the behavior of the subjects only during periods of rapid changes or social instability such as the beginning of the reproductive season or the establishment of a social hierarchy (see (Ramenofsky, 1984) for an early example in quail). At these times the individual correlations actually reflect different rates of adaptation to a new situation and are therefore indirectly correlations with changes across time.

This general existence of correlations in time but not across subjects between hormones and behavior caught my interest and actually turned out to be one of the topics that I investigated in different contexts during my entire career. I hypothesized that the lack of correlation reflected the existence of individual differences in sensitivity of the brain to steroid action. Two candidates were at that time (late seventies) the prime suspects as agents modulating this sensitivity: the concentration or affinity of steroid receptors and the activity of steroid metabolizing enzymes. I set out to test these ideas in avian models known for displaying a discrepancy between changes in behavior and in plasma concentrations of sex steroids.

During a post-doc at the Institute of Animal Behavior, I collaborated with Jeff Blaustein to test whether changes in progesterone receptors (PR) in the male brain could explain why progesterone activates incubation behavior in both female and male ring doves (*Streptopelia risoria*) but circulating progesterone concentrations remain stable during the entire breeding cycle in males whereas they increase before incubation in females (Silver *et al.*, 1974). We found that a 2-day treatment with testosterone was increasing PR concentration in the male brain making it potentially more responsive to a stable circulating concentration of the steroid (Balthazart *et al.*, 1980). We speculated that the peak in testosterone that follows the first encounter of the male with the female during the courtship phase (Feder *et al.*, 1977) was likely to induce this increase in PR during the “normal” breeding cycle, explaining why males will begin to incubate when the female lays her eggs after a few days of interaction. This increase of PR in the male brain was later confirmed to take place during the spontaneous breeding cycle in this species (Askew *et al.*, 1997).

Around that time, I came across a paper that was going to change my life or at least reorient my entire scientific career. Francesco Dessi-Fulgheri and his colleagues, Nazzareno Lucarini and Concetta Lupo di Prisco published a study demonstrating that differences in aggressive behavior in male mice were not correlated with a wide variety of measures of peripheral steroid production or concentration (including testicular or adrenal weight, plasma concentrations of testosterone or estradiol-17 β (E2), and gonadal or adrenal production of testosterone and androstenedione) but were in contrast significantly correlated with the concentration of E2 in the nuclear fraction of the whole brain homogenates (Dessi-Fulgheri *et al.*, 1976). This suggested that the concentration of E2 in the brain and thus probably its local production by aromatization of testosterone was the key factor determining individual

differences in behavior. This encouraged me to go to the laboratory of Professor Luciano Martini at the University of Milan where I learned in collaboration with Renato Massa techniques used for measuring this local metabolism of testosterone by brain cells and began testing whether this metabolism could control the behavioral action of testosterone (Balthazart *et al.*, 1979). The Japanese quail (*Coturnix japonica*) was selected as a model of choice to study these questions due to its wide availability, easy reproduction in the laboratory and aptitude to readily express a wide range of behaviors in captive condition (Ball & Balthazart, 2010).

Testosterone metabolism and behavioral effects of the metabolites

Work in the laboratory of Elizabeth Adkins-Regan at Cornell University had demonstrated that many actions of testosterone on reproductive behaviors in male quail, like in many other vertebrates, could be mimicked by its metabolites. In particular, two pre-copulatory behaviors, crowing and strutting were activated in castrated males by a systemic treatment with the non-aromatizable androgen 5 α -dihydrotestosterone (5 α -DHT) whereas the copulatory sequence *sensu stricto* was activated by E2 derived from testosterone aromatization eventually acting in synergy with 5 α -DHT (Adkins, 1977; Adkins & Pniewski, 1978; Adkins *et al.*, 1980; Adkins-Regan, 1981). We performed several experiments that confirmed and extended these findings by namely testing additional inhibitors of testosterone metabolizing enzymes as well as other specific agonists and antagonist of the androgen and estrogen receptors (Schumacher & Balthazart, 1983a; Balthazart *et al.*, 1984; 1985; Alexandre & Balthazart, 1986).

In a separate but related approach, we developed *in vitro* techniques to measure the activity of testosterone metabolizing enzymes based on the incubation of homogenates of brain samples with radioactive testosterone and then differential extraction and separation of the metabolites produced by thin layer chromatography (Balthazart *et al.*, 1983; Balthazart & Schumacher, 1984a; Schumacher *et al.*, 1984). These studies demonstrated that these metabolizing enzymes have a discrete anatomical distribution and that their activity changes as a function of the endocrine state of the animal and also its sex (Balthazart & Schumacher, 1984b; Schumacher & Balthazart, 1984; Balthazart & Schumacher, 1985; Balthazart, 1991).

Brain sites of testosterone action

In order to decipher the role of intracellular testosterone metabolism in the control of male sexual behaviors, it was first critical to identify the target structures where testosterone action plays a significant role in the activation of these behaviors. The neuroanatomical distribution of sex steroid receptors was at that time being identified for the first time by the technique of *in vivo* autoradiography (Sar & Stumpf, 1973; Grant & Stumpf, 1975; Morrell *et al.*, 1975) thus providing a list of potential target sites. In parallel, the technique of stereotaxic brain lesions and stereotaxic implantation of steroids in the brain had been implemented in mammalian studies (Lisk, 1962; Michael, 1962; Harris & Michael, 1964; Lisk, 1967) and its use in the study of male sexual behavior had identified the critical role of the medial part of the preoptic area (mPOA) in mammals (Davidson, 1966; Lisk, 1967),

birds (Barfield, 1969; Hutchison, 1971) and other vertebrate classes (see (Kelley & Pfaff, 1978) for review).

Following the discovery of a gross neuroanatomical sex difference in the volume of song control nuclei in canaries (*Serinus canaria*) and zebra finches (*Taeniopygia guttata*) (Nottebohm & Arnold, 1976), Roger Gorski and his collaborators re-investigated the structure of the mPOA of rats and identified a similar sex difference in the volume of a tiny nucleus located in this brain region that was 4–5 times larger in males than in females; they called this structure the sexually dimorphic nucleus (SDN-POA) (Gorski *et al.*, 1978). This led me to initiate a collaboration with Gian Carlo Panzica and Carla Viglietti-Panzica (University of Torino, Italy) and together we quantified the volume of various brain structures in Nissl-stained brain sections of male and female quail. We discovered in the mPOA a nucleus that was significantly larger in males than in females (Panzica *et al.*, 1987; Panzica *et al.*, 1996) that we named medial preoptic nucleus (POM) by homology to the similar nucleus previously described in the pigeon brain (Berk & Butler, 1981).

The quail POM is not homologous to the rat SDN-POA. The sexually differentiated quail nucleus is much larger than the rat SDN-POA, the sex difference in its volume is smaller (about 20 vs. 4–500%) and most importantly the sex difference in its volume is not induced by the exposure to a differential endocrine environment during ontogeny like in rats (Jacobson *et al.*, 1980; Jacobson *et al.*, 1981) but rather by the higher testosterone concentration of testosterone in adult males compared to adult females (Panzica *et al.*, 1987; Panzica *et al.*, 1991; Thompson & Adkins-Regan, 1992) (see (Panzica *et al.*, 1996; Balthazart & Ball, 2007) for review).

Androgen receptors had also been specifically identified in the quail mPOA by *in vivo* receptor autoradiography (Watson & Adkins-Regan, 1989b). A suite of experiments by Elizabeth Adkins-Regan and James Watson showed that testosterone propionate implants targeting the mPOA were able to activate copulatory behavior in castrated quail (Watson & Adkins-Regan, 1989a). These effects were mimicked by implants of estradiol benzoate (Watson & Adkins-Regan, 1989a). Furthermore effects of these testosterone propionate implants were blocked by a systemic treatment with an aromatase inhibitor thus indicating that the androgen had to be aromatized in this brain region in order to be behaviorally active (Watson & Adkins-Regan, 1989c).

Due to its sensitivity to testosterone in adulthood, the quail POM appeared as a likely site of testosterone action on male behavior within the mPOA. In parallel with the ongoing work of the Adkins-Regan laboratory, we had therefore initiated studies specifically focusing on this nucleus. This work demonstrated that lesions of the POM but not of adjacent regions of the mPOA delay or completely block activation of copulatory behavior by exogenous testosterone (Balthazart & Surlmont, 1990b). In parallel, very small stereotaxic implants of testosterone specifically targeting the POM were shown to activate copulatory behavior in castrates if and only if they were positioned within the cytoarchitectonic boundaries of the nucleus. Implants that were only 200–300 μm outside this nucleus were behaviorally ineffective (Balthazart & Surlmont, 1990b). Further studies demonstrated that implantation of an aromatase inhibitor specifically in the POM, but not outside, inhibits the activating

action of peripheral testosterone on behavior and additional experiments using estrogens, non aromatizable androgens as well as aromatase inhibitors, antiestrogens and antiandrogens confirmed that effects of testosterone on copulatory behavior are to a large extent mediated by estrogens derived from local aromatization of testosterone specifically in the POM (Balthazart *et al.*, 1990a; Balthazart & Surlemont, 1990a).

Together, all these studies demonstrate that the actions of testosterone and its aromatized metabolites in the POM are necessary and sufficient to activate male copulatory behavior in castrated quail when an adequate sexual female stimulus is present. This conclusion does not however preclude other areas from being implicated in this process as discussed in a few review papers that were previously published (see (Balthazart, 1989; Ball & Balthazart, 2004; Wild & Balthazart, 2013).

Testosterone induces brain aromatase: functional implications

Biochemical and molecular controls of brain aromatase activity (AA) were at that time poorly understood but in 1981 John Hutchison and Thierry Steimer (Cambridge University, UK) had discovered that treatment for a few days with testosterone dramatically increased (200–300%) the preoptic AA (Steimer & Hutchison, 1981) in ring doves (*Streptopelia risoria*), a finding that was later confirmed in rats (Roselli *et al.*, 1984; Roselli & Resko, 1984). Kinetic studies further suggested that the increased AA observed in doves was the result of an increased maximum velocity of the enzyme but there was not change in its apparent affinity of its substrate thus suggesting that the enzymatic change resulted for an induction (increased synthesis) of the enzyme rather than a change in its catalytic properties.

Several studies had demonstrated that the preoptic AA is significantly higher in males than in females of several species (e.g. (Roselli *et al.*, 1984)) including quail (Schumacher & Balthazart, 1984). There was also an extensive literature showing that testosterone readily activates copulatory behavior in castrated male quail but is behaviorally ineffective in ovariectomized females (Adkins, 1975; Balthazart *et al.*, 1983; Schumacher & Balthazart, 1983a). Given the established critical role of aromatase expressed in the POM in the activation of male copulatory behavior (Watson & Adkins-Regan, 1989c; Balthazart *et al.*, 1990a; Balthazart & Surlemont, 1990a), we wondered whether the sexually differentiated behavioral responses to testosterone were not caused by a differential basal expression and/or induction by testosterone of AA in the POM. *In vitro* radioenzymatic assays of microdissected hypothalamic regions demonstrated that indeed AA is 5–6 times higher in sexually mature males than in females and this difference is maximal in the microdissected region corresponding to the mPOA (Schumacher & Balthazart, 1986). Interestingly AA decreased to basal levels in males and females after gonadectomy but treatment with exogenous testosterone restored AA to its pre-castration / ovariectomy level including the sex difference in enzymatic activity (Schumacher & Balthazart, 1986). Assays performed on POM samples specifically dissected by the Palkovits punch technique (Palkovits, 1973) demonstrated that the preoptic AA is almost exclusively located within the boundaries of the POM (Schumacher & Balthazart, 1987). The decrease of AA following castration and restoration by testosterone was confirmed to take place in the POM samples dissected by the micropunch technique and this study also confirmed that AA in POM was higher in

gonadally intact males than in gonadally intact females although this sex difference was no longer present in gonadectomized subjects treated with testosterone (Balthazart *et al.*, 1990e).

Globally, these data were thus consistent with the idea that a low AA could be responsible of the lack of activation by testosterone of male-typical copulatory behavior in female quail. This is however inconsistent with the fact that treatment of ovariectomized females with exogenous E2, which should bypass the rate-limiting step constituted by aromatase, is still unable to activate male typical copulatory behavior in females (Schumacher & Balthazart, 1983b). Other limiting factors must therefore be present.

Cellular and subcellular distribution and regulation of aromatase

We found in 1990 that one antiserum raised against human placental aromatase (Harada, 1988) reliably identifies aromatase-expressing neurons in the brain of quail and other avian species (Balthazart *et al.*, 1990b; Balthazart *et al.*, 1990d). Cloning of the quail aromatase messenger RNA (mRNA) subsequently allowed the development of antibodies to a specific recombinant quail aromatase (Foidart *et al.* 1995). With these tools it became possible to analyze very precisely the neuroanatomical distribution of the enzyme and its control by steroids, and to define (by double-labeling studies) the neurochemical phenotype of aromatase-immunoreactive (ARO-ir) cells and the nature of their afferent inputs (for detailed reviews, (Panzica *et al.*, 1996; Absil *et al.*, 2001a; Balthazart *et al.*, 2004)). This demonstrated, among other things, that most if not all ARO-ir neurons of the preoptic area are located within the POM and actually allow defining the cytoarchitectonic boundaries of the nucleus (Foidart *et al.*, 1995; Balthazart *et al.*, 1996).

Based on the sequence of the quail aromatase (Harada *et al.*, 1992; Balthazart *et al.*, 2003) specific primers were also designed to quantify this mRNA by either reverse transcription polymerase chain reaction (RT-PCR) (Harada *et al.*, 1992; Harada *et al.*, 1993) or *in situ* hybridization (Aste *et al.*, 1998; Voigt *et al.*, 2009; 2011). This made it possible to study aromatase in the quail brain at three different levels: the concentration of the mRNA, the concentration of the enzymatic protein assessed semi-quantitatively by counting in standardized conditions the numbers of ARO-ir cells, and the level of enzymatic activity quantified by *in vitro* assays, either the product formation assay (Schumacher & Balthazart, 1986) or the tritiated water assay (Baillien & Balthazart, 1997). These three approaches demonstrated that testosterone increases AA in the POM mostly by increasing the concentration of the enzyme and its mRNA, suggesting that the steroid directly regulates transcription of the aromatase gene (for review: (Balthazart & Foidart, 1993; Balthazart & Ball, 1998; Absil *et al.*, 2001a)).

The steroid specificity of the effect of testosterone on AA was also assessed at these three levels of analysis. A treatment with estrogens reproduced almost entirely the effects of testosterone at the mRNA, protein, and enzymatic levels (Absil *et al.*, 2001a) whereas nonaromatizable androgens such as 5 α -DHT had almost no effect. There was however a clear synergism between androgens and estrogens in the activation of aromatase: inactive doses of 5 α -DHT markedly increased the effects of estrogens on levels of aromatase mRNA,

protein, and activity as also observed in the activation of copulatory behavior (Absil *et al.*, 2001a).

Immunocytological labeling of aromatase indicated that the enzymatic protein is largely located in the perikarya of positive neurons but was also filling the full length of cellular processes including dendrites and fine processes that looked like axons (Balthazart *et al.*, 1992; Balthazart & Foidart, 1993). Analysis of this material at the electron microscopic level confirmed the presence of immunoreactive aromatase in the axons and pre-synaptic boutons in the quail but also in the African green monkey (*Cercopithecus aethiops*) and human brain (Naftolin *et al.*, 1996). This conclusion was confirmed and extended to the finch brain where it could additionally be shown that the presynaptic aromatase is differentially expressed in the male and female brain (Rohmann *et al.*, 2007).

Time course of induction

The activation of copulatory behavior by testosterone was known to be a relatively slow process taking at least a few days to develop (Balthazart *et al.*, 1990c). If the behavioral activation is mediated at the cellular level by estrogens derived from local aromatization in the POM, it could then be expected that the induction of AA in the POM should precede the activation of behavior. A time course study accordingly demonstrated that castrates treated with exogenous testosterone display an increase in preoptic AA after only 8 hours (50% increase on average) that becomes statistically significant after 16 hours even if the full induction is only observed after 2 days (Figure 2A). The first evidence of weak sexual activity was in contrast observed after 24 hours but only became statistically significant after 4 days (Figure 2B) (Balthazart *et al.*, 1990c). Furthermore, blocking aromatase activity by a treatment with the aromatase inhibitor ATD (androstrienedione) delayed behavioral activation (Balthazart *et al.*, 1990c). Data were thus entirely consistent with the notion that an increased transcription of the aromatase gene is one of the first steps leading to the activation of male copulatory behavior by testosterone.

More recently, the availability of a reliable immunohistochemical visualization of brain aromatase in quail allowed us to revisit this question focusing more precisely on the POM. We asked whether testosterone-dependent induction of aromatase in POM specifically could be observed at short latencies. Brains from castrated male quail were collected after 1, 2, 7 and 14 days of testosterone treatment (CX+T) and compared to brains of untreated castrates (CX) collected after 1 or 14 days. POM volumes defined either by Nissl staining or by the cluster of aromatase-positive neurons increased in a time-dependent fashion in CX+T subjects and almost doubled after 14 days of treatment with testosterone while no change was observed in CX birds. A significant increase in the average POM volume was already detected after only one day of testosterone treatment. The optical density of Nissl and aromatase staining as well as the fractional area covered by ARO-ir material (Figure 2C, E) was also increased after one or two days of testosterone treatment. Activation of male copulatory behavior followed these morphological changes with a latency of approximately one day (Figure 2D). This rapid neurochemical and neuroanatomical plasticity observed in the quail POM thus presumably limits the activation of male sexual behavior and offers an

excellent model to analyze features of steroid-regulated brain structure and function that determine behavior expression (Charlier *et al.*, 2008).

Rapid inhibition of preoptic aromatase *in vitro*

Studies on brain homogenates and cell cultures

The activity of an enzyme can change in two fundamentally different ways: via modifications of its concentration often resulting from an increased transcription or alternatively via changes in its catalytic properties resulting for example from phosphorylations of the enzymatic protein (Hemmings *et al.*, 1989; Nestler & Greengard, 1999). We wondered whether the preoptic AA would be affected by changes in phosphorylating conditions associated with alterations of the concentrations of adenosine triphosphate (ATP) and divalent cations (Ca^{2+} and Mg^{2+}) as had been previously described for other enzymes (Albert *et al.*, 1984) and suggested for aromatase itself (Hochberg *et al.*, 1986; Onagbesan & Podie, 1989; Steimer & Hutchison, 1991).

A profound inhibition of AA was observed in quail preoptic homogenates that had been pre-incubated with increased but physiological concentrations of ATP, Mg^{2+} and Ca^{2+} . This inhibition was blocked by agents that chelate divalent ions such as EGTA or EDTA or by kinase inhibitors indicating that the enzymatic inhibition was indeed caused by Ca^{2+} -dependent phosphorylation processes (Balthazart *et al.*, 2001; Balthazart *et al.*, 2003).

Analysis of the quail aromatase sequence demonstrated the presence of multiple phosphorylation consensus sites ($n=15$) including two sites (threonine 455 and 486) corresponding to the specificity of protein kinases A and C that had been shown in pharmacological experiments to be implicated in the inhibition of AA by phosphorylation processes (Balthazart *et al.*, 2003).

More recently we also demonstrated that a similar rapid inhibition of AA is observed in quail ovary homogenates and in various cell lines (HEK293, Neuro2A and C6) transfected with human aromatase. Enzymatic activity was also rapidly inhibited following depolarization of aromatase-expressing HEK293 cells with 100 mM KCl and activity was fully restored when cells returned to control conditions (Charlier *et al.*, 2011a). Western blot analysis demonstrated that the reduction of enzymatic activity is not due to protein degradation and results from the phosphorylation of the enzymatic protein itself and not from the phosphorylation of another regulatory protein that would secondarily modulate AA. The transfer of HEK293 cells expressing human aromatase in phosphorylating conditions that inhibit the enzyme activity indeed enhanced the incorporation of P^{32} -ATP and the concentration of phospho-serine on the Western blot band that is immunoreactive for aromatase (Charlier *et al.*, 2011a). Attempts to identify by site-directed mutagenesis the specific phosphorylation sites on the aromatase protein that are affected by these phosphorylating conditions did however not meet with success. Mutation to alanine of the amino acids S118, S247, S267, T462, T493 or S497, alone or in combination, did not block the rapid inhibition of enzymatic activity induced by phosphorylating conditions but basal AA was markedly decreased in the S118A mutant. Altogether, these results demonstrate that the rapid inhibition of AA is a widespread and fully reversible process and that

phosphorylation of specific residues modulate AA. These experiments thus identified a new general mechanism by which local estrogen concentrations could potentially be rapidly altered in the brain and other tissues (Charlier *et al.*, 2011a).

Studies on preoptic *in vitro* explants

To evaluate the physiological relevance of these mechanisms in a biological system that would be closer to an intact brain, we also established an culture system in which one half of the preoptic/hypothalamic block (HPOA) could be maintained *in vitro* for a few hours and its aromatase activity dynamically quantified by the measure (every 5 or 30 min) of the release of the tritiated water produced during aromatization of [$1\beta^3$ -H]-androstenedione (Balthazart *et al.*, 2001). In these explants, AA reached steady state conditions after approximately 20 min. The enzymatic activity then remained relatively stable for several hours and effects of various experimental manipulations in one half HPOA block could be tested against a stable baseline measured in the other half HPOA (Balthazart *et al.*, 2001; Balthazart *et al.*, 2003). Modulations of the intracellular Ca^{2+} concentration by a K^+ -induced depolarization or addition of thapsigargin, a lactone known for its capacity to mobilize intracellular pools of Ca^{2+} , inhibited in a rapid (within 5 min) and reversible manner AA in these explants (Balthazart *et al.*, 2001).

Calcium release can also be triggered by glutamate. Additional experiments demonstrated that a transient addition (for 10 min) to the incubation medium of glutamate agonists such as -amino-methyl-4-isoxazole propionic acid [AMPA], kainate or, to a lower extent, N-methyl-D-aspartic acid [NMDA] significantly depress AA in these explants. These effects were fully reversible and the kainate-induced inhibition of AA was blocked by the preincubation with antagonists of the receptor, NBQX (1,2,3,4-tetrahydro-6-nitro-2,3-di-oxo-benzo[f]quinoxaline-7-sulfonamide disodium) and CNQX (6-cyano-7-nitroquinoxaline-2,3-dione disodium) (Balthazart *et al.*, 2006). These effects are presumably mediated by direct actions of the transmitters on aromatase expressing cells since electrophysiological studies showed that most ARO-ir cells in the POM are directly sensitive to AMPA, kainate and NMDA (Cornil *et al.*, 2004). AA in these explants is also rapidly and reversibly affected by dopaminergic agonist and antagonists but the specificity of the mechanisms underlying these effects remains poorly understood at present (Balthazart *et al.*, 2002).

These experiments demonstrated that *in vitro* AA can be rapidly regulated in brain tissue as a function of changes in neurotransmitter activity presumably acting via calcium-dependent phosphorylations of the enzyme. It remained however to be seen whether such rapid changes in AA were taking place *in vivo*.

Socio-sexual stimuli and restraint stress modulate aromatase activity in live subjects

Effects of socio-sexual interactions

To test this idea, we first investigated whether aromatase activity is affected immediately following expression of sexual behavior. Adult male quail were given visual access to or copulated with a receptive female for 1, 5 or 15 min. Their brain was then immediately

collected and AA was quantified in the HPOA by the tritiated water assay (Roselli & Resko, 1991; Baillien & Balthazart, 1997). A decrease in enzymatic activity was already detectable after 1 min of interaction with the female; this decrease reached a maximum after 5 min and then returned to control levels after 15 min of interaction (Cornil *et al.*, 2005). This drop in enzymatic activity had a smaller amplitude ($\pm 15\%$) than that observed *in vitro* but could still potentially influence physiological and behavioral processes.

Aromatase-positive cells are however clustered into several discrete groups leaving a large amount of aromatase-negative space in the HPOA (Foidart *et al.*, 1995). Furthermore, these different cell groups are differentially implicated in the control of sexual behavior and thus possibly experience different modulations of AA following sexual interactions. The limited changes in enzymatic activity observed *in vivo* might thus reflect a dilution of more prominent effects located in discrete regions by non responsive tissue or even the summation of changes occurring in opposite directions. This idea was tested in follow-up experiments that quantified changes in AA following sexual interactions in specific nuclei of the HPOA dissected by the Palkovits punch technique (Palkovits, 1973) adapted for quail brain (Schumacher & Balthazart, 1987; Cornil *et al.*, 2011a).

Male quail were allowed to freely interact with a sexually mature female or just see that female for various durations (0, 2, 5, 10 or 15 min) and AA was quantified in six microdissected brain regions: the medial preoptic nucleus (POM), the bed nucleus of the stria terminalis (BST), the ventromedial (VMN) and tuberal hypothalamus, the nucleus taeniae of the amygdala and the periaqueductal gray (de Bournonville *et al.*, 2013). A significant decrease in AA was already detected after 2 min of interaction (-25%) in the tuberal hypothalamus. After 5 min, a marked decrease was detected in the POM. Both effects progressively developed for at least 15 min at which point the maximal inhibition of activity reached 40% in the POM and tuberal hypothalamus. No significant change was detected in the other brain region although a transient average decrease was seen in the BST. In contrast, visual interaction with the female (no physical contact) resulted in a significant reduction of enzymatic activity in the BST only even if average decreases were also seen in the POM. Enzymatic activities were back to baseline two hours after the interactions (de Bournonville *et al.*, 2013).

Effect of stress

Another set of experiments investigated whether stress and the associated rise in plasma corticosterone also acutely influence brain aromatase activity. It is generally assumed that activation of the stress response system rapidly affects reproductive processes including behavior (Wingfield & Sapolsky, 2003). Since corticosterone had been shown to acutely reduce glutamatergic transmission in hypothalamic neurons (Di *et al.*, 2009) whereas copulation increases extracellular glutamate concentration in male rats (Dominguez *et al.*, 2006), based on the *in vitro* work summarized in the previous section, it was anticipated that if stress affects AA it would be in the opposite direction of the change induced by copulation.

In a preliminary experiment we exposed male quail to acute stress either by placing them in a small opaque bag for 15 min or injecting them with corticosterone 30 min prior to brain

collection. Control groups consisted of birds left undisturbed in their home cage or injected with the vehicle. Brains were collected immediately after the procedure and their HPOA dissected for enzymatic assays. As hypothesized, both these manipulations induced a significant rise in aromatase activity (Balthazart *et al.*, 2009).

The time course, regional and sex-specificity of this enzymatic response was then examined. Males and females were exposed to acute restraint stress for 0, 15 or 30 min and an additional group of birds was exposed to 30 min of stress followed by 30 min of recovery (return in home cage) to assess the reversibility of the effects. AA was then assayed in the six micro-dissected brain nuclei described above. The time-dependent effects of stress on AA were sex- and region-specific (Dickens *et al.*, 2011). In males, a rapid up-regulation of AA was detected in the POM (within 5 min) and in the VMN (significant at 15 min) after the initiation of stress and enzymatic activity returned to baseline at the 60 min time point i.e. after the 30 min recovery. In contrast, females exhibited a moderate increase in aromatase activity in the POM but a very profound enzymatic down-regulation in the tuberal hypothalamus that did not recover even at the 60 min time point after 30 min or recovery in their home cage.

These stress-induced changes in AA were however not correlated with circulating corticosterone concentrations, in all brain regions considered (Dickens *et al.*, 2011). Additional experiments were therefore conducted to test whether other stress hormones including arginine-vasotocin (AVT) and corticosterone-releasing factor (CRF) were mediating the enzymatic changes induced by acute stress. In males, AA in the POM responded to changes in corticosterone (at high presumably supra-physiological levels), as well as changes in AVT and CRF. In contrast, AA in the female POM appeared to increase only in response to increases CRF although more work would be needed to confirm the negative results observed with the other hormones (Dickens *et al.*, 2013).

Interestingly, when presented in sequence, sexual interactions reversed the stress-induced up-regulation of aromatization in the male POM, suggesting that copulation may rapidly restore the pre-stress enzymatic activity to actively preserve sexual behavior. Likewise, in the female tuberal hypothalamus sexual interactions seem to “rescue” basal enzymatic activity that had been decreased by acute stress. Surprisingly, behavioral endpoints (rates of copulatory interactions) were unaltered by exposure to the acute stress before behavioral testing although fertilization was reduced in females that had been stressed before copulation (Dickens *et al.*, 2012).

Changes in AA are often considered as a proxy of changes in the concentration of estrogens. If this notion is fairly well established when looking at long term (e.g. seasonal) changes, there was some suspicion that the correlation between the two variables might not be as good as expected when considering short-term changes (e.g. see (Charlier *et al.*, 2011b)). Utilizing two types of stimuli (sexual interactions and acute restraint stress) that had been demonstrated to reliably alter AA within minutes in opposite directions, we tested in Japanese quail whether rapid changes in AA are paralleled by changes in E2 concentrations in discrete brain areas (Dickens *et al.*, 2014). In males, E2 concentration in the pooled medial preoptic nucleus/medial 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 concentrations in POM/BST and measurements of female sexual behavior suggested a role for local E2 synthesis in female receptivity and specific tests of this idea provided supporting evidence (de Bournonville *et al.*, 2016a). These data thus highlight the complex mechanisms regulating local E2 concentrations in the brain and their rapid changes. Potential reasons explaining the discrepancy between changes in AA and in local E2 concentrations are presented in the concluding section of this review.

Glutamate modulates aromatase activity during copulation

Experiments in the previous section had demonstrated that in the male quail brain AA is rapidly inhibited within minutes in discrete brain regions, including the medial preoptic nucleus (POM), following copulation with a female (de Bournonville *et al.*, 2013). Earlier studies had also indicated that glutamate rapidly inhibits AA in preoptic-hypothalamic explants maintained *in vitro* in culture (Balthazart *et al.*, 2006). Given that (a) glutamate receptors are expressed in regions of the avian brain containing aromatase cells (Cornil *et al.*, 2000; Saldanha *et al.*, 2004), (b) the quail preoptic aromatase cells themselves are sensitive to AMPA, kainate and NMDA (Cornil *et al.*, 2004), (c) glutamate is released in rat medial preoptic area (MPOA) during sexual behavior (Dominguez *et al.*, 2006) and finally (d) infusion of glutamate in the auditory telencephalic region of zebra finches containing aromatase leads to a transient decrease of estradiol concentration (Remage-Healey *et al.*, 2008), we decided to investigate whether the rapid changes in AA observed after copulation are mediated by the release of endogenous glutamate.

A first group of experiments showed that the unilateral acute injection of the glutamatergic agonist kainate into the POM of anesthetized male quail inhibits after 20 minutes AA on the injected side as compared to the contralateral side that is either not injected or injected with the vehicle (de Bournonville *et al.*, 2017). A second group of experiments then measured by *in vivo* dialysis variations of glutamate concentration in the POM of male quail while they were copulating with a female. A clear increase in extracellular glutamate concentration was observed in birds that had the dialysis probe located in the POM and who actively copulated with the female. Detailed analysis of the glutamate profiles also indicated that the glutamate concentration increased most markedly in the 3 min dialysis sample that was collected immediately after copulation. These increases were however not seen if birds did not copulate or if their probe was located outside the boundaries of the POM (de Bournonville *et al.*, 2017). Together these *in vivo* data strongly suggest that glutamate release mediates the changes in AA observed in the POM after males have copulated with a female.

In summary, these experiments clearly demonstrate that sexual interactions decrease while acute stress increases AA in the POM of male quail. These acute responses of AA to stress and mating importantly show a clear anatomical specificity. In some regions, such as the male POM or the female tuber, aromatase seems responsive to both stimuli. However, the

enzyme appears exclusively sensitive to either mating or stress in other regions. Although the significance of these rapid modulations in brain AA, that take place in directions opposite to what would be naively expected, still remains to be determined, these data clearly demonstrate that *in vivo* aromatase activity is rapidly modulated in behaviorally relevant situations and in specific brain areas. This consequently raises the question of the function of these rapid changes in estrogen production and thus potentially in bioavailability of estrogens.

Functional significance: rapid behavioral effects of neuroestrogens

Experiments described in the previous section suggested that changes in the local bioavailability of estrogens should take place in the brain within minutes or even seconds, i.e., in a time frame that is compatible with the induction of rapid membrane-initiated effects of estrogens on cell physiology. Estrogens have indeed been shown to modify the electrical activity of neurons within seconds after their application which rules out the possibility of a classic steroid receptor-mediated effect on protein synthesis (for additional discussion see: (Schumacher, 1990; McEwen, 1994; Ramirez *et al.*, 1996; McEwen & Alves, 1999; Kelly & Ronnekleiv, 2002)).

When we initiated these studies, a handful of experiments only had identified rapid effects of estrogens on behavior (see (Cornil *et al.*, 2012) for review). In particular, one study on rats had demonstrated effects of E2 injections on the expression of male sexual behavior after latencies of 20–30 min (Cross & Roselli, 1999). We therefore decided to test whether the rapid changes in brain AA that had been identified in the experiments summarized above would have an impact on the expression of sexual behavior in male quail.

A first experiment tested whether a single injection of E2 would rapidly increase the expression of sexual behavior in castrated male quail primed with a sub-threshold dose of testosterone that is unable to restore this behavior by itself. A positive response was obtained: the injection of 0.5 mg/kg of E2 increased the rate of mount attempts and cloacal contact movements in tests performed 15 min later but the effect was no longer significant at 30 min (Figure 4A)(Cornil *et al.*, 2006a).

A second set of experiments used a reverse approach and tested whether the systemic inhibition of AA would rapidly affect the expression of sexual behavior in gonadally intact males or castrated males chronically treated with a high concentration of testosterone. A single injection of the non-steroidal aromatase inhibitor Vorozole™ decreased within 30–45 min the frequencies of copulatory behaviors (Cornil *et al.*, 2006b) as well as the expression of two measures of appetitive sexual behavior known to depend on testosterone aromatization: the learned social proximity response (LSPR) and the frequency of rhythmic cloacal sphincter movements (RCSM) (Ball & Balthazart, 2010). Effects on these measures of appetitive behavior were actually more important than effects on measures on the copulatory behavior itself. The decrease in RCSM frequencies was significant within 30–45 min and was no longer present 15 min later. The steroidal aromatase inhibitor androstatrienedione (ATD) that is acting by a different mechanism induced a similar but weaker inhibition of RCSM frequency (Cornil *et al.*, 2006b).

All these rapid effects of the manipulation of E2 availability had however a limited amplitude and were not fully reproducible (Cornil *et al.*, 2006b). Additional studies therefore tested whether more prominent effects would be observed after treatments modulating estrogens availability by injections through an intracerebroventricular (i.c.v.) cannula located in the third ventricle and thus in the close proximity to the POM.

Castrated males were first chronically treated with testosterone associated with i.p. injections of Vorozole™ administered twice daily for the duration of the experiment. This treatment was designed to allow expression of the full range of androgenic effects while profoundly limiting the possibility of estrogenic effects. As predicted by previous work (Taziaux *et al.*, 2004), this treatment nearly eliminated copulatory behavior and reduced RCSM frequency. The central administration of E2 to these males chronically deprived of estrogens increased within 15 min the expression of sexual motivation, as assessed by the two measures of appetitive sexual behavior (LSPR and RCSM) produced in response to the visual presentation of a female. However sexual performance (i.e. copulatory behavior *sensu stricto*) was not affected in the small arenas where tests took place (Seredynski *et al.*, 2013) suggesting that the slower transcriptional activity of estrogens is required to activate this aspect of sexual behavior (Vasudevan *et al.*, 2001; Kow & Pfaff, 2004).

These effects were mimicked by E2 coupled to bovine serum albumin (BSA), a membrane-impermeable analog of E2, indicating that they were initiated at the cell membrane. Conversely, blocking the action of estrogens by an i.c.v. injection of an antiestrogen (tamoxifen or ICI182,780) or blocking their synthesis by a single i.c.v. injection of an aromatase inhibitor (Vorozole or ATD) decreased sexual motivation (RCSM or LSPR) within 30 minutes again without affecting performance (Seredynski *et al.*, 2013). However if behavior tests were performed in a larger arena, treatment with these compounds then decreased the frequency of copulatory behavior, suggesting that in the smaller arena where all other tests had taken place copulation occurred in a reflexive manner when the male encountered the female, independently of the decrease of sexual motivation. In addition, the inhibition of RCSM induced by a single injection of Vorozole™ was blocked if birds were injected 15 minutes earlier with E2-biotin, another membrane-impermeable analog of E2 (Figure 4C) (Seredynski *et al.*, 2013).

These experiments thus demonstrated that the functions of E2 have evolved to regulate different behavioral components (motivation vs. performance) in distinct temporal domains (long- vs. short-term) so that diverse reproductive activities can be properly coordinated to ensure successive reproduction. A review of the existing literature also indicated that other responses controlled by estrogens might similarly depend on a slow genomic regulation of neuronal plasticity underlying behavioral activation and an acute control of motivation to engage in behavior thus leading to the formulation of the dual action of estrogen hypothesis (Cornil *et al.*, 2015).

Identification of the membrane E2 receptors involved

Several estrogen receptors (ER) located at the cell membrane could potentially mediate membrane-initiated non-genomic effects of estrogens. These receptors include G-protein

coupled receptors such as GPR30 (Filardo & Thomas, 2005), Gq-mER (Roepke *et al.*, 2009) or the less characterized ER-X (Toran-Allerand *et al.*, 2002) and also the classical nuclear estrogen receptors ER α and ER β translocated to the cell membrane (Micevych & Dominguez, 2009). Another series of experiments was therefore carried out to identify the receptor(s) involved in the rapid control of sexual motivation in male quail by injecting through an i.c.v. cannula located in the third ventricle specific agonists and antagonists of the different membrane ER in males that had previously received an acute injection of an aromatase inhibitor to transiently suppress estrogens bioavailability (Seredynski *et al.*, 2015).

The acute inhibition of aromatase maximally inhibited sexual motivation as measured by the frequency of RSCM in response to the visual access to a female when tests took place 30 min after the injection and this effect could be rescued by an injection of E2 15 min before the test. Using the same temporal paradigm, diarylpropionitrile (DPN), an ER β -specific agonist prevented this VorozoleTM-induced inhibition when injected at doses ranging from 2 to 50 μ g/birds (Figure 4C). In contrast, agonists of ER α (PPT), GPR30 (G1), and the Gq-mER (STX) did not rescue the response inhibited by VorozoleTM and 17 α -E2, possibly acting through ER-X only had a marginal effect (Seredynski *et al.*, 2015).

Estrogens binding to membrane ERs have been shown to activate cellular responses through the transactivation of associated metabotropic glutamate receptors (mGluRs) (Dewing *et al.*, 2007; Meitzen *et al.*, 2013). The next set of experiments therefore investigated the possible involvement of mGluRs in the acute behavioral response triggered by E2.

The i.c.v. injection of the mGluR1a antagonist LY367385 significantly inhibited sexual motivation but mGluR2/3 and mGluR5 antagonists were ineffective. This suggested the possible participation of mGluR1 to the control of sexual motivation but told nothing about their possible relationship with membrane ER. Additional studies showed that the transactivation of mGluR1a is actually a downstream event taking place after the activation of the ER β . LY367385 was indeed able to block the behavioral restoration of RSCM frequencies induced by E2 or DPN following the acute inhibition by VorozoleTM (Figure 4D) (Seredynski *et al.*, 2015).

Together these results show that membrane ER β play a key role in sexual behavior regulation and the cooperation between mERs and mGluRs recently uncovered in female rodents (Meitzen *et al.*, 2013) is functional in males where it mediates the acute effects of estrogens produced centrally in response to social stimuli. The presence of an ER–mGluR interaction in birds also suggests that similar mechanisms emerged relatively early in vertebrate history and are well conserved.

Conclusions

This review summarizes the keynote lecture that I presented during the meeting of the Society for Behavioral Neuroendocrinology in Montreal in August 2016. The text covers the main theme that was the backbone of the research performed in my laboratory over the past 45 years. A number of related topics were also investigated in parallel including the analysis

of the sexual differentiation of brain and behavior, the identification of the neural circuit mediating appetitive and consummatory sexual behavior and the description of steroid-induced brain plasticity but they were not considered here to preserve the focus of this presentation.

These investigations of the role of steroid metabolism, in particular testosterone aromatization, have uncovered a number of new mechanisms that are implicated in the control of male sexual behavior. We identified the main metabolic pathways for brain testosterone metabolism, their neuroanatomical localization and their control by various endogenous or exogenous stimuli. We also uncovered mechanisms that regulate in a rapid manner the preoptic aromatase activity and found that the resulting rapid changes in neuroestrogens bioavailability modulate male sexual motivation through their binding to membrane estrogen receptors beta. At several points in time we thought that the question of testosterone metabolism was solved and the research could move on to another topic but each time there was a rebound due to unexpected findings or new technological developments.

Many questions remain at this time:

- How can we reconcile the fact that glutamate at the same time increases expression of sexual behavior based on mammalian studies (Dominguez *et al.*, 2006) but also inhibits aromatase activity producing neuroestrogens which activate the motivation for this same behavior (Balthazart *et al.*, 2006)?

Both types of effects are presumably not mediated by the same mechanism (receptors?) and do not occur within the same time frame. Indeed, facilitative effects of glutamate on copulation based on studies in rats appear to be mediated mainly through the activation of NMDA receptors (Dominguez *et al.*, 2007; Hull *et al.*, 2007; Vigdorchik *et al.*, 2012). In contrast, NMDA only has marginal effects *in vitro* on AA in quail preoptic explants while the glutamatergic agonists, kainate and AMPA, induce a rapid, transient inhibition of the enzymatic activity (Balthazart *et al.*, 2006). Estradiol concentrations measured by *in vivo* dialysis in the zebra finch caudomedial nidopallium are also decreased by glutamate but not by NMDA retrodialysis (Remage-Healey *et al.*, 2008). Actions of glutamate on behavior are also likely to be faster (and thus potentially concomitant with behavior expression) than actions on AA that were shown to develop only after a few minutes (Balthazart *et al.*, 2006) even if technical limitations obviously prevent quantifications at earlier time points. Glutamate release observed during copulation could thus lead first to a rapid NMDA receptor-dependent improvement of sexual behavior and then to a slower AMPA or kainate receptor-dependent decrease in AA. The higher affinity of glutamate for NMDA receptors as compared to AMPA and kainate receptors (Hollmann & Heinemann, 1994) could contribute to the differential timing of these two effects. Facilitatory effects on sexual behavior would occur when glutamate concentrations begin to increase and the inhibition of AA would take place when high concentrations of glutamate have accumulated.

- Why are changes in aromatase observed *ex vivo* after brain collection systematically in a direction that is opposite to what would have been “naively”

anticipated (decreases after engaging in sexual behavior but increases after acute stress)?

The decrease in AA after engaging in sexual behavior fits with predictions made based on the rapid inhibition of AA induced by glutamate in quail HPOA explants (Balthazart *et al.*, 2006) and the rise in extracellular glutamate concentration demonstrated in the POA of copulating male rats (Dominguez *et al.*, 2006) and quail (de Bournonville *et al.*, 2017). Yet, this observation seems difficult to reconcile with the rapid stimulatory role of estrogens on sexual behavior illustrated by the effects of i.c.v. injections described here (Seredynski *et al.*, 2013; Seredynski *et al.*, 2015). Part of the explanation may lie in the fact that, due to technical limitations, AA is measured *in vitro* after behavior has been expressed following brain collection and tissue homogenization. AA in these conditions may thus not fully reflect the enzymatic activity present *in vivo*. Note, however, that aromatase activity is already inhibited in males visually exposed to a female who did not have a chance to physically interact and copulate with her (de Bournonville *et al.*, 2013). It thus seems unlikely that the enzymatic inhibition reflects the satiation processes that occurs after the behavior and mediates the refractory period. Alternatively, a form of negative feedback might decrease the activity of the enzymatic molecules immediately after they have been active due for example to a modification of the subcellular localization of the enzyme and to an action of the synthesized estrogens on the enzyme itself. It is however comforting that enzymatic changes induced by stress and copulation occur in opposite directions in agreement with the general assumption that acute stress impairs reproduction. More work is needed to reconcile changes in AA measured *ex vivo* with the changes to possibly take *in vivo*.

- Why are rapid changes of AA measured in microdissected brain regions not always reflecting the local concentrations in estradiol? A study of zebra finches showed that simulated territorial intrusions differentially affect AA and E2 concentrations in microdissected brain nuclei (Charlier *et al.*, 2011b). Similarly an experiment compared effects of acute stress on AA and E2 concentration in the quail brain and found completely discordant results: in the POA, AA increased but E2 concentration decreased after acute stress (Dickens *et al.*, 2014). Furthermore we also recently discovered during *in vivo* dialysis experiments that the extracellular E2 concentration in the POM increases during and after copulation in male quail whereas AA measured in this brain region as soon as 2 min after copulation is significantly decreased (de Bournonville *et al.*, 2016b). It is plausible as discussed in the previous section that AA measured *ex vivo* no longer reflects AA that was present before the dissection and homogenization of the tissue and the addition of optimal concentrations of ions and co-factors. It is however also possible that additional mechanisms regulate the concentration of E2 (e.g., its catabolism to other steroids) and/or regulate AA *in vivo* (e.g., competition with other steroid metabolism enzymes for the androgenic substrate, control of enzymatic by local intracellular changes in pH) (Osawa *et al.*, 1993) and these should be investigated.
- Where in the brain are neuroestrogens exerting their rapid effects on sexual motivation? It is well established that the genomic effects of estrogens on sexual

behavior are mediated to a large extent by an action in the POM or at least that action at this site is sufficient to activate behavior in castrated subjects (Balthazart *et al.*, 1990a; Balthazart & Surlemont, 1990a). One could assume that the faster membrane-initiated effects on sexual motivation are obtained by an action at the same neuroanatomical site but this is not necessarily the case. Part or most of the action might take place in pre-synaptic boutons where aromatase is present and active (Schlinger & Callard, 1989; Naftolin *et al.*, 1996; Rohmann *et al.*, 2007). Non-genomic actions of estrogens identified so far are compatible with both pre- and post-synaptic modulation of synaptic transmission (Cornil, 2009). Recent studies conducted in songbirds suggest that presynaptic boutons might constitute a specific site for acute regulation of estrogen production (Remage-Healey *et al.*, 2009; Cornil & Charlier, 2010; Cornil *et al.*, 2011b; Saldanha *et al.*, 2011). The aromatase-positive cells in the POM extend projections to many brain regions including namely the periaqueductal central grey (Absil *et al.*, 2001b; Carere *et al.*, 2007) and the control by neuroestrogens of sexual motivation might be effected at these sites. This could only be tested by additional experiments involving stereotaxic implantation of estrogens, antiestrogens and aromatase inhibitors and evaluations of the behavioral consequences. All these questions are clearly testable and we have specific hypotheses that are currently being evaluated but obviously the work of testosterone metabolism and behavior is not over!

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References

- Absil P, Baillien M, Ball GF, Carlo Panzica G, Balthazart J. The control of preoptic aromatase activity by afferent inputs in Japanese quail. *Brain Research Reviews*. 2001a; 37:38–58. [PubMed: 11744073]
- Absil P, Riters LV, Balthazart J. Preoptic aromatase cells project to the mesencephalic central gray in the male Japanese quail (*Coturnix japonica*). *Horm Behav*. 2001b; 40:369–383. [PubMed: 11673910]

- Adkins EK. Hormonal basis of sexual differentiation in the Japanese quail. *J Comp Physiol Psychol.* 1975; 89:61–71. [PubMed: 1159113]
- Adkins EK. Effects of diverse androgens on the sexual behavior and morphology of castrated male quail. *Horm Behav.* 1977; 8:201–207. [PubMed: 863401]
- Adkins EK, Boop JJ, Koutnik DL, Morris JB, Pniewski EE. Further evidence that androgen aromatization is essential for the activation of copulation in male quail. *Physiol Behav.* 1980; 24:441–446. [PubMed: 7375563]
- Adkins EK, Pniewski EE. Control of reproductive behavior by sex steroids in male quail. *J Comp Physiol Psychol.* 1978; 92:1169–1178.
- Adkins-Regan E. Hormone specificity, androgen metabolism, and social behavior. *Amer Zool.* 1981; 21:257–271.
- Albert KA, Helmer-Matyjek E, Nairn AA, Müller TH, Haycock JW, Greene LA, Goldstein M, Greengard P. Calcium/phospholipid-dependent protein kinase (protein kinase C) phosphorylates and activates tyrosine hydroxylase. *Proc Natl Acad Sci USA.* 1984; 81:7713–7717. [PubMed: 6151178]
- Alexandre C, Balthazart J. Effects of metabolism inhibitors, antiestrogens and antiandrogens on the androgen and estrogen induced sexual behavior in Japanese quail. *Physiol Behav.* 1986; 38:581–591. [PubMed: 2950530]
- Askew JA, Georgiou GC, Sharp PJ, Lea RW. Localization of progesterone receptor in brain and pituitary of the ring dove: Influence of breeding cycle and estrogen. *Horm Behav.* 1997; 32:105–113. [PubMed: 9367718]
- Aste N, Panzica GC, Viglietti-Panzica C, Harada N, Balthazart J. Distribution and effects of testosterone on aromatase mRNA in the quail forebrain: A nonradioactive in situ hybridization study. *J Chem Neuroanat.* 1998; 14:103–115. [PubMed: 9625355]
- 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]
- Ball GF, Balthazart J. Hormonal regulation of brain circuits mediating male sexual behavior in birds. *Physiology and Behavior.* 2004; 83:329–346. [PubMed: 15488549]
- Ball GF, Balthazart J. Japanese quail as a model system for studying the neuroendocrine control of reproductive and social behaviors. *ILAR Journal.* 2010; 51:310–325. [PubMed: 21131709]
- Balthazart J. Short-term effects of testosterone propionate on the behaviour of young intact male domestic ducks (*Anas platyrhynchos*). *Psychologica Belgica.* 1974; 14:1–10.
- Balthazart J. Daily variations of behavioural activities and of plasma testosterone levels in the domestic duck *Anas platyrhynchos*. *J Zool Lond.* 1976; 180:155–173.
- Balthazart, J. Steroid metabolism and the activation of social behavior. In: Balthazart, J., editor. *Advances in Comparative and Environmental Physiology.* Vol. 3. Springer Verlag; Berlin: 1989. p. 105–159.
- Balthazart J. Testosterone metabolism in the avian hypothalamus. *J Steroid Biochem Mol Biol.* 1991; 40:557–570. [PubMed: 1958558]
- Balthazart J, Baillien M, Ball GF. Rapid and reversible inhibition of brain aromatase activity. *Journal of Neuroendocrinology.* 2001; 13:63–73. [PubMed: 11123516]
- Balthazart J, Baillien M, Ball GF. Interactions between aromatase (estrogen synthase) and dopamine in the control of male sexual behavior in quail. *Comparative Biochemistry and Physiology - B Biochemistry and Molecular Biology.* 2002; 132:37–55. [PubMed: 11997208]
- Balthazart J, Baillien M, Ball GF. Rapid control of brain aromatase activity by glutamatergic inputs. *Endocrinology.* 2006; 147:359–366. [PubMed: 16195408]
- Balthazart J, Baillien M, Charlier TD, Ball GF. Calcium-dependent phosphorylation processes control brain aromatase in quail. *European Journal of Neuroscience.* 2003; 17:1591–1606. [PubMed: 12752377]
- Balthazart J, Baillien M, Cornil CA, Ball GF. Preoptic aromatase modulates male sexual behavior: slow and fast mechanisms of action. *Physiol Behav.* 2004; 83:247–270. [PubMed: 15488543]
- Balthazart J, Ball GF. New insights into the regulation and function of brain estrogen synthase (aromatase). *Trends in Neurosciences.* 1998; 21:243–249. [PubMed: 9641536]

- Balthazart J, Ball GF. Topography in the preoptic region: Differential regulation of appetitive and consummatory male sexual behaviors. *Frontiers in Neuroendocrinology*. 2007; 28:161–178. [PubMed: 17624413]
- Balthazart J, Blaustein JD, Cheng MF, Feder HH. Hormones modulate the concentration of cytoplasmic progesterone receptors in the brain of male ring doves (*Streptopelia risoria*). *Journal of Endocrinology*. 1980; 86:251–261. [PubMed: 7191878]
- 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. *J Exp Zool A Ecol Genet Physiol*. 2009; 311:323–345. [PubMed: 18481266]
- Balthazart J, Deviche P, Hendrick J. Effects of exogenous hormones on the reproductive behaviour of adult male domestic ducks. II Correlation with morphology and hormone plasma levels Behavioural Processes. 1977a; 2:147–161. [PubMed: 24896434]
- Balthazart J, Evrard L, Surlemont C. Effects of the non-steroidal aromatase inhibitor, R76713 on testosterone-induced sexual behavior in the Japanese quail (*Coturnix coturnix japonica*). *Horm Behav*. 1990a; 24:510–531. [PubMed: 2286366]
- Balthazart J, Foidart A. Brain aromatase and the control of male sexual behavior. *Journal of Steroid Biochemistry and Molecular Biology*. 1993; 44:521–540. [PubMed: 8476766]
- Balthazart J, Foidart A, Harada N. Immunocytochemical localization of aromatase in the brain. *Brain Research*. 1990b; 514:327–333. [PubMed: 2357545]
- Balthazart J, Foidart A, Hendrick JC. The induction by testosterone of aromatase activity in the preoptic area and activation of copulatory behavior. *Physiology and Behavior*. 1990c; 47:83–94. [PubMed: 2326347]
- Balthazart J, Foidart A, Surlemont C, Harada N, Naftolin F. Neuroanatomical specificity in the autoregulation of aromatase-immunoreactive neurons by androgens and estrogens: An immunocytochemical study. *Brain Res*. 1992; 574:280–290. [PubMed: 1638401]
- Balthazart J, Foidart A, Surlemont C, Vockel A, Harada N. Distribution of aromatase in the brain of the Japanese quail, ring dove, and zebra finch: An immunocytochemical study. *J Comp Neurol*. 1990d; 301:276–288. [PubMed: 2262592]
- Balthazart J, Hendrick J. Annual variation in reproductive behavior, testosterone, and plasma FSH levels in the Rouen duck, *Anas platyrhynchos*. *General and Comparative Endocrinology*. 1976; 28:171–183. [PubMed: 944669]
- Balthazart J, Hendrick JC, Deviche P. Diurnal variations of plasma gonadotrophins in male domestic ducks during the sexual cycle. *General and Comparative Endocrinology*. 1977b; 32:376–389. [PubMed: 561732]
- Balthazart J, Massa R, Negri-Cesi P. Photoperiodic control of testosterone metabolism, plasma gonadotrophins, cloacal gland growth, and reproductive behavior in the Japanese quail. *Gen Comp Endocrinol*. 1979; 39:222–235. [PubMed: 499750]
- Balthazart J, Schoffeniels E. Pheromones are involved in the control of sexual behaviour in birds. *Naturwissenschaften*. 1979; 66:55–56. [PubMed: 370614]
- Balthazart J, Schumacher M. Changes in testosterone metabolism by the brain and cloacal gland during sexual maturation in the Japanese quail (*Coturnix coturnix japonica*). *Journal of Endocrinology*. 1984a; 100:13–18. [PubMed: 6317782]
- Balthazart J, Schumacher M. Organization and activation of behavior in quail: Role of testosterone metabolism. *Journal of Experimental Zoology*. 1984b; 232:595–604. [PubMed: 6394700]
- Balthazart J, Schumacher M. Role of testosterone metabolism in the activation of sexual behaviour in birds. In: Gilles, R., Balthazart, J., editors. *Neurobiology. Current comparative Approaches*. Springer-Verlag; Berlin: 1985. p. 121–140.
- Balthazart J, Schumacher M, Evrard L. Sex differences and steroid control of testosterone-metabolizing enzyme activity in the quail brain. *J Neuroendocrinol*. 1990e; 2:675–683. [PubMed: 19215405]
- Balthazart J, Schumacher M, Malacarne G. Relative potencies of testosterone and 5 α -dihydrotestosterone on crowing and cloacal gland growth in the Japanese quail (*Coturnix coturnix japonica*). *J Endocrinol*. 1984; 100:19–23. [PubMed: 6690644]

- Balthazart J, Schumacher M, Malacarne G. Interaction of androgens and estrogens in the control of sexual behavior in male Japanese quail. *Physiol Behav.* 1985; 35:157–166. [PubMed: 3877943]
- Balthazart J, Schumacher M, Ottinger MA. Sexual differences in the Japanese quail: behavior, morphology and intracellular metabolism of testosterone. *Gen Comp Endocrinol.* 1983; 51:191–207. [PubMed: 6413294]
- Balthazart J, Stevens M. Effects of testosterone propionate on the social behaviour of groups of male domestic ducklings *Anas platyrhynchos* L. *Anim Behav.* 1975; 23:926–931. [PubMed: 1239228]
- Balthazart J, Surlemont C. Androgen and estrogen action in the preoptic area and activation of copulatory behavior in quail. *Physiology and Behavior.* 1990a; 48:599–609. [PubMed: 2082358]
- Balthazart J, Surlemont C. Copulatory behavior is controlled by the sexually dimorphic nucleus of the quail POA. *Brain Research Bulletin.* 1990b; 25:7–14. [PubMed: 2207718]
- Balthazart J, Taziaux M. The underestimated role of olfaction in avian reproduction? *Behavioural Brain Research.* 2009; 200:248–259. [PubMed: 18804490]
- Balthazart J, Tlemçani O, Harada N. Localization of testosterone-sensitive and sexually dimorphic aromatase-immunoreactive cells in the quail preoptic area. *Journal of Chemical Neuroanatomy.* 1996; 11:147–171. [PubMed: 8906458]
- Barfield RJ. Activation of copulatory behavior by androgen implanted into the preoptic area of the male fowl. *Horm Behav.* 1969; 1:37–52.
- Berk ML, Butler AB. Efferent projections of the medial preoptic nucleus and medial hypothalamus in the pigeon. *J Comp Neurol.* 1981; 203:379–399. [PubMed: 6274919]
- Bonadonna F, Nevitt GA. Partner-specific odor recognition in an Antarctic seabird. *Science.* 2004; 306:835. [PubMed: 15514149]
- Carere C, Ball GF, Balthazart J. Sex differences in projections from preoptic area aromatase cells to the periaqueductal gray in Japanese quail. *Journal of Comparative Neurology.* 2007; 500:894–907. [PubMed: 17177261]
- Charlier TD, Ball GF, Balthazart J. Rapid action on neuroplasticity precedes behavioral activation by testosterone. *Hormones and Behavior.* 2008; 54:488–495. [PubMed: 18452920]
- 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, Po KW, Newman AE, Shah AH, Saldanha CJ, Soma KK. 17beta-Estradiol levels in male zebra finch brain: combining Palkovits punch and an ultrasensitive radioimmunoassay. *Gen Comp Endocrinol.* 2011b; 167:18–26.
- Cornil C, Foidart A, Minet A, Balthazart J. Immunocytochemical localization of ionotropic glutamate receptors subunits in the adult quail forebrain. *J Comp Neurol.* 2000; 428:577–608. [PubMed: 11077414]
- 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. 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. The dual action of estrogen hypothesis. *Trends Neurosci.* 2015; 38:408–416. [PubMed: 26089224]
- Cornil CA, Ball GF, Balthazart J, Charlier TD. Organizing effects of sex steroids on brain aromatase activity in quail. *PLoS ONE.* 2011a; 6
- 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, Balthazart J. Estradiol rapidly activates male sexual behavior and affects brain monoamine levels in the quail brain. *Behav Brain Res.* 2006a; 166:110–123. [PubMed: 16159671]
- 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]

- Cornil CA, Leung CH, Pletcher ER, Naranjo KC, Saldanha CJ. Synaptosomal aromatase is more rapidly inhibited by calcium dependent phosphorylations than microsomal aromatase in the zebra finch brain. Abstract of Society For Neuroscience. 2011b; 37:501–503.
- Cornil CA, Seutin V, Motte P, Balthazart J. Electrophysiological and neurochemical characterization of neurons of the medial preoptic area in Japanese quail (*Coturnix japonica*). Brain Research. 2004; 1029:224–240. [PubMed: 15542078]
- Cornil CA, Taziaux M, Baillien M, Ball GF, Balthazart J. Rapid effects of aromatase inhibition on male reproductive behaviors in Japanese quail. Horm Behav. 2006b; 49:45–67. [PubMed: 15963995]
- Croix D, Hendrick JC, Balthazart J, Franchimont P. Radioimmunologic assay of follicle stimulating hormone of duck hypophysis by a mammalian system. Comptes Rendus des Seances de la Societe de Biologie et de Ses Filiales. 1974; 168:136–140. [PubMed: 4279732]
- Cross E, Roselli CE. 17beta-estradiol rapidly facilitates chemoinvestigation and mounting in castrated male rats. The American journal of physiology. 1999; 276:R1346–1350. [PubMed: 10233026]
- Davidson JM. Activation of the male rat's sexual behavior by intracerebral implantation of androgen. Endocrinology. 1966; 79:783–794. [PubMed: 5957191]
- de Bournonville C, Balthazart J, Ball GF, Cornil CA. Non-ovarian aromatization is required to activate female sexual motivation in testosterone-treated ovariectomized quail. Horm Behav. 2016a; 83:45–59. [PubMed: 27189762]
- de Bournonville C, de Bournonville MP, Aourz N, Van Eeckhaut A, Smolders I, Ball GF, Balthazart J, Cornil CA. Preoptic glutamate and estradiol release during male sexual behavior. Soc Neurosci Abst. 2016b 539.503/FFF519.
- 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; 38:789–799. [PubMed: 22999655]
- de Bournonville C, Smolders I, Van Eeckhaut A, Ball GF, Balthazart J, Cornil CA. Glutamate released in the preoptic area during sexual behavior controls local estrogen synthesis in male quail. Psychoneuroendocrinology. 2017; 79:49–58. [PubMed: 28259043]
- Dessi-Fulgheri F, Lucarini N, Lupo di Prisco C. Relationships between testosterone metabolism in the brain, other endocrine variables and intermale aggression in mice. Aggr Behav. 1976; 2:223–231.
- Dewing P, Boulware MI, Sinchak K, Christensen A, Mermelstein PG, Micevych P. Membrane estrogen receptor-alpha interactions with metabotropic glutamate receptor 1a modulate female sexual receptivity in rats. J Neurosci. 2007; 27:9294–9300. [PubMed: 17728443]
- Di S, Maxson MM, Franco A, Tasker JG. Glucocorticoids regulate glutamate and GABA synapse-specific retrograde transmission via divergent nongenomic signaling pathways. J Neurosci. 2009; 29:393–401. [PubMed: 19144839]
- 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. Journal of Neuroendocrinology. 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; 152:4242–4251. [PubMed: 21878510]
- Dickens MJ, Cornil CA, Balthazart J. Neurochemical control of rapid stress-induced changes in brain aromatase activity. Journal of Neuroendocrinology. 2013; 25:329–339. [PubMed: 23253172]
- Dickens MJ, de Bournonville C, Balthazart J, Cornil CA. Relationships between rapid changes in local aromatase activity and estradiol concentrations in male and female quail brain. Horm Behav. 2014; 65:154–164. [PubMed: 24368290]
- Dominguez JM, Balfour ME, Lee HS, Brown JL, Davis BA, Coolen LM. Mating activates NMDA receptors in the medial preoptic area of male rats. Behavioral neuroscience. 2007; 121:1023–1031. [PubMed: 17907833]
- Dominguez JM, Gil M, Hull EM. Preoptic glutamate facilitates male sexual behavior. J Neurosci. 2006; 26:1699–1703. [PubMed: 16467517]

- Feder HH, Storey A, Goodwin D, Reboulleau C, Silver R. Testosterone and “5 α -dihydrotestosterone” levels in peripheral plasma of male and female ring doves (*Streptopelia risoria*) during the reproductive cycle. *Biol Reprod.* 1977; 16:666–677. [PubMed: 861338]
- Filardo EJ, Thomas P. GPR30: a seven-transmembrane-spanning estrogen receptor that triggers EGF release. *Trends Endocrinol Metab.* 2005; 16:362–367. [PubMed: 16125968]
- Foidart A, Reid J, Absil P, Yoshimura N, Harada N, Balthazart J. Critical reexamination 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]
- Follett BK, Scanes CG, Cunningham FJ. A radioimmunoassay for avian luteinizing hormone. *J Endocrinol.* 1972; 52:359–398. [PubMed: 5015389]
- Gorski RA, Gordon JH, Shryne JE, Southam AM. Evidence for a morphological sex difference within the medial preoptic area of the rat brain. *Brain Res.* 1978; 148:333–346. [PubMed: 656937]
- Grant, LD., Stumpf, WE. Hormone uptake sites in CNS biogenic amines systems. In: Stumpf, WE., Grant, LD., editors. *Anatomical neuroendocrinology*. Karger; Basel: 1975. p. 445-463.
- Grunt JA, Young WC. Consistency of sexual behavior patterns in individual male guinea pigs following castration and androgen therapy. *J Comp Physiol Psychol.* 1953; 46:138–144. [PubMed: 13044875]
- Hagelin, JC. Odors and chemical signaling. In: Jamieson, BGM., editor. *Reproductive biology and phylogeny of birds, Part B*. Science Publishers, Enfield (NH) Jersey; Plymouth: 2007. p. 75-119.
- Hagelin JC, Jones IL, Rasmussen LE. A tangerine-scented social odour in a monogamous seabird. *Proc R Soc Lond [Biol]*. 2003; 270:1323–1329.
- Harada N. Novel properties of human placental aromatase as cytochrome P-450: purification and characterization of a unique form of aromatase. *J Biochem.* 1988; 103:106–113. [PubMed: 3129418]
- Harada N, Abe-Dohmae S, Loeffen R, Foidart A, Balthazart J. Synergism between androgens and estrogens in the induction of aromatase and its messenger RNA in the brain. *Brain Research.* 1993; 622:243–256. [PubMed: 8242362]
- Harada N, Yamada K, Foidart A, Balthazart J. Regulation of aromatase cytochrome P-450 (estrogen synthetase) transcripts in the quail brain by testosterone. *Mol Brain Res.* 1992; 15:19–26. [PubMed: 1331667]
- Harris GW, Michael RP. The activation of sexual behaviour by hypothalamic implants of oestrogen. *The Journal of physiology.* 1964; 171:275–301. [PubMed: 14191480]
- Hemmings HCJ, Nairn AC, McGuinness TL, Hagan RL, Greengard P. Role of protein phosphorylation in neuronal signal transduction. *FASEB J.* 1989; 3:1583–1580. [PubMed: 2493406]
- Hochberg Z, Bick T, Pelman R, Brandes JM, Barzilai D. The dual effect of calcium on aromatization by cultured human trophoblast. *J Steroid Biochem.* 1986; 24:1217–1219. [PubMed: 3016410]
- Hollmann M, Heinemann S. Cloned glutamate receptors. *Annual review of neuroscience.* 1994; 17:31–108.
- Hull, EM., Dominguez, JM., Muschamp, JW. Neurochemistry of male sexual behavior. In: Lajtha, A., editor. *Handbook of Neurochemistry and Molecular Neurobiology: Behavioral Neurochemistry, Neuroendocrinology and Molecular Neurobiology* (Volume Editor J Blaustein). Springer; New York: 2007. p. 38-94.
- Hutchison JB. Effects of hypothalamic implants of gonadal steroids on courtship behaviour in Barbary doves (*Streptopelia risoria*). *J Endocrinol.* 1971; 50:97–113. [PubMed: 4931797]
- Jacob J, Balthazart J, Schoffeniels E. Sex differences in the chemical composition of uropygial gland waxes in domestic ducks. *Biochem Syst Ecol.* 1979; 7:149–153.
- Jacobson CD, Csernus VJ, Shryne JE, Gorski RA. The influence of gonadectomy, androgen exposure, or a gonadal graft in the neonatal rat on the volume of the sexually dimorphic nucleus of the preoptic area. *J Neurosci.* 1981; 1:1142–1147. [PubMed: 7288477]
- Jacobson CD, Shryne JE, Shapiro F, Gorski RA. Ontogeny of the sexually dimorphic nucleus of the preoptic area. *J Comp Neurol.* 1980; 193:541–548. [PubMed: 7440782]

- Kelley, DB., Pfaff, DW. Generalizations from comparative studies on neuroanatomical and endocrine mechanisms of sexual behaviour. In: Hutchison, JB., editor. *Biological determinants of sexual behaviour*. John Wiley & Sons; Chichester: 1978. p. 225-254.
- Kelly, MJ., Ronnekleiv, OK. Rapid membrane effects of estrogen in the central nervous system. In: Pfaff, DW., Arnold, AP., Etgen, AM., Fahrback, SE., Rubin, RT., editors. *Hormones, Brain and Behavior*. Academic press; San Diego: 2002. p. 361-380.
- Kow LM, Pfaff DW. The membrane actions of estrogens can potentiate their lordosis behavior-facilitating genomic actions. *PNAS*. 2004; 101:12354–12357. [PubMed: 15302933]
- Lisk RD. Diencephalic placement of estradiol and sexual receptivity in the female rat. *Am J Physiol*. 1962; 203:493–496. [PubMed: 14465798]
- Lisk RD. Neural localization for androgen activation of copulatory behavior in the male rat. *Endocrinology*. 1967; 80:754–761. [PubMed: 6022056]
- McEwen BS. Steroid hormone actions on the brain: When is the genome involved? *Horm Behav*. 1994; 28:396–405. [PubMed: 7729808]
- McEwen BS, Alves SE. Estrogen actions in the central nervous system. *Endocr Rev*. 1999; 20:279–307. [PubMed: 10368772]
- Meitzen J, Luoma JI, Boulware MI, Hedges VL, Peterson BM, Tuomela K, Britson KA, Mermelstein PG. Palmitoylation of estrogen receptors is essential for neuronal membrane signaling. *Endocrinology*. 2013; 154:4293–4304. [PubMed: 24008343]
- Micevych P, Dominguez R. Membrane estradiol signaling in the brain. *Front Neuroendocrinol*. 2009; 30:315–327. [PubMed: 19416735]
- Michael RP. Estrogen-sensitive neurons and sexual behavior in female cats. *Science*. 1962; 136:322–323. [PubMed: 14473322]
- Morrell, JI., Kelley, DB., Pfaff, DW. Sex steroid binding in the brain of vertebrates. In: Knigge, KM., Scott, DE., Kobayashi, H., Miura, S., Ishii, S., editors. *Brain-endocrine interactions II*. Karger; Basel: 1975. p. 230-256.
- Naftolin F, Horvath TL, Jakab RL, Leranth C, Harada N, Balthazart J. Aromatase immunoreactivity in axon terminals of the vertebrate brain. *Neuroendocrinology*. 1996; 63:149–155. [PubMed: 9053779]
- Nestler, EJ., Greengard, P. Serine and threonine phosphorylation. In: Siegel, GJ., Agranoff, BW., Albers, RW., Fisher, SK., Uhler, MD., editors. *Basic Neurochemistry Molecular, Cellular and Medical Aspects*. Lippincott-Raven; Philadelphia: 1999. p. 471-495.
- Nottebohm F, Arnold AP. Sexual dimorphism in the vocal control areas in the song bird brain. *Science*. 1976; 194:211–213. [PubMed: 959852]
- Onagbesan OM, Podie MJ. Calcium-dependent stimulation of estrogen secretion by FSH from theca cells of the domestic hen (*Gallus domesticus*). *Gen Comp Endocrinol*. 1989; 75:177–186. [PubMed: 2509282]
- 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]
- Panzica G, Viglietti-Panzica C, Sanchez F, Sante P, Balthazart J. Effects of testosterone on a selected neuronal population within the preoptic sexually dimorphic nucleus of the Japanese quail. *Journal of Comparative Neurology*. 1991; 303:443–456. [PubMed: 2007660]
- Panzica GC, Viglietti-Panzica C, Balthazart J. The sexually dimorphic medial preoptic nucleus of quail: A key brain area mediating steroid action on male sexual behavior. *Frontiers in Neuroendocrinology*. 1996; 17:51–125. [PubMed: 8788569]
- Panzica GC, Viglietti-Panzica C, Calcagni M, Anselmetti GC, Schumacher M, Balthazart J. Sexual differentiation and hormonal control of the sexually dimorphic preoptic medial nucleus in quail. *Brain Res*. 1987; 416:59–68. [PubMed: 3620956]
- Ramenofsky M. Agonistic behaviour and endogenous plasma hormones in male Japanese quail. *Anim Behav*. 1984; 32:698–708.

- Ramirez VD, Zheng JB, Siddique KM. Membrane receptors for estrogen, progesterone, and testosterone in the rat brain: Fantasy or reality. *Cell Mol Neurobiol.* 1996; 16:175–198. [PubMed: 8743968]
- Remage-Healey L, Maidment NT, Schlinger BA. Forebrain steroid levels fluctuate rapidly during social interactions. *Nat Neurosci.* 2008; 11:1327–1334. [PubMed: 18820691]
- Remage-Healey L, Oyama RK, Schlinger BA. Elevated aromatase activity in forebrain synaptic terminals during song. *J Neuroendocrinol.* 2009; 21:191–199. [PubMed: 19207827]
- Roepke TA, Qiu J, Bosch MA, Ronnekleiv OK, Kelly MJ. Cross-talk between membrane-initiated and nuclear-initiated oestrogen signalling in the hypothalamus. *J Neuroendocrinol.* 2009; 21:263–270. [PubMed: 19187465]
- Rohmann KN, Schlinger BA, Saldanha CJ. Subcellular compartmentalization of aromatase is sexually dimorphic in the adult zebra finch brain. *Dev Neurobiol.* 2007; 67:1–9. [PubMed: 17443767]
- Roselli CE, Ellinwood WE, Resko JA. Regulation of brain aromatase activity in rats. *Endocrinology.* 1984; 114:192–200. [PubMed: 6537806]
- Roselli CE, Resko JA. Androgens regulate brain aromatase activity in adult male rats through a receptor mechanism. *Endocrinology.* 1984; 114:2183–2189. [PubMed: 6723579]
- Roselli, CE., Resko, JA. In vitro assay of aromatase activity in the central nervous system. In: Greenstein, B., editor. *Neuroendocrine research methods*. Vol. 2. Harwood Academic Publishers; Chur, Switzerland: 1991. p. 937-951.p. 930
- Saldanha CJ, Remage-Healey L, Schlinger BA. Synaptocrine signaling: steroid synthesis and action at the synapse. *Endocrine reviews.* 2011; 32:532–549. [PubMed: 21622487]
- Saldanha CJ, Schlinger BA, Micevych PE, Horvath TL. Presynaptic N-methyl-D-aspartate receptor expression is increased by estrogen in an aromatase-rich area of the songbird hippocampus. *J Comp Neurol.* 2004; 469:522–534. [PubMed: 14755533]
- Sar M, Stumpf WE. Autoradiographic localization of radioactivity in the rat brain after the injection of 1,2-³H-testosterone. *Endocrinology.* 1973; 92:251–256. [PubMed: 4681930]
- Schlinger BA, Callard GV. Localization of aromatase in synaptosomal and microsomal subfractions of quail (*Coturnix coturnix japonica*) brain. *Neuroendocrinol.* 1989; 49:434–441.
- Schumacher M. Rapid membrane effects of steroid hormones: an emerging concept in neuroendocrinology. *Trends Neurosci.* 1990; 13:359–362. [PubMed: 1699322]
- Schumacher M, Balthazart J. The effects of testosterone and its metabolites on sexual behavior and morphology in male and female Japanese quail. *Physiol Behav.* 1983a; 30:335–339. [PubMed: 6867131]
- Schumacher M, Balthazart J. The effects of testosterone and its metabolites on sexual behavior and morphology in male and female Japanese quail. *Physiology and Behavior.* 1983b; 30:335–339. [PubMed: 6867131]
- Schumacher M, Balthazart J. Sexual dimorphism of the hypothalamic metabolism of testosterone in the Japanese quail (*Coturnix coturnix japonica*). *Prog Brain Res.* 1984; 61:53–61. [PubMed: 6396714]
- Schumacher M, Balthazart J. Testosterone-induced brain aromatase is sexually dimorphic. *Brain Research.* 1986; 370:285–293. [PubMed: 3708327]
- Schumacher M, Balthazart J. Neuroanatomical distribution of testosterone metabolizing enzymes in the Japanese quail. *Brain Res.* 1987; 422:137–148. [PubMed: 3676776]
- Schumacher M, Contenti E, Balthazart J. Partial characterization of testosterone-metabolizing enzymes in the quail brain. *Brain Res.* 1984; 305:51–59. [PubMed: 6744060]
- Seredynski AL, Balthazart J, Ball GF, Cornil CA. Estrogen Receptor beta Activation Rapidly Modulates Male Sexual Motivation through the Transactivation of Metabotropic Glutamate Receptor 1a. *J Neurosci.* 2015; 35:13110–13123. [PubMed: 26400941]
- Seredynski AL, Balthazart J, Christophe VJ, Ball GF, Cornil CA. Neuroestrogens rapidly regulate sexual motivation but not performance. *Journal of Neuroscience.* 2013; 33:164–174. [PubMed: 23283331]
- Silver R, Reboulleau C, Lehrman DS, Feder HH. Radioimmunoassay of plasma progesterone during the reproductive cycle of male and female ring doves (*Streptopelia risoria*). *Endocrinology.* 1974; 94:1547–1554. [PubMed: 4831120]

- Steimer T, Hutchison JB. Androgen increases formation of behaviourally effective oestrogen in dove brain. *Nature*. 1981; 292:345–347. [PubMed: 7254332]
- Steimer, T., Hutchison, JB. Micromethods for the in vitro study of steroid metabolism in the brain using radiolabelled tracers. In: Greenstein, B., editor. *Neuroendocrine research methods*. Vol. 2. Harwood Academic Publishers; Chur, Switzerland: 1991. p. 875-919.
- Taziaux M, Cornil CA, Balthazart J. Aromatase inhibition blocks the expression of sexually-motivated cloacal gland movements in male quail. *Behavioural Processes*. 2004; 67:461–469. [PubMed: 15518995]
- Thompson RR, Adkins-Regan E. Ontogeny of a sexually dimorphic nucleus in the preoptic area of the Japanese quail (*Coturnix japonica*). *Dev Brain Res*. 1992; 70:231–237. [PubMed: 1477957]
- Toran-Allerand CD, Guan XP, MacLusky NJ, Horvath TL, Diano S, Singh M, Connolly ES Jr, Nathrapalli IS, Tinnikov AA. ER-X: A novel, plasma membrane-associated, putative estrogen receptor that is regulated during development and after ischemic brain injury. *J Neurosci*. 2002; 22:8391–8401. [PubMed: 12351713]
- Vasudevan N, Kow LM, Pfaff DW. Early membrane estrogenic effects required for full expression of slower genomic actions in a nerve cell line. *PNAS*. 2001; 98:12267–12271. [PubMed: 11572951]
- Vigdorichik AV, Parrish BP, Lagoda GA, McHenry JA, Hull EM. An NMDA antagonist in the MPOA impairs copulation and stimulus sensitization in male rats. *Behavioral neuroscience*. 2012; 126:186–195. [PubMed: 22289046]
- Voigt C, Ball GF, Balthazart J. Sex differences in the expression of sex steroid receptor mRNA in the quail brain. *J Neuroendocrinol*. 2009; 21:1045–1062. [PubMed: 19845834]
- Voigt C, Ball GF, Balthazart J. Effects of sex steroids on aromatase mRNA expression in the male and female quail brain. *Gen Comp Endocrinol*. 2011; 170:180–188. [PubMed: 20951703]
- Watson JT, Adkins-Regan E. Activation of sexual behavior by implantation of testosterone propionate and estradiol benzoate into the preoptic area of the male Japanese quail (*Coturnix japonica*). *Horm Behav*. 1989a; 23:251–268. [PubMed: 2744741]
- Watson JT, Adkins-Regan E. Neuroanatomical localization of sex steroid-concentrating cells in the Japanese quail (*Coturnix japonica*): Autoradiography with [3H]-testosterone, [3H]-estradiol, and [3H]-dihydrotestosterone. *Neuroendocrinol*. 1989b; 49:51–64.
- Watson JT, Adkins-Regan E. Testosterone implanted in the preoptic area of male Japanese quail must be aromatized to activate copulation. *Horm Behav*. 1989c; 23:432–447. [PubMed: 2793083]
- Wild JM, Balthazart J. Neural pathways mediating control of reproductive behavior in male Japanese quail. *Journal of Comparative Neurology*. 2013; 521:2067–2087. [PubMed: 23225613]
- Wingfield JC, Sapolsky RM. Reproduction and resistance to stress: when and how. *J Neuroendocrinol*. 2003; 15:711–724. [PubMed: 12834431]

Highlights

- Preoptic aromatization of testosterone modulates activation of male sexual behavior
- Calcium-dependent phosphorylations inhibit in vitro aromatase activity
- Sexual interactions with a female rapidly down-regulate preoptic aromatase activity
- Membrane-initiated actions of neuroestrogens activate male sexual motivation
- Estrogens exert genomic and non-genomic effects on behavior in different time frames

Figure 1. Annual variations in plasma testosterone concentrations and frequencies of sexual behaviors or social displays (A) and correlations between individual frequencies of social displays and plasma testosterone concentrations at the peak of spring behavioral activity (B) in male domestic ducks

During the annual cycle, changes in plasma testosterone are clearly predictive of the changes in frequencies of the two types of behaviors but when the behavior is fully established in spring, the individual differences in behavior and testosterone are not related. The Spearman rank correlation between social displays and testosterone is only -0.28 (Not significant; see panel B for individual points) and is even smaller between sexual behaviors and testosterone

($R_s=0.05$). Redrawn from data in (Balthazart & Hendrick, 1976) and (Balthazart *et al.*, 1977a)

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Figure 2. Time course of the activation of sexual behavior and of aromatase induction by testosterone in castrated male quail

A. Percentage of birds displaying copulatory behavior, as indicated by mount attempts, following 1 to 8 days of treatment with testosterone. **B.** Aromatase activity in the preoptic area of the same males as in panel A. A detectable increase is seen after 8 hours but the effect only becomes significant after 16 hours. **C.** Activation of sexual behavior, as illustrated here by the frequency of neck grabs (means±SEM), following 1 to 14 days of treatment with testosterone (CX+T 1 to 14); castrated birds not treated with testosterone were also studied on days 1 and 14 (CX 1/14). **D.** Induction of aromatase immunoreactivity in the medial preoptic nucleus as measured by the fractional area covered by aromatase-immunoreactive (ARO-ir) material (means±SEM) in the same males as in panel C. **E.** Photomicrographs illustrating the induction of aromatase immunoreactivity after 1 or 14 days of exposure to testosterone as compared with control birds on day 1. Redrawn and modified from data in (Balthazart *et al.*, 1990c) (panels A,B) and in (Charlier *et al.*, 2008) (panels C–E).

Figure 3. Rapid changes in aromatase activity in quail preoptic area-hypothalamus implants maintained in vitro (A) and in the medial preoptic nucleus (POM) and tuberal hypothalamus (Tuber) of male quail after sexual interactions with a female (B)

A–B. After 20 min of equilibration, one set of explants was exposed to either an increased concentration of potassium (K⁺ in A) or to 100 mM Kainate (in B) for 10 min and then the initial incubation medium was restored while the contralateral explants stayed throughout in the initial medium (Control). In B, the dotted line indicates the stability of aromatase activity after exposure to kainate if the explants are continuously exposed to an excess of the kainate receptor blocker NBQX. Aromatase activity (mean±SEM) is expressed in percentage of the stabilized activity during the 15 to 20 min period. **C.** Effect of the sexual interaction with a female during 0 (control) or 2 to 15 min on aromatase activity. Redrawn and modified from data in (Balthazart *et al.*, 2001) (Panel A), (Balthazart *et al.*, 2006) (Panel B) and (de Bournonville *et al.*, 2013) (Panel C)

Figure 4. Rapid effects of estrogens on male sexual behavior in quail

A. Injection of a bolus of estradiol increases by comparison with control birds (gray area) the frequency of cloacal contact movements (CCM) performed 15 but not 5 or 30 minutes later. **B.** The i.c.v. injection of the aromatase inhibitor Vorozole™ (VOR) inhibits the frequency of rhythmic cloacal sphincter movements (RCSM) 30 minutes later in comparison with injection of the vehicle (Veh) and this effect is blocked by the injection 15 minutes before the test of estradiol coupled to biotin (E2 Biot.), which prevents entry of the steroid into cells. Castrated testosterone-treated males were tested repeatedly in a randomized order after the different injections as well as without injection before (Pre) and after (Post) the treatments to ensure that there was no long-lasting effects of the injections. **C.** A similar experimental approach showed that the inhibitory effects of VOR can be blocked by the ER β agonist DPN but not by the ER α agonist PPT and these two compounds have no synergistic effect. **D.** The blockade by DPN of the VOR-induced inhibition is no longer present when males are injected with the antagonist of the metabotropic glutamate receptor 1 called LY367385. Redrawn from data in (Cornil *et al.*, 2006a) (panels A), (Seredynski *et al.*, 2013) (panel B) and (Seredynski *et al.*, 2015) (panels C–D).