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Dual action of neuro-estrogens in the regulation of male sexual behavior

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Abstract

Estrogens derived from brain testosterone aromatization (neuro-estrogens) are critical for the activation of male sexual behavior. Their effects on this behavior are typically associated with longterm changes in circulating levels of testosterone and the transcriptional activity of their liganded nuclear receptors. According to this view, neuro-estrogens would prime the neural circuits controlling the long-term expression of behavior, which would then be acutely regulated by neurotransmitter systems conveying information from the social environment. In parallel, neuro-estrogens are also able to produce much faster effects than previously anticipated. Our recent investigations in Japanese quail revealed an interesting dichotomy in the regulation of male sexual behavior by membrane- and nuclear-initiated estrogen signaling providing respectively an acute modulation of sexual motivation and a long-term control of the capacity to display the copulatory sequence. In parallel, a similar dichotomy applies to the regulation of brain aromatase whose expression depends on the transcriptional activity of testosterone metabolites while its enzymatic activity is rapidly regulated in a region- and context-dependent manner. Recent evidences suggest that rapid changes in sexual motivation result from rapid changes in local estrogen production. Together, these data support the idea that the acute regulation of some aspects of male sexual behavior depends not only on classical neurotransmitter systems, but also on rapid and spatially restricted changes in local estrogen availability. The existing literature suggests that this acute regulation by neuro-estrogens of the motivational aspects of behavior could be generalized to other systems such as singing behavior in songbirds.

Keywords

aromatase; Japanese quail; glutamate; estradiol; preoptic area; sexual motivation

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1. Introduction – the classical view of sex steroid action on behavior

Steroid hormones and neurotransmitters are defined as very distinct chemical messengers based on their site of synthesis, the type of receptors they activate and the type of response they produce. Steroids are secreted by glands such as the gonads and the adrenals and travel long distances in the bloodstream to reach their targets. They act on nuclear receptors which are ligand activated transcription factors that bind to specific sequences in the promoter region of target genes (McEwen and Alves, 1999). The resulting changes in gene transcription lead at the organismal level to relatively slow effects (hours to days) that persist over extended periods of time. This mode of action is often qualified as genomic but the term nucleus-initiated signaling is often preferred now to distinguish it from membrane-initiated actions (see below).

On the other hand, neurotransmitters are produced within the central nervous system and secreted in the synapse where they travel infinitely short distances to reach their targets. They activate membrane ligand gated ion channels or G protein coupled receptors producing extremely rapid cellular effects resulting in organismal effects observed within seconds to minutes.

Steroid action is often studied in the context of long-term changes of their circulating concentration whose time course is in good agreement with the time course provided by their transcriptional mode of action. The modulation of the concentration of proteins involved in neurotransmission, axonal growth, or neurogenesis explains well the long-term variations in behavior expression. By contrast, the acute regulation of behavior is often considered to depend exclusively on neurotransmitter systems. But steroid hormones, estrogens in particular, can modulate cellular function much more rapidly than predicted based on their genomic actions (Kelly and Ronnekleiv, 2002). These effects are explained by the activation of membrane estrogen receptors that activate a variety of intracellular cascades leading to changes in cell activity translating in rapid modulations of behavior (Cornil et al., 2012a; Remage-Healey, 2014; Heimovics et al., 2015; Rudolph et al., 2016). Here, we will summarize the work conducted in our lab, which has contributed along with the work of others to a better understanding of the regulatory mechanisms of male sexual behavior by brain-derived estrogens.

2. The brain as a source of estrogens (neuro-estrogens)

The gonads and the adrenals are commonly known sources of steroid hormones. Steroid hormones are also produced in the brain either from *de novo* synthesis or through conversion from a precursor coming from the periphery. This is in particular the case for estrogens whose production by aromatization of testosterone in the brain is required to activate the full spectrum of effects of testosterone on behavior (Balthazart and Ball, 2013). Aromatase is expressed in the brain of all vertebrates where it is abundantly found in the hypothalamus and the preoptic area, although it is also present in many other areas including the cortex. Aromatase is constitutively expressed in neurons where it is found in cell bodies and processes but also in synaptic terminals (Schlinger and Callard, 1989; Naftolin et al., 1996; Saldanha et al., 2000; Peterson et al., 2005). One function of central aromatization is the

control of male sexual behavior in the preoptic area (Balthazart et al., 2004; Roselli et al., 2009).

3. Neuro-estrogens acutely modulate sexual motivation

The acute effects of estrogens on sexual behavior were first documented in male rats (Cross and Roselli, 1999). Using Japanese quail as an animal model, we later confirmed and extended this observation. First, systemic administration of 17 β -estradiol (E₂) facilitates copulatory behavior within 30 to 45 minutes in castrated males chronically treated with a dose of testosterone that is not sufficient to activate sexual behavior by itself (Cornil et al., 2006c). Conversely, systemic treatment with aromatase inhibitors rapidly inhibited measures of sexual motivation as well as copulation with a more prominent effect on sexual motivation (Cornil et al., 2006b). This distinction was subsequently confirmed by blocking estrogen action or synthesis in the brain. Indeed, acute intracerebroventricular injections of the steroid or non-steroid aromatase inhibitors, ATD and vorozole respectively, robustly reduced within 15 minutes the frequency of female evoked rhythmic cloacal sphincter movements (RCSM) and the learned social proximity response, two measures of sexual motivation (Seredynski et al., 2013; Seredynski et al., 2015). Yet, these treatments were without effect on copulation unless it was measured in a context where the male had to actively pursue the female. Similarly, tamoxifen and ICI 182,780, two general estrogen antagonists, rapidly decreased RCSM frequency but not copulation (Seredynski et al., 2013). Together these observations thus indicate that brain derived estrogens participate in the rapid control of sexual motivation but not in the ability to perform the stereotyped copulatory sequence.

Furthermore, a concurrent injection of E₂ along with the aromatase inhibitor prevented the behavioral effects induced by estrogen depletion. The restorative effect of E₂ vanished within 2 hours and was mimicked by biotinylated-E₂ demonstrating that this effect is transient and initiated at the cell membrane (Seredynski et al., 2013; Seredynski et al., 2015). Finally, central E₂ injection without aromatase blockade did not raise RCSM frequency above baseline levels suggesting that untreated males may maintain under these conditions a maximal production of neuro-estrogens which might help maintain a high level of motivation (Seredynski et al., 2015). The rapid regulation of behavior by estrogens identified here above would thus depend on membrane-initiated events but the receptor involved in these effects was unknown until recently.

To determine the identity of the receptor involved in this effect, the same experimental paradigm was used in which E₂ or the general estrogen antagonist was respectively replaced by an agonist or an antagonist specific to one of the known estrogen receptors (ER). In the past 15 years, three novel ERs have been identified: G-protein coupled estrogen receptor 1 (GPER1, also known as GPR30), ER-X and Gq-mER (Micevych et al., 2015). These receptors are all considered to be membrane specific based on their molecular structure and/or their physiological properties (Toran-Allerand et al., 2002; Revankar et al., 2005; Qiu et al., 2008). It should be noted however that the localization of GPER1 to the membrane has been challenged (Langer et al., 2010; Micevych et al., 2015) and that the molecular structure as well as the corresponding gene coding for ER-X and Gq-mER remains unknown. Based

on our finding that E₂ effect was mimicked by membrane impermeant estrogens (see also below), it could have been anticipated that this effect involved one of the newly discovered membrane estrogen receptors (mER). But none of the drugs specifically targeting these receptors were able to mimic the effect of E₂. Indeed, the specific agonists of GPER1 (G1), Gq-mER (STX) and ER-X (17 α -ER) could not prevent the reduction in RSCM frequency induced by central aromatase inhibition and the specific GPER1 antagonist (G15) did not affect behavior either (Seredynski et al., 2015).

Recent evidence had however shown that the classical nuclear receptors were able to translocate to the membrane where they can signal like G-protein coupled receptors (Micevych and Mermelstein, 2008) and we had shown that the general antagonists of nuclear ER acutely reduced RSCM frequency (Seredynski et al., 2013). Using drugs targeting ER α and ER β , we found no effect of ER α specific drugs, but DPN, the ER β agonist, did prevent the behavioral inhibition induced by acute estrogen depletion in a manner similar to E₂ (Levin, 2011; Seredynski et al., 2015).

At the membrane, nuclear ERs associate with a variety of proteins including receptors for growth factors, chemokines and metabotropic receptors for neurotransmitters (Mermelstein, 2009; Le Romancer et al., 2011; Levin, 2011). In particular, both ER α and ER β couple to different subtypes of metabotropic glutamate receptors (mGluR), resulting in the activation of distinct intracellular cascades in a region specific manner (Boulware et al., 2005; Grove-Strawser et al., 2010; Meitzen and Mermelstein, 2011) and the regulation of different behaviors (Dewing et al., 2007; Boulware et al., 2013; Martinez et al., 2014). Testing the possible implication of mGluRs in the modulation of sexual motivation, we first found that blocking mGluR1a but not mGluR2/3 and mGluR5 decreases the frequency of RSCM. More importantly, antagonizing this specific mGluR subtype blocked the effect of E₂ and DPN, the ER β agonist, on RSCM thus demonstrating that the acute modulation of male sexual behavior by brain-derived estrogens likely depends on the transactivation of mGluR1 by ER β (Seredynski et al., 2015).

4. Copulatory behavior depends on the transcriptional activity of estrogens

The results discussed here above indicate that sexual motivation is regulated in a rapid and transient manner by membrane-initiated effects of neuro-estrogens. No such effect was observed on copulation unless the males were tested in a context in which they had to actively chase the female to achieve copulation (Seredynski et al., 2013). Indeed, if tested in a small enclosure in which they had an easy access to the female, males were perfectly able to display the stereotyped copulatory response regardless of the treatment. Thus although approach behavior and RSCM frequency are severely reduced by central aromatase inhibition, the ability to perform a coordinated motor response is not altered by an acute depletion in local brain estrogens. Interestingly, the acute restorative effect of E₂ (or the membrane impermeant bovine serum albumin conjugated estrogen, E₂-BSA) on RSCM frequency was also observed following chronic aromatase blockade while again no such effect was found on copulatory behavior (Seredynski et al., 2013). As opposed to sexual motivation, copulatory behavior thus seems to exclusively depend on the long-term activity of brain-derived estrogens. Interestingly, knocking down SRC-2, a key regulator of steroid-

dependent transcription, significantly down-regulates copulatory behavior but has no effect on sexual motivation (Niessen et al., 2011). Therefore, the transcriptional activity of estrogens and androgens appears necessary for sexual performance, but not motivation. Among the potential target genes of these effects is aromatase. Indeed, the regulation of its expression limits the effect of testosterone on behavior activation (Balthazart et al., 2009). Proteins involved in changes in neural plasticity such as the growth of the medial preoptic nucleus (POM), a key site for the activation of male sexual behavior by testosterone, are likely targets as well (Balthazart and Ball, 2007).

5. Dual action of estrogens on male sexual behavior

This dichotomy in the effects of membrane- and nucleus-initiated actions of estrogens on male sexual behavior led to the formulation of the dual action of estrogen hypothesis which postulates that estrogens exert distinct but complementary effects operating in different timeframes and on different aspects of the same behavior (Cornil et al., 2015). On the one hand, their nucleus-initiated effects exert a long-term control on the circuits regulating copulatory behavior. Such processes involving the synthesis but also the integration in functional complexes of proteins implicated in neurotransmission, axonal growth and/or neurogenesis takes time to fully mature. Such a time course matches the time course provided by transcriptional activity of steroids. On the other hand, membrane-initiated effects modulate sexual motivation in a timeframe generally associated to neurotransmitters. In sexually mature males whose circuits controlling sexual behavior are fully activated following long-term exposure to both estrogens and androgens, these membrane-initiated effects would thus acutely regulate their motivation to engage in sexual encounters. Yet, the acute changes in behavior induced by membrane-initiated effects of estrogens are only possible if local estrogen levels rapidly change prior to or during the sexual encounter.

6. Mechanisms of acute regulation of aromatase activity (AA)

As alluded to earlier, aromatase itself is a target of both estrogens and androgens and extensive work demonstrated that increased aromatase expression is one of the key events in the activation of male sexual behavior by testosterone (Balthazart et al., 2009). Taking advantage of the higher expression of aromatase in the brain of birds compared to mammals, it was serendipitously found that aromatase activity (AA) is rapidly and transiently inhibited in quail preoptic hypothalamic (HPOA) explants by potassium induced depolarizations or exposure to glutamate agonists (Balthazart et al., 2001; Balthazart et al., 2006). This suggested that estrogen synthesis is acutely regulated by changes in neuronal activity. The same conclusion was reached a few years later based on acute changes in brain estrogen levels following similar manipulations conducted *in vivo* in the caudal medial nidopallium (NCM) of zebra finches (Remage-Healey et al., 2008; Remage-Healey et al., 2011). In the avian brain, acute estrogen provision is therefore inversely related to neuronal activation. In rat hippocampus, however, activation of kainate or NMDA glutamate receptors increases AA (Hojo et al., 2004; Sato and Woolley, 2016). Whether this discrepancy reflects a sub-regional or a species difference remains unclear at present.

Extensive *in vitro* work mainly conducted in quail HPOA homogenates was then undertaken to determine what were the mechanisms underlying these rapid enzymatic changes. This work testing the effect of high but physiological concentrations of ATP, Mg^{2+} and Ca^{2+} revealed that avian AA is rapidly inhibited by calcium dependent phosphorylations of the enzyme itself (Balthazart et al., 2001; Balthazart et al., 2003; Charlier et al., 2011a; Konkle and Balthazart, 2011). Similar work conducted in zebra finch extended this conclusion to the nidopallium and hippocampus but also revealed potentially important species and region differences such as a more prominent role of Mg^{2+} compared to Ca^{2+} in zebra finch as well as stimulatory effects of ATP at higher doses in the hippocampus and the auditory cortex of zebra finch (Cornil et al., 2012b; Comito et al., 2015). Interestingly, *in vitro* and *in vivo* work conducted in zebra finch telencephalon suggested that this regulation preferentially takes place at the synaptic level thus providing a mechanism for acute regulation of local E_2 levels with a very high spatial and temporal resolution (Remage-Healey et al., 2011; Cornil et al., 2012b).

7. Aromatase activity is acutely regulated by behaviorally relevant cues

As alluded to here above, retrodialysis of glutamate resulted in reduced local extracellular E_2 levels in zebra finch nidopallium as measured by *in vivo* microdialysis (Remage-Healey et al., 2008). This was recently confirmed in quail where an *in vivo* injection of kainate targeted to the POM of one hemisphere resulted in a significant decrease of AA measured post-mortem in POM microdissections that received kainate compared to the vehicle-injected side (de Bournonville et al., 2017). Only injections that successfully hit POM affected AA thus demonstrating the site specificity of this effect.

Previous studies had shown an increase in the extracellular concentration of glutamate during sexual encounters that culminates at ejaculation (Dominguez et al., 2006). Using *in vivo* dialysis, we measured variations in extracellular concentration of glutamate in samples collected every 3 minutes from the POM of freely behaving males. The presence of a female induced a rise of glutamate only in males that displayed at least one copulatory bout and had a probe located in the POM, compared to males with a probe outside POM. Interestingly, the highest peaks of glutamate were observed in samples immediately following a copulatory event (de Bournonville et al., 2017). Therefore, in quail as in rats, glutamate is released at the end of the copulatory sequence. In addition to its direct role on behavior (Dominguez et al., 2007; Vigdorchik et al., 2012), glutamate might thus also regulate local E_2 synthesis.

Based on the inhibitory role of glutamate on AA and its profile of release during sexual interactions, it can be hypothesized that brain AA should be reduced after copulation. This hypothesis is supported by *ex vivo* AA assays on HPOA or POM collected shortly after mating (Cornil et al., 2005; de Bournonville et al., 2013). Decreased AA is detected as fast as 5 min in POM and within 2 min in the mediobasal hypothalamus and for less than 2 hours suggesting this acute change in AA could occur via post-translational modifications such as phosphorylations (de Bournonville et al., 2013). These observations thus support the notion that AA is down-regulated after copulation and the associated reduction in local estrogen concentration could be associated to the initiation of a post-ejaculatory refractory period. Yet, these enzymatic changes follow rather than precede the behavioral down-regulation and

are specifically tied to the presence of the female suggesting that an elevation of AA, and presumably of local E₂ level, is important during the appetitive (motivation) rather than the consummatory (performance) phase of the behavior (de Bournonville et al., 2013). Surprisingly, AA was also rapidly reduced in the in the bed nucleus of the stria terminalis (BST) of males visually exposed to a female compared to unexposed males (Cornil et al., 2005; de Bournonville et al., 2013). These observations do not seem to match with E₂ effects on motivation (see section 3).

To make things more complicated, mild restrain stress up-regulates AA within 5 minutes in a sex- and region-dependent manner (Dickens et al., 2011, 2013) and the effect of 15 min of stress in the POM is counteracted within 5 minutes by copulation thus demonstrating how labile these enzymatic changes are (Dickens et al., 2012). Importantly, the down-regulation of preoptic AA induced by sexual encounters is paralleled by a similar reduction in estrogen content. This is however not the case for stress which results in an up-regulation of AA but a down-regulation in E₂ content (Dickens et al., 2014). There are several explanations for these discrepancies including differential effects of the treatments on the availability of aromatase substrates or on estrogen elimination; but the bottom line is that post-mortem AA cannot be used as a proxy for local E₂ concentration. This conclusion is supported by the observation in white crowned sparrows of rapid changes in local E₂ content in response to territorial intrusion with no parallel changes in AA (Charlier et al., 2011b).

The variations in local E₂ levels should thus be studied in live animals. We thus used microdialysis to investigate the changes in extracellular E₂ concentration in the POM of freely behaving quail with samples collected every 10 minutes and assayed for E₂ by radioimmunoassay using the method pioneered in zebra finch (Remage-Healey et al., 2008). Surprisingly, a dramatic increase in extracellular E₂ concentration was detected in the first sampling period during which males copulated with the female, while no such change was observed during the pre-copulatory visual interaction (de Bournonville et al., 2016). This rise peaked at 350% of the pre-interaction baseline after 30 min and remained elevated for 1 hour. This observation contradicts the *ex vivo* data described here above and raises numerous questions. What is the origin of this discrepancy? Is it real or does it reflect a technical artifact? What is the functional significance of this rise in local E₂ levels during copulation?

It should be noted that the *ex vivo* AA assays were carried out in conditions optimized to detect low levels of enzymatic activity (e.g. saturating concentrations of enzymatic substrate and co-factor). The availability of these factors likely fluctuates *in vivo* thus contributing to changes in the estrogen output independent of changes in AA. In particular, the social/sexual context can induce changes in local androgen availability resulting from changes in gonadal secretion (For review see: (Harding, 1981; Wingfield and Silverin, 2009; Cornil et al., 2012a)) or brain synthesis (Pradhan et al., 2010a). In parallel, changes in E₂ degradation by hydroxylation or conjugation could result in variations in local E₂ levels (Cornil et al., 2006a). Interestingly, besides its role in E₂ synthesis, aromatase appears to also regulate estrogen hydroxylation via changes in local pH and might thus provide a fine and local regulation of E₂ levels (Osawa et al., 1993; Balthazart et al., 1994).

Finally, despite these discrepancies that will require further investigation to be understood, our work along with this of others converges to provide evidence that sexual/social interactions can drive changes in the activity of steroid synthesizing enzymes in the brain which in parallel with changes in steroid elimination result in rapid fluctuations in local E₂ levels (Cornil et al., 2005; Remage-Healey et al., 2008; Remage-Healey et al., 2009; Pradhan et al., 2010b; Charlier et al., 2011b; de Bournonville et al., 2013; Dickens et al., 2013; Dickens et al., 2014; de Bournonville et al., 2016).

8. Summary and conclusions

In summary, in quail HPOA, AA is regulated by two distinct mechanisms that operate in two distinct timeframes: the transcriptional activity of both androgens and estrogens provides a long-term regulation of the enzyme concentration, while post-translational modifications acutely control its enzymatic activity. These two timeframes fit in well with the time course of the two modes of action of estrogens which cooperate to the fine regulation of male sexual behavior: while nucleus-initiated signaling is necessary to activate the circuits underlying the display of the coordinated motor sequence, membrane-initiated signaling acutely controls the motivation to engage in behavior, a regulation that was previously thought to exclusively depend on neurotransmitters. Interestingly, the dichotomy in the regulation of the two phases of behavior may also concern different receptors and brain sites. Indeed, the rapid regulation of sexual motivation depends on ER β (Seredynski et al., 2015) while studies in mice lacking the classical ERs point at ER α as the major player in the regulation of the transcriptional activity of estrogens on reproductive behaviors and physiology (Reviewed in (Rissman, 2008)). Previous work based on brain lesions and behavior induced immediate early gene expression suggests the existence of a dichotomy in the sub-regions of the MPOA controlling the two aspects of the behavior (Balthazart et al., 1998; Taziaux et al., 2005).

Recent evidence revealed that testosterone regulates the motivation to sing and song quality at two distinct sites, POM and HVC respectively (Alward et al., 2013). These nuclei express ERs (Balthazart et al., 1996; Metzdorf et al., 1999) and are the sole nuclei of the song system supplied by local E₂ production (Saldanha et al., 2000). Chronic aromatase blockade in zebra finch inhibits song production (Walters and Harding, 1988), while acute aromatase blockade rapidly alters song production and quality (Alward et al., 2016). A dual regulation of behavior by estrogens could thus also apply to singing behavior. Over the past ten years, estrogens have been found to rapidly regulate a variety of physiological and behavioral processes from social behaviors to cognition. A similar dichotomy in the regulation of distinct aspects of a same physiological or behavioral response may thus apply to many other responses (Cornil et al., 2012a; Heimovics et al., 2015; Micevych et al., 2015).

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Abbreviations

AA	aromatase activity
ATD	androstatrienedione
ER	estrogen receptors
HPOA	hypothalamus and preoptic area
mER	, membrane estrogen receptors
mGluR	metabotropic glutamate receptors
MPOA	medial preoptic area
POM	medial preoptic nucleus
NCM	caudal medial nidopallium (NCM)
RCSM	rhythmic cloacal sphincter movements
SRC-2	steroid receptor coactivator 2

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Highlights

- Neuro-estrogens acutely stimulate male sexual motivation but not performance
- The transcriptional activity of steroids exclusively control copulatory performance
- Brain aromatase activity (AA) is acutely regulated by changes in neuronal activity
- Preoptic AA and brain E₂ levels fluctuate rapidly during sexual interactions