Rapid Modulation of Aromatase Activity in the Vertebrate Brain

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Abstract: Numerous steroid hormones, including 17β-estradiol (E2), activate rapid and transient cellular, physiological, and behavioral changes in addition to their well-described genomic effects. Aromatase is the key-limiting enzyme in the production of estrogens, and the rapid modulation of this enzymatic activity could produce rapid changes in local E2 concentrations. The mechanisms that might mediate such rapid enzymatic changes are not fully understood but are currently under intense scrutiny. Recent studies in our laboratory indicate that brain aromatase activity is rapidly inhibited by an increase in intracellular calcium concentration resulting from potassium-induced depolarization or from the activation of glutamatergic receptors. Phosphorylating conditions also reduce aromatase activity within minutes, and this inhibition is blocked by the addition of multiple protein kinase inhibitors. This rapid modulation of aromatase activity by phosphorylating conditions is a general mechanism observed in different cell types and tissues derived from a variety of species, including human aromatase expressed in various cell lines. Phosphorylation processes affect aromatase itself and do not involve changes in aromatase protein concentration. The control of aromatase activity by multiple kinases suggests that several amino acids must be concomitantly phosphorylated to modify enzymatic activity but site-directed mutagenesis of several amino acids alone or in combination has not to date revealed the identity of the targeted residue(s). Altogether, the phosphorylation processes affecting aromatase activity provide a new general mechanism by which the concentration of estrogens can be rapidly altered in the brain.

Keywords: testosterone, estrogens, Japanese quail, hypothalamus, 17β-estradiol, phosphorylation, medial preoptic nucleus, songbird, caudal medial nidopallium
Introduction
Many of the biological effects of steroid hormones are mediated through the activation of their respective nuclear receptors to regulate gene transcription. Modulation of gene transcription in turn affects the targeted cell physiology and ultimately modifies the organism’s physiology and behavior. These effects usually develop relatively slowly after hormone exposure but usually last for a long period. However, several laboratories, including ours, have now described major physiological changes triggered by steroid hormones that are too rapid to result from de novo mRNA transcription and protein synthesis. For example, the acute elevation of steroid hormones in vitro triggers the activation of numerous intracellular signaling pathways, including the modulation of intracellular calcium concentrations and the phosphorylation of a variety of proteins such as the mitogen-activated protein kinase (MAPK) and cAMP response element binding protein (CREB).5–6 Importantly, these fast changes induced by steroid hormones at the molecular levels were also shown to rapidly modulate neuronal activation in various brain regions, and, in some cases, were shown to acutely affect behavior.7–12 In particular, one of these steroid hormones, 17β-estradiol (E2), has been the focus of an extensive research, and our lab, amongst others, has shown that the specific activation of estrogen receptors leads to rapid modulation of behavior, including motivation to approach and copulate with a female.13–14 While the rapid effects of steroid hormones have received a lot of attention, there remain numerous questions concerning how this rapid change in steroid concentration can occur. If steroid hormone action is in numerous instances similar to a neurotransmitter or at least to a neuromodulator,15 steroids cannot, however, be stored in synaptic vesicles before rapid release due to their lipophilic nature. Our work focused on the mechanism that could rapidly affect E2 concentrations, and we hypothesized that a rapid control of estrogen synthesis via local changes in estrogen synthase, or aromatase, activity mediates the fast effect of estrogens on physiology and behavior. This review will present evidence that aromatase activity (AA) can be controlled rapidly by posttranslational modifications, allowing for a potential rapid and local control of estrogen concentrations.

Control of Brain Aromatase Activity
The enzyme aromatase catalyzes the synthesis of estrogens from androgens and is present in numerous well-defined brain regions. The presence of aromatase in specific brain nuclei is likely to control steroid potency (production and thus action of estrogens as opposed to androgens) and increases the local concentration of estrogens within a specific brain region (Fig. 1).16–20 Changes in aromatase activity often reflect changes in aromatase concentration resulting from the slow variation of the synthesis of this protein at the transcriptional level. However, the rapid effects of E2 introduced above request a more rapid regulation of estrogen concentration and require mechanisms rapidly affecting the synthesis of the steroid. It is only recently that rapid changes in AA were demonstrated to occur in vivo in response to changes in the environment. Performance of sexual behavior can indeed rapidly affect AA in specific brain regions: a rapid and transient change in AA was detected in preoptic-hypothalamic area of quail after a 5 to 30 minute sexual encounter with a receptive female (decrease21,22) and after acute restraint stress (increase23). These rapid changes in enzymatic activity could thus produce fast changes of local estrogen concentration in behaviorally relevant situations. Similarly in zebra finches, a brief exposure to songs (30 minutes) resulted in an increase of AA in NCM brain region (Fig. 1), principally in synaptic terminals.24

Interestingly, these rapid changes in enzymatic activity can also be triggered in preoptic/hypothalamic explants from Japanese quail by a change in extracellular K+ concentration, glutamate receptor activation, or intracellular calcium concentration.25–28 Similarly in the zebra finch telencephalon (a K+-induced depolarization via the activation of voltage-gated calcium channels at the presynaptic level) and glutamate exposure were also shown to reduce significantly the concentration of estradiol, likely through the rapid inhibition of AA.29 The direct links between the activation of glutamatergic receptor, calcium release, and the exact intracellular pathway(s) involved in the rapid modulation of AA, however, require further investigation.

Importance of Phosphorylations
We hypothesized that these rapid modulations of aromatase activity are not mediated by transcription-
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dependent changes but rather involve rapid post-translational modifications of the protein, such as phosphorylation. We used experimental conditions known to induce phosphorylation and showed that exposure of Japanese quail preoptic area/hypothalamus homogenates to high but physiological concentrations of ATP, Ca^{++}, and Mg^{++} (ATP/Mg/Ca) significantly inhibited AA within 15 minutes. These conditions affected both male and female hypothalamus, although some of these effects in females differed from what was observed in males. In addition, phosphorylating conditions rapidly reduced AA from zebra finch telencephalon although the different subcellular compartments (synaptosome vs microsome) showed different sensitivity to the phosphorylation events.

The Role of Kinases and Phosphatases

To test whether ATP/Mg/Ca conditions induced kinase-dependent protein phosphorylations or whether these conditions lead to a nonspecific inactivation of AA, we tested the addition of various protein kinase activators and inhibitors on the AA of preoptic-hypothalamic homogenates in the presence or absence of ATP/Mg/Ca. Several of these inhibitors, such as staurosporine (a general serine/threonine (Ser/Thr) kinase inhibitor) and genistein (general tyrosine (Tyr) kinase inhibitor), significantly blocked the inhibition produced by ATP/Mg/Ca while others had no effect. These data therefore indicate that the activity of aromatase is controlled by the phosphorylation of both Tyr and Ser/Thr residues. It should be noted that the effects of phosphatase inhibitors were not clear-cut and suggested the implication of phosphorylation of some residues to reduce aromatase activity while the phosphorylation of other residues might be required to sustain this enzymatic activity (see Balthazart, et al. for in-depth discussion). We also showed that calmodulin significantly inhibited quail preoptic-hypothalamic AA both in the presence and in absence of phosphorylating conditions, suggesting that calmodulin itself interacts directly with

Figure 1. Schematic diagram representing the mechanisms involved in the rapid control of aromatase activity. Phosphorylations (P) or calmodulin rapidly reduce aromatase activity, inhibiting the transformation of testosterone (T) into 17β-estradiol (E2). It is likely that these modifications are induced by calcium-voltage channels and/or by glutamatergic receptors, although the link has not been experimentally tested. The increase of intracellular calcium (Ca^{++}), either from intracellular storage or from the activation of voltage-gated channel is in most cases a prerequisite for the inhibition of aromatase activity.

Abbreviations: Cb, cerebellum; GCL, mesencephalic central gray (periaqueductal gray); Hp, hippocampus; HVC, used as a proper name; N, nidopallium; NCM, caudal medial nidopallium; OL, optic lobe; OM, occipito-mesencephalic tract; POM, medial preoptic nucleus; RA, robust nucleus of arcopallium; Sp, Septum; nucleus; TnA, nucleus taeniae of the amygdala; V lat., lateral ventricle. B represents aromatase-immunoreactive cells in the POM, magnification bar is 50 µm.
aromatase rather than through a modulation of Ca\(^{++}\)/calmodulin-dependent protein kinases.\(^{28}\) The rapid control of brain AA thus appears to include at least 2 mechanisms: (1) a regulatory process that involves the Ca\(^{++}\)/calmodulin binding site and (2) a phosphorylation by several protein kinases (PKC, PKA as well as Ca\(^{++}\)/calmodulin kinase[s]) of the aromatase molecule. These processes are reviewed in Figure 2.

The Rapid Reduction of AA by Phosphorylations is a General Phenomenon

Investigations of the rapid modulation of AA had until recently been carried out only on quail brain tissue so that the importance of phosphorylations in the rapid control AA in other tissues or species could not be evaluated. To investigate whether the rapid inhibition of AA by ATP/Mg/Ca-dependent phosphorylation processes is specific to the neuronal environment or can be observed in other aromatase-rich tissues, effects of phosphorylating conditions were quantified in ovary and ovarian follicles homogenates. These experiments demonstrated a drastic decrease in enzymatic activity within 15 minutes.\(^{33}\) We also stably expressed human aromatase in several cell lines, including HEK293 (human embryonic kidney), C6 (rat glioma) and Neuro2A (mouse neuroblastoma). Similarly to what was observed in the preoptic area/hypothalamus of Japanese quail and telencephalon of zebra finch, a KCl-induced depolarization triggered a pronounced inhibition of AA expressed in HEK293 cells. This effect was transient and could be fully reversed when cell cultures returned to control conditions. Importantly, we also demonstrated that the rapid inhibition of human AA in HEK293 cells does not involve aromatase degradation since the concentration of the protein was not affected: the amount of aromatase protein quantified by Western blot analysis with actin used as an internal standard was similar after depolarization and in control conditions. Interestingly, the rapid enzymatic inhibition induced by depolarization involved the activity of protein kinases. Addition of staurosporine (Ser/Thr kinase inhibitor) or genistein (Tyr kinase inhibitor) blocked the effect of KCl-induced depolarizations on AA.\(^{33}\) The importance of protein phosphorylation was further confirmed by the demonstration that a 15-minute preincubation in phosphorylating conditions (ATP/Ca/Mg) significantly reduced the activity of human aromatase from cell lysates as compared with matched control samples (in HEK293, C6 and Neuro2A). These results indicate that the modulation of AA by phosphorylations is a general process,

![Diagram](image-url)
present not only in birds, but also presumably in humans and other mammals. Interestingly, we also showed that phosphorylating conditions do not affect the apparent enzyme affinity for its substrate but only change the maximum velocity of reaction.

**AA Inhibition is Associated with Phosphorylations of the Aromatase Protein**

The experiments summarized above strongly suggest that phosphorylation processes rapidly and transiently regulate AA. However, because all assays were carried out on cell lines, brain homogenates or in vivo and not on purified aromatase protein, they did not address the question of whether phosphorylations controlling enzymatic activity directly affect the aromatase itself or another coexisting protein that could secondarily regulate aromatase. To test whether phosphorylations underlying the rapid modulation of AA target the aromatase protein itself, we engineered a modified human aromatase containing a c-myc tag that allows its immunoprecipitation. HEK293 cells transfected with this construct were incubated with \( \gamma^{32}P \)-ATP in phosphorylating or nonphosphorylating (control) conditions. A \( 32P \)-labeled protein was detected at the expected molecular weight for aromatase c-myc in phosphorylating conditions while only a faint band was present in control conditions. In parallel experiments, the immunoprecipitated protein visualized with antiphosphoserine similarly showed an immunoreactive band at the expected molecular weight after 5 minutes of incubation of the cell lysate in phosphorylating conditions (ATP/Ca/Mg\(^{33}\)), confirming previous experiments on immunoprecipitated quail aromatase. Altogether, these experiments demonstrate that the aromatase protein itself is rapidly phosphorylated in the presence of ATP/Mg/Ca and strongly suggest that these phosphorylations directly cause the rapid decrease of enzymatic activity.

**Identification of Aromatase Residues Involved in the Rapid Control of Activity**

Pharmacological experiments on quail hypothalamus homogenates and HEK293 expressing human aromatase indicated that the inhibition of AA by phosphorylation is mainly catalyzed by the activity of 2 Ser/Thr kinases: protein kinase A (PKA) and protein kinase C (PKC). Based on this knowledge, we used bioinformatic tools (NetPhos 2.0 and NetPhosK 1.0) to analyze the quail and human aromatase coding sequences and identified several potential phosphorylation sites, highly conserved among different avian and mammalian species. From these results, we focused our attention on 6 different residues: S247, S267, and S497, (which had high scores in both the predictive phosphorylation sites, and PKA or PKC recognition consensus sequences), T462 and T493 (which correspond to positions S455 and S486 in quail aromatase, 2 residues that were predicted to be involved in the phosphorylation of quail aromatase), and serine S118 based on previous data suggesting that phosphorylation of that residue affect the stability or activity of the enzyme.

Using the human aromatase as template, 6 different mutants S/T to alanine (A) were produced to determine the potential importance of these amino acids in the rapid modulation of AA by phosphorylating conditions. All mutants still expressed AA and phosphorylating conditions markedly reduced this enzymatic activity in the 6 different mutants alone or in combination roughly to the same extent as in wild type enzyme. In all cases, inhibition was more pronounced after exposure to a higher concentration of ATP. Against all expectations, these single or combined mutations did not block the rapid inhibition of aromatase by phosphorylating conditions. It is possible that a combination of several phosphorylated residues that was not tested here is required to control AA. Multiples residues of a protein are often phosphorylated in vivo, and the control of AA by multiple kinases reinforces the idea that several amino acids must concomitantly be phosphorylated to modify AA. Although we mutated amino acids with the highest phosphorylation and kinase recognition prediction scores, other consensus sites for phosphorylations and for other types of kinases were also predicted on the quail and human aromatase sequences suggesting that other amino acids could be involved. The lack of effects of these mutations on the rapid control of AA by phosphorylations could be due to numerous reasons that are discussed in more detail by Charlier et al.

Apart from the implication of specific residues in the rapid control of AA by phosphorylation, we also
observed that 2 mutants, S118A and S497A, affected basal AA. More specifically, S118A aromatase had a markedly reduced enzymatic activity, while S497A mutant showed a higher AA than the wild type and other mutants. To our knowledge, these 2 residues have not been directly implicated in substrate binding or reaction catalysis. Miller and colleagues suggested that S118 phosphorylation by PKC could be required for stabilization of aromatase, but the exact function of these 2 residues in the control of basal AA and protein stability remains to be determined.

Conclusions
In summary, numerous biochemical and pharmacological studies from our laboratory and others confirm that AA can be rapidly modulated via posttranslational modifications (see current model, Fig. 2). The rapid modulation of AA by phosphorylating conditions is a widespread mechanism, observed in several different tissues that express aromatase, and has been shown to regulate aromatase in a variety of species, including humans. Although the mechanisms leading to these modfications remain only partially understood, the experiments reviewed here demonstrate that rapid changes in AA take place in the brain and these changes will result in a local rapid modulation of estrogen production, and thus presumably availability, that will ultimately affect cellular events in the absence of changes in protein synthesis.

Abnormally high levels of aromatase activity in estrogen-dependent organs such as the uterus and breasts are associated with the development of cancer. Aromatase is therefore a target of choice for several anticancer drugs, including anastrozole (Arimidex), letrozole (Femara) or exemestane (Aromasin). Unfortunately, these treatments have many serious side effects associated with their systemic action. In addition to the reduction of aromatase activity in cancerous tissue such as the breast, bones and brain will also be affected resulting in undesirable side effects (osteoporosis, mood shifts, and hot flushes). These additional effects often lead to failure to take the correct dose or even discontinuation of the treatment by a large percentage of patients (up to 25% in some studies) Although posttranslational modifications seem to be a general mechanism controlling aromatase activity, the specific kinase(s) involved in this modification could be tissue-specific and therefore offer a target of choice to affect aromatase activity in a more specific manner. Future work should thus define in more detail the molecular mechanisms associated with aromatase phosphorylation in the brain and other tissues.

Author Contributions
Conceived and designed the experiments: TDC. Analyzed the data: TDC, CAC, JB. Wrote the first draft of the manuscript: TDC. Contributed to the writing of the manuscript: TDC, CAC, JB. Agree with manuscript results and conclusions: TDC, CAC, JB. Jointly developed the structure and arguments for the paper: TDC, CAC, JB. Made critical revisions and approved final version: TDC, CAC, JB.

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Competing Interests
Author(s) disclose no potential conflicts of interest.

Disclosures and Ethics
As a requirement of publication the authors have provided signed confirmation of their compliance with ethical and legal obligations including but not limited to compliance with ICMJE authorship and competing interests guidelines, that the article is neither under consideration for publication nor published elsewhere, of their compliance with legal and ethical guidelines concerning human and animal research participants (if applicable), and that permission has been obtained for reproduction of any copyrighted material. This article was subject to blind, independent, expert peer review. The reviewers reported no competing interests. Provenance: the authors were invited to submit this paper.

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