

Review

# The control of preoptic aromatase activity by afferent inputs in Japanese quail<sup>☆</sup>

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## Abstract

This review summarizes current knowledge on the mechanisms that control aromatase activity in the quail preoptic area, a brain region that plays a key role in the control of reproduction. Aromatase and aromatase mRNA synthesis in the preoptic area are enhanced by testosterone and its metabolite estradiol, but estradiol receptors of the alpha subtype are not regularly colocalized with aromatase. Estradiol receptors of the beta subtype are present in the preoptic area but it is not yet known whether these receptors are colocalized with aromatase. The regulation by estrogen of aromatase activity may be, in part, trans-synaptically mediated, in a manner that is reminiscent of the ways in which steroids control the activity of gonadotropic hormone releasing hormone neurons. Aromatase-immunoreactive neurons are surrounded by dense networks of vasotocin-immunoreactive and tyrosine hydroxylase-immunoreactive fibers and punctate structures. These inputs are in part steroid-sensitive and could therefore mediate the effects of steroids on aromatase activity. In vivo pharmacological experiments indicate that catecholaminergic depletions significantly affect aromatase activity presumably by modulating aromatase transcription. In addition, in vitro studies on brain homogenates or on preoptic–hypothalamic explants show that aromatase activity can be rapidly modulated by a variety of dopaminergic compounds. These effects do not appear to be mediated by the membrane dopamine receptors and could involve changes in the phosphorylation state of the enzyme. Together, these results provide converging evidence for a direct control of aromatase activity by catecholamines consistent with the anatomical data indicating the presence of a catecholaminergic innervation of aromatase cells. These dopamine-induced changes in aromatase activity are observed after several hours or days and presumably result from changes in aromatase transcription but rapid non-genomic controls have also been identified. The potential significance of these processes for the physiology of reproduction is critically evaluated. © 2001 Elsevier Science B.V. All rights reserved.

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## 1. Introduction

Sex steroid hormones act in the brain to coordinate the production of a wide variety of behavioral and physiological endpoints. The ability of steroids to exert such effects can differ between individuals of the same species (e.g. there are sex differences in the physiological response to steroids [27]) or within individuals when they are in different environments [66]. It was proposed over 15 years ago that one-way for steroid action to be affected by context would be for different environments to influence the availability of sex steroid receptors [88]. Data have now accumulated indicating that ascending catecholamine inputs to diencephalic brain nuclei containing sex steroid receptor types such as estrogen receptors (ER) and progesterone receptors (PR) can regulate the availability of ER and PR as measured by in vitro binding assays [45].

Another important component of the neural substrate that mediates the response to steroid hormones involves the local metabolism of steroids. For example, in males of many vertebrate species testosterone (T) exerts its effects at the cellular level after first being metabolized to an androgenic (via the action of the  $5\alpha$ -reductase) or estrogenic (via the action of aromatase) metabolite of testosterone [16,78]. In some cases this metabolic conversion is rate limiting for a particular physiological process. One such example is the activation of male sexual behavior in many species. In Japanese quail (*Coturnix japonica*) the aromatization of testosterone to  $17\beta$ -estradiol is necessary and sufficient for the activation of male sexual behavior [28]. Thus the regulation of aromatase activity (AA) is another route by which the environmental context could influence the effectiveness of steroids.

In this paper, we first review data from our laboratory illustrating the key role played by the aromatization of T in the medial preoptic area (POA) in the activation of male sexual behavior. We shall then describe the mechanisms that regulate the activity of this enzyme. These mechanisms are in part genomic and regulate the concentration of the enzyme. In addition, recent studies also indicate that aromatase activity can be modulated in a rapid manner by mechanisms that do not involve changes in the concentration of the enzymatic protein. These rapid regulatory

mechanisms could therefore modify the bioavailability of estrogens in the brain within minutes or even seconds. Rapid effects of estrogens on neuronal physiology or behavior have been described recently. Such changes in estrogen availability through the modification of the estrogen synthase (aromatase) could therefore provide a physiologically relevant mechanism that would control these rapid non-genomic effects of estrogens. In this context, aromatase could produce locally high concentrations of estrogens acting at the membrane level. These estrogens would then have to be considered as neuro(active)-steroids.

## 2. Aromatase and sexual behavior in quail

Male quail reliably display the full sequence of copulatory behavior including grabbing the female's neck feathers, mount and performing cloacal contact movements in strictly controlled laboratory conditions. This entire behavioral sequence is steroid-dependent. Castration eliminates these behaviors within a few days and treatment of castrated males with exogenous T rapidly restores the behaviors in most, if not all, birds in less than 1 week [3,109].

In quail as in other vertebrate species, T can be irreversibly metabolized into a variety of compounds within the central nervous system and these transformations play a key role in the activation of male behavior [16,111]. The enzyme  $5\alpha$ -reductase transforms T into  $5\alpha$ -dihydrotestosterone, a potent androgen (like T) that appears to be responsible for the development of male secondary sexual characteristics such as the growth of the cloacal gland in quail or the growth of the comb and wattles in chickens. In contrast,  $5\beta$ -reductase, the enzyme that transforms T into  $5\beta$ -dihydrotestosterone represents an inactivation pathway for the androgen at least as far as reproductive behavior is concerned. To our knowledge,  $5\beta$ -dihydrotestosterone has no or very little behavioral activity and does not bind with high affinity to androgen receptors. A third enzyme, aromatase (or estrogen synthase or  $P450_{\text{aro}}$ ) metabolizes T into estradiol. This enzyme is a member of the P450 enzyme family encoded by gene

CYP19 [75,119] and in Japanese quail, brain AA plays a critical limiting role in the activation of male sexual behavior by T. This conclusion has been derived from a variety of experiments in which either the AA was blocked by specific inhibitors or the access of locally formed estrogens to their receptor was blocked by drugs known as anti-estrogens (e.g. nitromifene citrate or tamoxifen). In both cases, pharmacological interference with the production or activity of estrogens almost completely suppressed the behavioral effects of T [28].

Morphometric analyses of the POA, a brain region known to be involved in the control of male-typical copulatory behavior in most, if not all vertebrate species, identified the presence of a sexually dimorphic nucleus. This structure, called the medial preoptic nucleus (POM) is significantly larger in volume in males than in females [132]. Furthermore the POM volume is sensitive to the circulating levels of androgens: it regresses in castrated birds and is restored to the volume typical of sexually mature males by a two week treatment with exogenous T [99,100]. These variations nicely parallel the mechanisms that control copulatory behavior in quail and it was hypothesized that the POM may be an important part of the neural pathway mediating the activation of male-typical reproductive behavior by T [25,98]. This conclusion has been fully supported by a series of behavioral experiments. Lesions of the POM decrease or abolish the expression of male copulatory behavior and the behavioral deficits are proportional to the volume of POM lesioned but not to the absolute size of the lesion. Furthermore, stereotaxic implantation of T within the cytoarchitectonic boundaries of the POM activates male copulatory behavior in castrated males but implants located elsewhere in the POA are behaviorally ineffective. Stereotaxic implants of an aromatase inhibitor or of an antiestrogen within the POM substantially decrease or suppress the activating effects of a systemic treatment with T in castrated males.

Taken together, these data indicate that T must be aromatized and locally-produced estrogens must act within the POM to activate male copulation. The preoptic aromatase in quail thus plays a key role in the control of male reproductive behavior and a number of studies were therefore devoted to the analysis of the neuroanatomical distribution of this enzyme and of the mechanisms that control its activity [25,26,98].

### 3. Control of aromatase transcription by steroids

In quail, AA is higher in the POM than in any other brain region [113]. Accordingly, the boundaries of the POM can be delineated by the high density of cells expressing aromatase as identified at the protein or mRNA levels by immunocytochemistry or in situ hybridization, respectively [9,31].

T increases AA in the POA of all species of higher

vertebrates examined so far. This effect appears to be largely mediated by the interaction of the steroid with androgen receptors in rats [106,107] but by an action of locally produced estrogens in birds [68] and in quail in particular [28]. There is a synergism between non-aromatizable androgens and estrogens in the mechanism that regulates aromatase and this synergism can be observed at the three different levels at which aromatase has been studied (the mRNA, the protein and the enzyme activity) [36,59,112] (Fig. 1).

The most parsimonious model that could explain this control of aromatase synthesis by estrogens assumes that when entering its target cells, T is aromatized in the cytoplasmic compartment into an estrogen which would then bind to estrogen receptors (ER) and activate the transcription of the aromatase gene. Surprisingly however, immunocytochemical studies revealed that the estrogen-synthesizing enzyme and the ER, although they are present

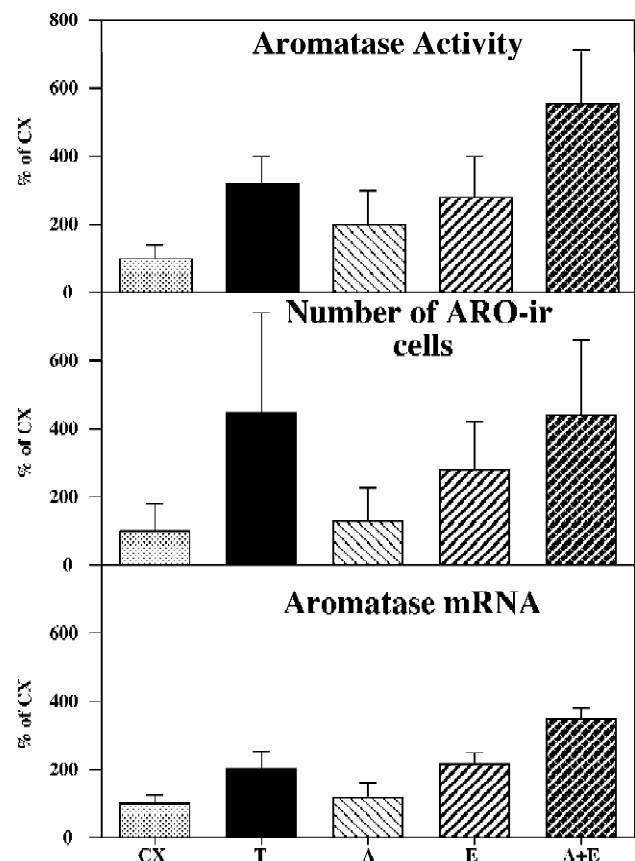


Fig. 1. Effects of testosterone (T), of a non-aromatizable androgen (A) and of an estrogen (E) alone or in combination (A+E) on aromatase activity (top), on the number of aromatase-immunoreactive cells in the POM (middle) and on the aromatase mRNA concentration (bottom) in the POA in male quail. The non aromatizable androgen was methyltrienolone in top panel and 5 $\alpha$ -dihydrotestosterone in the other two panels. The estrogen used in top panel was diethylstilbestrol. Estradiol-17 $\beta$  was used in the other panels. All data (means $\pm$ S.D.) were expressed as a percentage of corresponding values in castrates (CX) to permit direct comparisons. Recalculated and plotted from data in Refs. [59,112].

in the same brain areas (e.g. the POA, the bed nucleus striae terminalis, the ventro-medial nucleus of the hypothalamus), are not always co-localized in the same cells [34]. A large fraction (about 70–80%) of the aromatase-immunoreactive (ARO-ir) neurons in the ventro-medial hypothalamus contain immunoreactive ER, but the percentage of cells exhibiting a pattern of co-localization is far lower in the POM (approximately 18%) and bed nucleus striae terminalis (approximately 4%). A similar finding (ER co-localized with aromatase in the ventro-medial hypothalamus but not in the POA) has been reported in the neonatal mouse and rat brain [123,124]. This lack of co-localization suggests that estrogens do not control aromatase expression by binding to receptors located in the cells where they are produced. Other modes of estrogen action on AA should thus be considered.

A second type of estrogen receptor, called ER $\beta$  [72,73] to distinguish it from the previously identified receptor now renamed ER $\alpha$ , was recently described. ER $\beta$  could be located in aromatase-containing neurons and directly mediate the actions of the steroid in these neurons. Attempts to localize the ER $\beta$  in the quail brain by immunocytochemistry with commercially available antibodies raised against mammalian ER $\beta$  sequences were originally unsuccessful and it was therefore decided to clone the quail ER $\beta$  to be able to study by *in situ* hybridization the distribution of the corresponding messenger RNA.

A partial cDNA for ER $\beta$  was first isolated from testicular RNA by nested RT-PCR [74] and a combination of 3'- and 5'-RACE was subsequently used to identify the full-length cDNA sequence showing high homology with the ER $\beta$  sequenced in mammals [54]. Antisense oligonucleotide probes corresponding to different domains of the ER $\beta$  cDNA were then used for *in situ* hybridization studies in quail [54]. An extensive distribution of the ER $\beta$  mRNA was detected throughout the rostral–caudal extent of the hypothalamus, in the mesencephalic nucleus intercollicularis and in the telencephalic nucleus taeniae. An intense hybridization signal was, in particular, found in the POM. High densities of ER $\beta$  mRNA also outlined the medial part of the nucleus striae terminalis (BST) as defined by Aste and collaborators [7].

These studies indicate that ER $\beta$  is especially prevalent in the lateral part of the POM where aromatase is also expressed at high levels. It is thus possible that ER $\beta$  is colocalized with aromatase in the lateral POM and that estrogens control aromatase synthesis in an intracrine manner after binding to ER $\beta$  that are localized within the aromatase-expressing cells. However the coexistence of ER $\beta$  and aromatase in the same brain area does not necessarily prove that these two antigens are colocalized in the same cells, as already observed for ER $\alpha$ . Additional studies combining on the same sections a detection of ER $\beta$  by *in situ* hybridization with the visualization of the aromatase protein or mRNA are currently in progress to determine whether this mode of aromatase control is

compatible with the anatomical organization of the enzyme and the receptor. Data available so far suggest that ER $\beta$  may, like ER $\alpha$ , not be colocalized with aromatase in the POM but techniques should be improved to obtain full support of this negative conclusion.

If ARO-ir cells do not contain estrogen receptors (of the alpha or beta subtypes), a control of aromatase synthesis by a direct intracrine action with ER acting as transcription factors within the aromatase cells becomes difficult to entertain. Estrogens could diffuse to adjacent cells that contain ER but alternatively estrogens could also modify AA by affecting inputs to aromatase cells in a manner that would be reminiscent of what has been described in much more detail for the regulation of GnRH neurons. Although steroids exert a strong influence on the synthesis and release of GnRH, neurons producing this peptide usually contain no estrogen receptors [62,63,115] and they are controlled by an estrogen-sensitive neuronal network that involves several neurotransmitters and neuropeptides [42,52,134]. This suggestion is supported by anatomical and pharmacological results.

#### 4. Catecholaminergic and peptidergic inputs to aromatase-containing brain areas

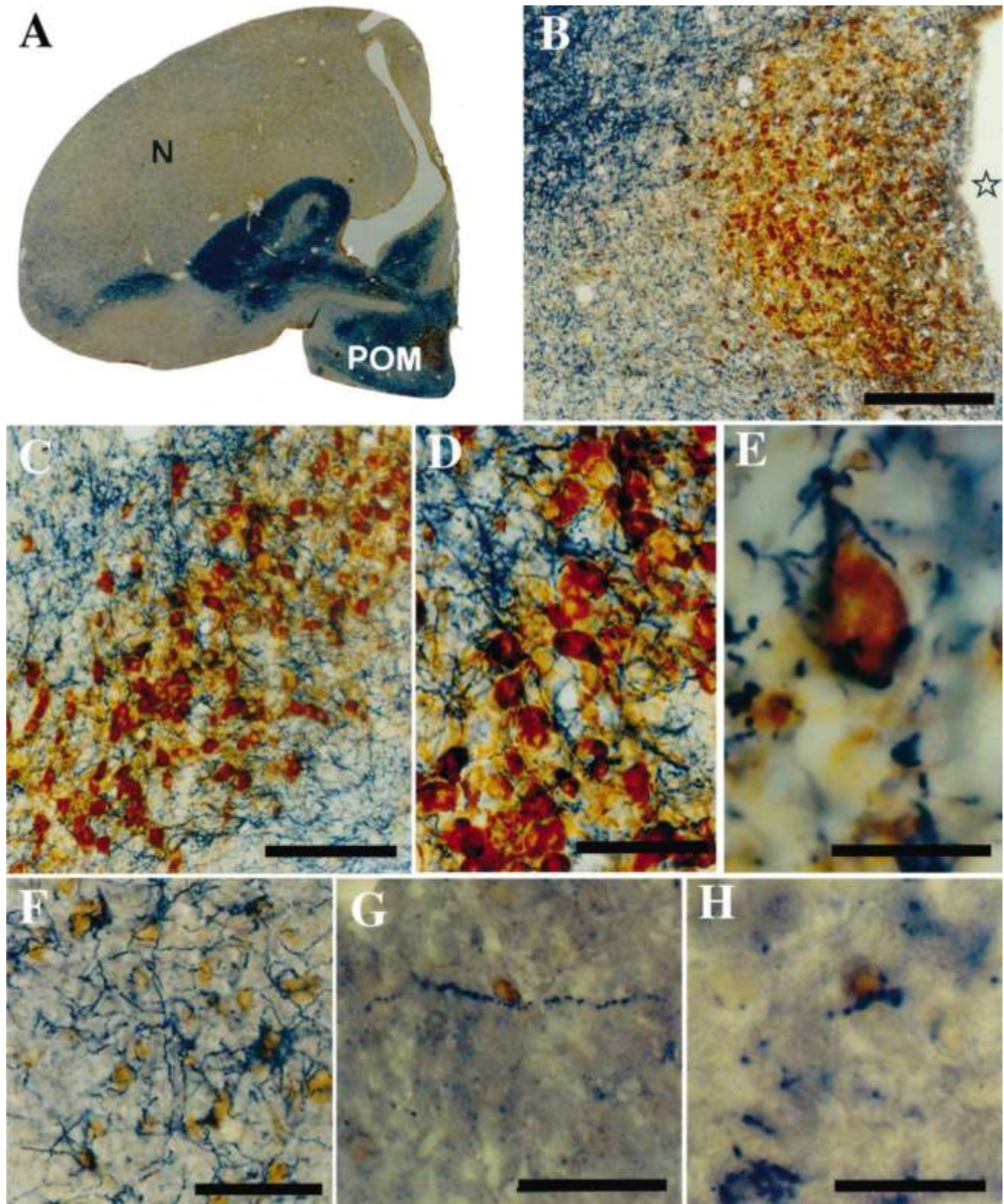
A host of studies have demonstrated that the medial preoptic region and the nucleus of the stria terminalis in birds receive dense plexus of catecholaminergic and peptidergic inputs [10,70,96,131]. In quail specifically, biochemical assays based on spectrofluorometry or on high-performance liquid chromatography (HPLC) associated with electrochemical detection have identified high concentrations of both norepinephrine (NE) and dopamine (DA) in the POA and more specifically in the POM [33,93,94]. This presence of catecholaminergic inputs in cell groups expressing aromatase was also confirmed by immunocytochemistry for the DA and NE synthesizing enzymes, tyrosine hydroxylase (TH) and dopamine beta-hydroxylase (DBH) [11].

Catecholaminergic activity has been shown to be controlled by estrogens in a variety of animal models and accordingly estrogen receptors are present and colocalized with TH in some of the catecholaminergic cell groups in the avian and mammalian brain [56,64,77,110]. There is also evidence from *in vitro* studies that second messenger systems such as cAMP can influence AA [15,24,46,48,84,125,127] and many catecholamine receptors act via this second messenger system [24,29].

We therefore speculated that AA could be modulated by afferent catecholaminergic inputs and, as a first step in testing this hypothesis, we analyzed by double-label immunocytochemistry the anatomical relationships between aromatase-immunoreactive cells and catecholaminergic fibers as identified by the synthesizing enzymes, TH and DBH [12,30]. These studies revealed an extensive overlap

between the distribution of TH and aromatase in many brain areas (Fig. 2). In all densely labeled ARO-ir cell groups, including the POM, BST, and medio-basal hypothalamus, ARO-ir cells were found in close association with TH-ir fibers. These TH-ir fibers often converged on

an ARO-ir cell, and one or more TH-ir punctate structure(s) were found in close contact with nearly every densely labeled ARO-ir cell. In the telencephalon (mostly the neostriatum), weakly labeled ARO-ir cells were also detected. They were always surrounded by dense plexus of



TH-ir fibers. TH and aromatase were, however, not found to be colocalized except in a few cells located at the rostro-medial pole of the anterior hypothalamus in a TH-ir cell cluster that appears to be homologous to the anteroventral periventricular (AVPv) catecholaminergic cell group described in the rat brain [116–118].

Attempts were also made to double label sections through the quail brain for aromatase and DBH. Because the only available antibodies that permit visualization of these antigens have been both raised in rabbits, cross-reactions were frequently observed so that meaningful observations could not be performed. However, in selected cases where all antigenic sites from the first immunocytochemical sequence had been effectively blocked by the diaminobenzidine precipitate, specific labels could be obtained as confirmed by the subcellular localization of the chromogens (in perikarya for aromatase, in fibers and terminals for DBH). DBH-ir punctate structures and fibers were then found in close association with ARO-ir cell bodies in all brain regions where these are present namely the POM, BST and the ventro-medial and tuberal hypothalamus. These data thus support the notion that noradrenergic fibers also innervate ARO-ir cells.

The origin of the TH-ir fibers innervating the POM and BST was also identified by retrograde tract-tracing combined with immunocytochemistry for TH [17]. The TH-ir fibers that are found in the POM originate mostly in dopaminergic neurons located in the periventricular hypothalamus and in the area ventralis of Tsai (AVT, catecholaminergic cell group A10). Most of the catecholaminergic neurons of the hypothalamus that project to the POM are located at its rostral end at the level of nucleus AVPv (Fig. 3). In contrast, the catecholaminergic cells projecting to the BST are mostly located in the substantia nigra (catecholaminergic group A9) and to a lesser extent in the retrorubral field (A8) and AVT (A9). A noradrenergic contribution to these catecholaminergic inputs to POM and BST was also detected during these tract-tracing studies. Retrogradely labelled TH-ir positive neurons were detected in the nucleus ceruleus and subceruleus after injection of tracer in the POM or BST.

It is also interesting to note that estrogen receptors have been identified in several of these catecholaminergic cell groups that project to POM and/or BST. The colocaliza-

tion of ER $\alpha$  and TH has in mammals been mostly observed in the hypothalamic dopaminergic neurons [56,64,110] as well as in some brain stem and medullar nuclei [64]. More recently, similar data have been reported in one avian species, the canary. In a study comparing the distribution of the estrogen receptor-alpha (ER $\alpha$ ) mRNA expression with that of TH-ir structures in the brainstem of male canaries, it was found that dense ER $\alpha$  mRNA labeling overlaps with TH-ir structures in both the locus ceruleus and the area ventralis of Tsai [77]. Furthermore, immunocytochemical double label experiments have demonstrated the presence of immunoreactive ER $\alpha$  in the TH-ir neurons of the anterior hypothalamus at the level of the putative homologue of AVPv. The presence of ER $\alpha$  within these cell groups suggests that sex steroid hormones may affect catecholaminergic activity at the site of synthesis.

Taken together, these observations are consistent with the hypothesis that catecholamines regulate the synthesis of aromatase and potentially mediate the effects of estrogens on AA. Pharmacological experiments were therefore carried out to test this notion (see below).

Other afferent inputs have also been described in brain areas that contain high densities of aromatase-expressing cells. In particular, high densities of vasotocin-immunoreactive (VT-ir) fibers have been found to be associated with all brain areas that contain aromatase-immunoreactive cells such as the POM and BST. Sequential staining of the same sections for aromatase and vasotocin demonstrated that there is a close anatomical correlation between the extension of ARO-ir cells groups and VT-ir fibers throughout the quail brain [18]. All clusters of ARO-ir cells except the nucleus accumbens (sensu Kuenzel and Masson [71]) are embedded in a dense network of VT-ir fibers. Many of the VT-ir terminals appeared to end in the neuropile surrounding ARO-ir perikarya (Fig. 4).

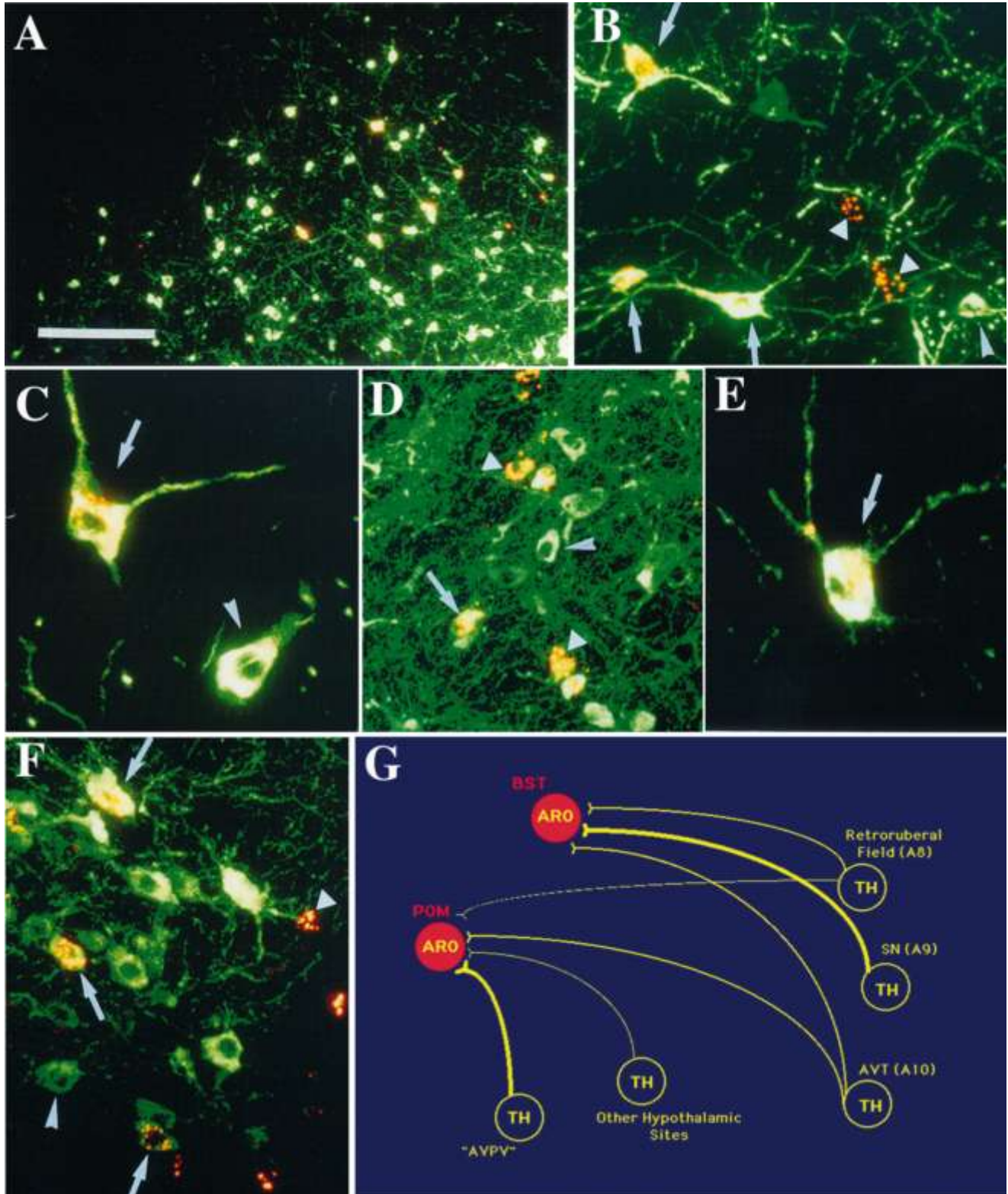
These vasotocinergic inputs are clearly steroid sensitive. Castration of male quail almost completely abolishes the VT-ir innervation of the POM, BST and septal region and treatment of castrated males with testosterone restores this innervation within 2 weeks [97,129,130]. Interestingly, these effects of testosterone appear to be mediated almost exclusively through its aromatization as previously demonstrated in mammals [52,53]. They are fully mimicked by a

Fig. 2. Photomicrographs illustrating the distribution of aromatase-immunoreactive (ARO-ir) cells (brown chromogen) and of tyrosine hydroxylase-immunoreactive (TH-ir) fibers (blue chromogen) in the rostral forebrain of male quail. (A) Photography of a section at the level of the preoptic area illustrating the dense group of ARO-ir cells outlining the entire nucleus preopticus medialis (POM). This entire region is densely innervated by TH-ir fibers. Weakly labeled ARO-ir cells are also present in the neostriatum (N). (B) Enlargement of the preoptic region showing the presence of TH-ir fibers within the limits of the POM. (C) Enlargement of the BST showing the presence of TH-ir fibers within the limits of the ARO-ir cell group. (D) Higher magnification of the BST showing the TH-ir fibers intermingled with ARO-ir cells. (E) Detail of one ARO-ir cell from the BST associated with TH-ir fibers and punctate structures. (F) High magnification of the weakly immunoreactive aromatase cells in the neostriatum illustrating their anatomical relationship with TH-ir fibers. (G,H) Sections labeled by double-label immunocytochemistry illustrating the anatomical interactions between ARO-ir cells in brown and dopamine beta-hydroxylase (DBH) immunoreactive fibers in blue. The two panels show a DBH-ir fiber in close contact with an ARO-ir cell in the POM (G) and BST (H). Magnification bars: 200  $\mu$ m in (B,C), 100  $\mu$ m in (D), 50  $\mu$ m in (F and G), and 20  $\mu$ m in (E and H). Modified from data in Ref. [30].

treatment with exogenous estradiol but not by a treatment with the non-aromatizable androgen 5 $\alpha$ -dihydrotestosterone [128]. Vasotocin is therefore another estrogen-sensitive potential candidate that could mediate effects of estrogens on AA. The intracellular transduction systems

that could mediate this effect are unclear at present but several hypothesis can be suggested.

Vasopressin (VP), the mammalian homologue of VT, acts mainly through its V1 receptors that are coupled to the inositol phosphate second messenger system [41]. VT-



induced changes in the inositol phosphate system or in the intracellular calcium concentration could therefore modulate aromatase activity given that calcium appears to play a key role at this level (see below).

Alternatively, it has also been demonstrated that VP modulates the effects of neurotransmitters such as DA and NE that affect themselves the intracellular cyclic AMP concentration [41] even though VP alone is unable to affect cyclic AMP concentration. It is therefore conceivable that VT/VP acts as a neuromodulator on the catecholaminergic synapse that controls aromatase activity.

It must also be noticed that these anatomical data do not necessarily imply a control of AA or synthesis by VT or vice versa a modulation of VT release by the aromatase

cell. Vasotocinergic inputs on the ARO-ir cells could modulate their functional (electrical) output but not affect the enzyme itself. This anatomical arrangement would achieve the convergence in one specific point of an endocrine signal (steroid) and of an environmental signal transduced by VT that would both influence reproductive behavior or any other estrogen-dependent brain function.

A large number of other neuropeptides are also present in fibers located in the POM and BST [98] and the expression of some of these peptides is steroid-sensitive [1]. Their specific function has however not been investigated. Experiments should be carried out to test the functional significance of the anatomical interactions between VT or other neuropeptides and aromatase.

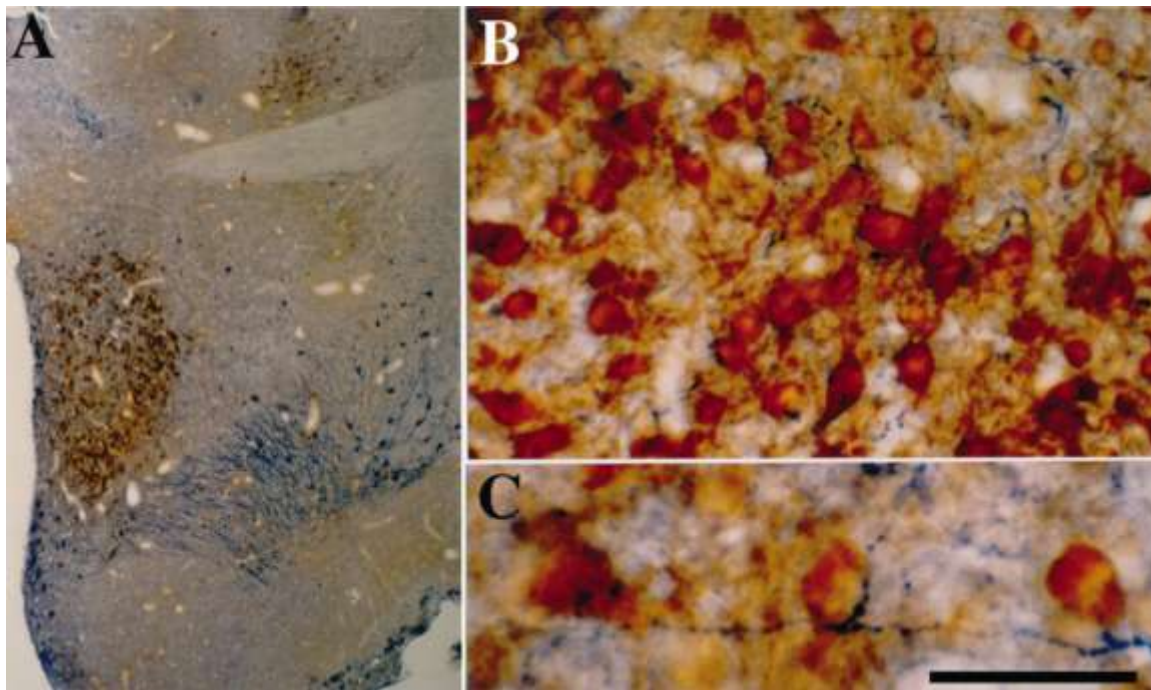


Fig. 4. Morphological interactions between aromatase-immunoreactive neurons (brown chromogen) and vasotocin-immunoreactive fibers (blue chromogen) in the preoptic area on male quail. (A) Low power enlargement of the preoptic area showing the sexually dimorphic medial preoptic nucleus identified by a dense cluster of aromatase-immunoreactive cells. Magnocellular vasotocinergic neurons and the associated fiber tracts are also visible in the ventro lateral preoptic region. (B) Higher enlargement showing close contacts between vasotocin fibers and aromatase cells. (C) At higher power enlargement, vasotocin-immunoreactive fibers and punctate structures (presumptive synapses) are seen in close contact with aromatase-immunoreactive cells. The magnification bars shown at the bottom right represents 1000  $\mu\text{m}$  in (A), 100  $\mu\text{m}$  in (B) and 50  $\mu\text{m}$  in (C). Based on data in Ref. [18].

Fig. 3. Identification by retrograde tract-tracing of the origin of catecholaminergic fibers innervating aromatase-expressing neurons in the medial preoptic nucleus (POM) and bed nucleus of the stria terminalis (BST). Panels (A–F) present color photomicrographs obtained by confocal microscopy illustrating catecholaminergic areas of the brain that had been injected in the BST with red latex fluospheres (red dots). Catecholaminergic cells were identified by immunocytochemistry for tyrosine hydroxylase with the use of fluoresceine (FITC; green fluorescence) as reporter fluorophore. Catecholaminergic cells (green label) that do or do not contain retrogradely transported red latex fluospheres (RLF; red dots) are observed in different panels as well as non-catecholaminergic cells projecting to the areas where the retrograde tracer was injected (accumulation of red dots outside of FITC labeled cells). Examples of double labeled cells are indicated by arrows, FITC positive but RLF negative cells are indicated by arrow heads and cells containing RLF but not FITC fluorescence are indicated by triangles in panels (A–F). (A) Retrochubral field; (B) substantia nigra; (C) higher magnification of a double labeled cell and an FITC positive-RLF negative cell in the substantia nigra; (D) area ventralis of Tsai; (E) high magnification of a double labeled cell in the area ventralis of Tsai; and (F) locus ceruleus. The magnification bar shown at the top left represents 200  $\mu\text{m}$  in (A), 50  $\mu\text{m}$  in (B,D,F) and 30  $\mu\text{m}$  in (C and E). (G) Schematic representation of the relative numbers of tyrosine hydroxylase-immunoreactive neurons that project to the two main groups of aromatase-immunoreactive cells located in the POM and BST. The thickness of arrows has been adjusted to reflect the relative numbers of neurons projecting from one catecholaminergic cell group to each aromatase cell cluster. Based on data in Ref. [17].

## 5. In vivo pharmacological evidence for a control by catecholamines of aromatase activity

### 5.1. Systemic treatments with catecholaminergic neurotoxins

In a series of experiments, castrated male quail that were submitted to a replacement therapy with T were systemically injected with the specific noradrenergic neurotoxin, DSP4. HPLC assays confirmed that the single injection of DSP4 significantly depleted NE concentrations in all brain areas including the POA-hypothalamus, and the POM in particular [22,35]. The preoptic AA was increased after the injection of DSP4 indicating that NE may exert a chronic inhibition of this enzymatic activity [22]. The DBH fibers originating from the locus ceruleus and subceruleus that are in close contact with ARO-ir cells in the POM and BST [12,17] presumably represent the morphological signature of this regulation.

It has been shown previously that noradrenergic agonists modulate aromatase activity in the pineal gland [48] or in the testes [125] and similar controls might take place in the mammalian hypothalamus [47,104,105]. Given that the noradrenergic activity in the brain appears to be under the control of estrogens [81], an estrogen-sensitive noradrenergic transmission might represent the neuroanatomical substrate underlying the regulation of aromatase activity by estrogens.

In male quail, a rapid decrease in DA concentration is observed after a single injection of the TH inhibitor,  $\alpha$ -methyl-para tyrosine ( $\alpha$ MPT) [33,93]. This DA disappearance is supposed to reflect the turnover of the amine (see however Ref. [133] for a critical discussion of this issue). In one experiment, brain DA levels were chronically depressed in castrated testosterone-treated male quail by daily injections of  $\alpha$ MPT [29]. This treatment produced, after 3 days, a significant inhibition of aromatase activity in the POA by comparison with the control T-treated birds injected with the solvent. It is clear that a chronic treatment with  $\alpha$ MPT decreases both NE (which presumably inhibits AA in the quail brain based on the DSP4 experiments described above) and DA [33]. It can therefore be expected that a selective dopaminergic inhibition would lead to an even larger decrease in enzymatic activity which would be consistent with the notion that DA up-regulates brain aromatase, in particular in the POM (see Ref. [24] for additional discussion).

### 5.2. Central treatment with 6-hydroxydopamine

To specify further the functional interactions between catecholamines and preoptic AA, we depleted catecholaminergic inputs to the POM by injecting into this nucleus the catecholamine neurotoxin 6-hydroxydopamine (6-

OHDA). Male quail were castrated and received, 2 weeks later, two 20 mm-long subcutaneous Silastic capsules filled with crystalline testosterone. The catecholaminergic neurotoxin, 6-hydroxydopamine hydrochloride (6-OHDA HCl) was dissolved in distilled water containing 9‰ NaCl and 1‰ ascorbic acid at the dose of 50  $\mu\text{g}/\mu\text{l}$  and each bird was injected stereotaxically in the medial part of the POA with 200 nl of this solution (10  $\mu\text{g}$  of the toxin per bird). Control animals received the corresponding volume of the vehicle solution (saline+ascorbic acid). Preliminary experiments had shown that this neurotoxin does not deplete tyrosine hydroxylase activity measured 5 days after the injection and does not affect aromatase activity if injected at a lower dose (100 nl of a solution at 20  $\mu\text{g}/\mu\text{l}$ , i.e. 2  $\mu\text{g}$  per bird). On another hand injection of 20  $\mu\text{g}$  6-OHDA per subject leads to a high rate of mortality. The 10  $\mu\text{g}$  dose selected for this experiment can therefore be considered as the highest dose that can be tolerated by adult quail.

Stereotaxic injections were targeted to the medial preoptic nucleus (POM), with coordinates derived from previous studies [37–39]. Pressure injections were made unilaterally through a glass micropipette (external tip diameter of about 50  $\mu\text{m}$ ) connected to a 0.5  $\mu\text{l}$  Hamilton syringe and a micropump. The micropipette was introduced vertically and 200 nl of the toxin solution were delivered in about 7 min. The micropipette was removed slowly 5 min later to avoid leakage of the drug through the pipette track. Birds were injected on the left or right side of the brain in equal numbers. No lateralization could be detected in the absolute level of the biochemical variables (depletion of aromatase activity or tyrosine hydroxylase activity, see below) that were measured in the present experiments and similarly no interaction between the experimental effects and the injection side was observed. The brain side was therefore ignored in the analysis of results that is presented here. A total of 12 birds were injected in this way with 6-OHDA while 11 birds received a control injection.

One day after the 6-OHDA injection, birds were killed by decapitation, their brain was quickly removed from the skull and frozen on powdered dry ice. The POA-hypothalamic block was dissected from the frozen brains by two coronal cuts at the level of the tractus septomesencephalicus (rostral edge of the POA) and of the oculomotor nerves (caudal edge of hypothalamus), two parasagittal cuts placed approximately 2 mm lateral to the brain midline and one horizontal cut about 2 mm above the floor of the brain. This isolated a block of tissue weighing 70–90 mg that contains the vast majority of the cells that express aromatase in the quail brain. This block was then separated into its left and right parts by a medial cut passing largely through the third ventricle so that it could be very accurately positioned. This isolated the brain site ipsilateral to the injection from its contra-lateral counterpart. Finally, the left and right preoptic area-hypothalamic blocks were cut in two equal halves by a coronal cut isolating the rostral and caudal parts corresponding roughly

to the preoptic area (POA, where injection of 6-OHDA/saline had been made) and to the hypothalamus (HYP), respectively.

AA was quantified in these four blocks by measuring the tritiated water assay method, as described by Roselli and Resko [108], with minor modifications [13]. Tyrosine hydroxylase activity (TH-A) was also assayed in the same samples in order to assess the extent of the catecholaminergic lesion by measuring the tritiated water production from 3,5- $^3\text{H}$ -L-tyrosine, as initially described by Nagatsu et al. [87] and later Levine et al. [76], with some further modifications [14]. These two methods have been previously validated for use in the quail brain [13,14].

Results described below were expressed as fmol/nmol per brain fraction, representing the total enzymatic activities by brain area instead of activities per mg protein or mg fresh weight. The reasons for this choice are explained by the anatomy of the systems under investigation. Aromatase-expressing cells and TH-containing structures only form a small fraction (5% or less) of the POA and HYP [8,11,30,40]. The micro-dissection into four blocks of the POA–HYP reliably separated the left and right hemispheres and isolated in a reliable manner the rostral aromatase cells that are primarily located in the POM and BST from the more caudal cells located in the medio-basal hypothalamus. However, the precise location of the lateral cuts was probably more variable. Thus tissue blocks presumably contained variable amounts of extraneous tissue that does not contain AA, given that all ARO-ir cells are located in periventricular position and are necessarily included in the dissection independent of the position of the lateral cuts. Therefore expressing data per mg protein or fresh weight introduces unexplained variance in the results, a problem that is not encountered when enzyme activities are expressed by entire brain area (POA or HYP). These data were analyzed directly by one or two-way analysis of variance (ANOVA) or as a ratio of the enzyme activities (AA or TH-A) measured on the injection side divided by the control values on the contralateral side (percentage (%) AA, %TH-A).

Both AA and TH-A were very similar in the two sides of the brain in birds that had been injected with the control saline solution, thus confirming the reliability of the brain dissections and accuracy of the enzymatic assays (Fig. 5). A noticeable decrease in AA was observed in the POA on the side receiving the 6-OHDA injection (Fig. 5). Analysis of these results by two-way ANOVA with the two experimental groups as an independent factor and the two brain sides as a repeated factor detected no significant effects of the two main factors (Treatment:  $F_{1,21}=0.138$ ,  $P=0.7141$ ; Side:  $F_{1,21}=3.653$ ,  $P=0.0697$ ) but indicated a significant interaction between them, therefore confirming a localized effect of the experimental treatment ( $F_{1,21}=4.525$ ,  $P=0.0454$ ). The observed decrease in AA in the brain side injected with 6-OHDA was admittedly of a limited amplitude but the assays of TH activity indicated a

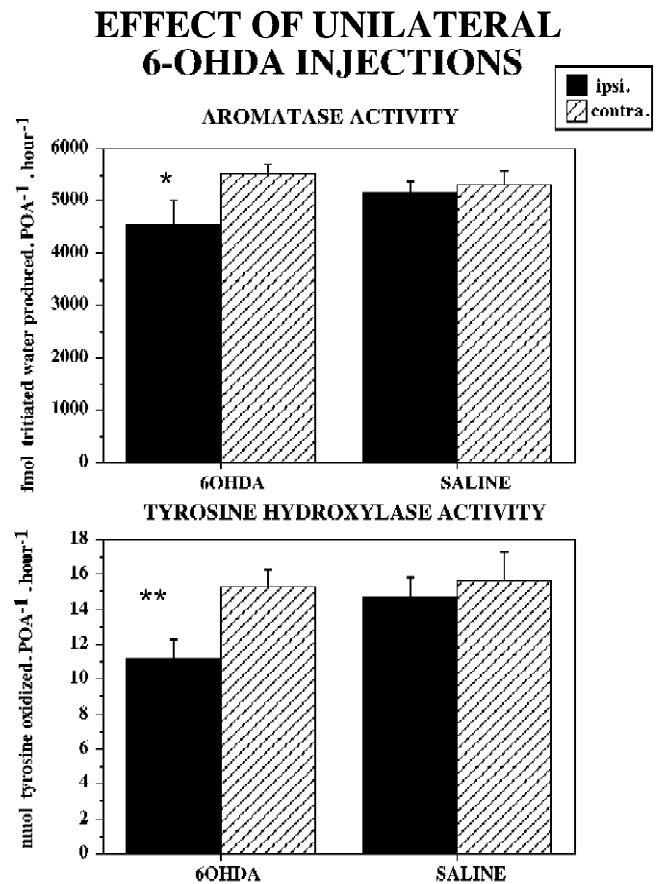


Fig. 5. Effects of a unilateral injection of 6-OHDA (10  $\mu\text{g}$ ) in the preoptic area on the aromatase activity and tyrosine hydroxylase activity in the preoptic area. Enzyme activities were measured on the injection (ipsi.) and on the contra-lateral (contra.) side that was used as internal control. Values obtained in saline-injected birds (SAL) are presented as additional controls. Data are means  $\pm$  S.E.M.;  $n=12$  for 6OHDA birds and  $n=11$  for saline controls. Analysis of these data by two-way ANOVA with one repeated factor (brain side) indicated the presence of significant interaction between brain side and experimental treatment (6OHDA vs. saline) for aromatase activity and of brain side for tyrosine hydroxylase activity (see text). Further analysis of these data in 6OHDA and saline birds separately by one-way repeated ANOVA confirmed the decline of aromatase activity (\* $P<0.05$ ) and of tyrosine hydroxylase activity (\*\* $P<0.01$ ) on the injection side in 6OHDA birds.

catecholaminergic depletion that was similarly small (Fig. 5).

TH-A was also slightly smaller on the injection than on the contralateral sides in saline-treated birds, so that no significant treatment effect ( $F_{1,21}=2.162$ ,  $P=0.1563$ ) and no interaction between side and treatment ( $F_{1,21}=2.445$ ,  $P=0.1328$ ) was detected in the analysis of these results. There was however a significant effect of the brain side on TH-A ( $F_{1,21}=15.263$ ,  $P=0.0008$ ).

In the HYP, caudal to the injection site, AA was not affected by the treatment, the side of the brain and their interaction (respectively:  $F_{1,21}=0.228$ ,  $P=0.6378$ ;  $F_{1,21}=1.151$ ,  $P=0.2955$  and  $F_{1,21}=0.023$ ,  $P=0.8809$ ). Interest-

ingly, TH-A was enhanced specifically on the injection side in the HYP of 6-OHDA treated birds. This resulted in a significant interaction of the treatment with brain side ( $F_{1,21}=6.865$ ,  $P=0.0160$ ) in the absence of effect of the two main factors (Treatment:  $F_{1,21}=0.429$ ,  $P=0.5196$ , Side:  $F_{1,21}=0.739$ ,  $P=0.3396$ ).

The extent of AA depletion in the POA side injected with 6-OHDA was found to be quite variable from one bird to the other and we therefore asked whether this AA depletion was related to the degree of catecholaminergic depletion measured by the TH-A decrease. Correlations were therefore calculated between the individual percentage of AA left in the injection side (compared with the contra-lateral side) and the percentage of TH-A computed in the same way. Interestingly, these two variables were found to be significantly correlated (Fig. 6) with large

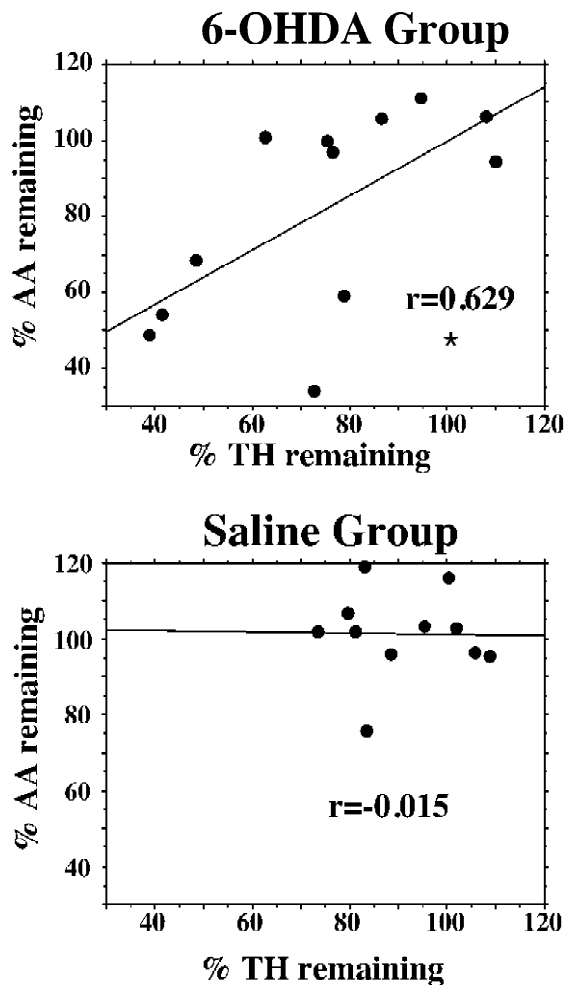


Fig. 6. Correlations between the depletions in aromatase activity (AA) and in tyrosine hydroxylase activity (TH-A) observed after unilateral injection in the preoptic area of 6-OHDA (top) or of saline solution (bottom) as a control procedure. All depletions have been expressed as percentage of the value measured in the injection side by comparison with the corresponding control contra-lateral side.

TH-A depletions being associated with the largest depletions in aromatase ( $r=0.629$ ,  $n=12$ ,  $P=0.0283$ ). By contrast there was no relationship between these two percentages of depletion in birds injected with saline ( $r=0.015$ ,  $n=11$ ,  $P=0.9662$ ).

These percentages of AA and TH-A depletion (by comparison with the non-injected side) were further studied by a one-way analysis of covariance using the experimental treatment (6-OHDA vs. saline) as the independent factor, the %AA remaining on the injection side (as a percentage of the non injected side) as dependent variable and the %TH-A remaining on the injection side as a covariate. An extremely significant effect of the experimental treatment was detected in this way ( $F_{1,20}=15.811$ ,  $P=0.0007$ ) as well as a significant effect of the interaction between the treatment and the covariate ( $F_{1,20}=12.539$ ,  $P=0.0021$ ).

These results were replicated in a second experiment testing in addition the effects of two catecholamine uptake inhibitors on the 6-OHDA induced depletion of AA, in an attempt to identify the specific nature of the catecholaminergic effect on AA. In this study, castrated male quail that had been treated with exogenous testosterone were again stereotaxically injected in the POM with 10  $\mu\text{g}$  6-OHDA or with the control solution but in addition, some of the birds were pretreated 50 min before with either the norepinephrine uptake inhibitor desipramine hydrochloride (DMI; 25 mg/kg ip) or the dopamine uptake inhibitor GBR-12909 hydrochloride (GBR; 40 mg/kg ip). The combination of these treatments thus defined six experimental groups: birds injected with 6-OHDA after a pre-treatment with saline (6-OHDA,  $n=12$ ), birds injected with saline after a pre-treatment with DMI (DMI,  $n=12$ ), birds injected with saline after a pre-treatment with GBR (GBR,  $n=12$ ), birds injected with 6-OHDA after a pre-treatment with DMI (DMI+OHDA,  $n=12$ ), birds injected with 6-OHDA after a pre-treatment with GBR (GBR+OHDA,  $n=12$ ) and finally birds injected with saline after a pre-treatment with saline (SAL,  $n=9$ ).

A two-way ANOVA of AA levels with an independent (six treatment groups) and a repeated (two brain sides) factor confirmed the absence of overall effects of the treatments ( $F_{5,63}=0.664$ ,  $P=0.6522$ ) but the side and, most importantly, the interaction of side with treatment had very significant effects on the enzyme activity (respectively  $F_{1,63}=7.456$ ,  $P=0.0082$  and  $F_{5,63}=3.635$ ,  $P=0.0059$ ). Analysis by one-way ANOVA of the % AA remaining in the injection side as compared to the contralateral side confirmed the overall effect of the treatments ( $F_{5,63}=3.579$ ,  $P=0.0065$ ) and post-hoc Fisher PLSD tests indicated that the %AA remaining on the injection side was significantly smaller in the 6-OHDA group than in the Saline, DMI and GBR groups ( $P<0.05$  in each case). No other comparison between groups was significant (Fig. 7). These results therefore confirmed the effect of 6-OHDA on

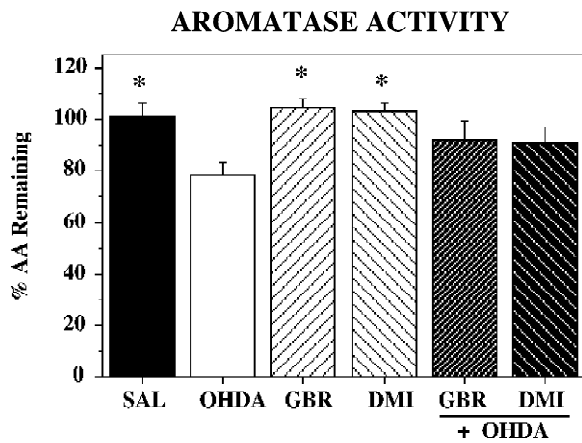


Fig. 7. Effects of a unilateral injection of 6-OHDA in the preoptic area combined or not with a pre-treatment by the catecholamine reuptake inhibitors GBR-12909 hydrochloride (GBR) and desipramine hydrochloride (DMI) on the aromatase activity (AA) in the preoptic area. Enzyme activity measured on the injection side was expressed as a percentage of the control value obtained on the contra-lateral side. The different treatments were compared two by two by Fisher PLSD tests whose results are reported by symbols at the top of corresponding columns (\*  $P < 0.05$  by comparison with the 6-OHDA group). Data presented are means  $\pm$  S.E.M.;  $n = 12$  subjects in each group except in the saline group where  $n = 9$ .

AA but failed to determine whether the effect is mediated by noradrenergic or dopaminergic receptors, presumably because the dose and/or timing selected for the reuptake inhibitors were not adequate to block the 6-OHDA induced lesion on specific receptor types.

A correlation was again observed between the AA depletion and the TH-A depletion (both expressed as a percentage of activity found in the injected side by comparison with the contra-lateral side) in the 6-OHDA injected birds but it fell short of significance ( $r = 0.523$ ,  $n = 12$ ,  $P = 0.0813$ ). No relationship between these variables was present in saline-injected subjects ( $r = -0.499$ ,  $n = 9$ ,  $P = 0.1719$ ).

The pooled data of both experiments expressed as a percentage of activity remaining on the injection vs. contralateral side were also submitted to an analysis of covariance with the six experimental groups as the independent factor, the %AA remaining as a dependent variable and the %TH-A remaining as a covariate. This global analysis confirmed the presence of an overall effect of treatments ( $F_{5,80} = 4.804$ ,  $P = 0.007$ ), of the covariate, i.e. the TH depletion ( $F_{1,80} = 8.186$ ,  $P = 0.0054$ ) and of a significant interaction between the main effect and the covariate ( $F_{5,80} = 3.479$ ,  $P = 0.0068$ ). Correlation analyses of these pooled data expressed in percentages also confirmed the positive relationship in 6-OHDA injected birds between AA remaining and TH-A remaining in the injection site ( $r = 0.499$ ,  $n = 24$ ,  $P = 0.0131$ ). These correlations were not observed in the pooled data of saline injected birds ( $r = 0.293$ ,  $P = 0.2093$ ).

## 6. Cellular mechanisms mediating changes by afferent inputs of aromatase concentration

Taken together, these anatomical data and experimental manipulations of the catecholaminergic inputs to the POA strongly suggest that catecholamines control AA in the brain POA. The single experiment carried out so far in quail, suggests that NE exerts a tonic inhibition on the preoptic AA. On the contrary, pharmacological depletions of DA result in a decrease of AA and the decreases of AA and TH-A are significantly correlated within individuals, further supporting the existence of a causal link between these two enzymatic changes. All these data thus point to a stimulatory effect of DA on AA. These studies are, however, all based on dopaminergic depletions (lesions) and it would be desirable to confirm the specificity of these effects by testing whether a chronic stimulation by D1 or D2 agonists really leads to an increase in AA. Such a goal is however technically difficult to achieve.

The cellular mechanisms that mediate the stimulation of AA by DA have not been investigated in the brain. It is clear however that the binding of DA to its membrane receptors modulates the production of cAMP (respectively increase and decrease in concentration mediated by the D1 and D2-like receptors). In addition, it has been shown that experimental manipulations of cAMP affect aromatase activity in a variety of tissues including the brain [46,84,95,125–127,135]. It is conceivable that the stimulation of the adenylyl cyclase activity caused by the binding of DA to its D1 receptors leads to the activation of protein kinase A resulting in the phosphorylation of the cAMP response element binding protein (CREB). Phosphorylated CREB is a transcriptional regulator that could then increase transcription of the aromatase gene and thus the concentration of the enzyme. In agreement with this scenario, CREB responsive elements (CRE or SF1) have been identified on forms of the aromatase gene exon 1 that are typically found in peripheral tissues but also to a lesser extent in the brain [55,65,102,120].

It must be stressed that this model of DA actions implies the presence of DA receptors at the surface of aromatase cells and no evidence for this presence has to date been collected due to the lack of suitable antibodies. It has actually been shown that the D1 receptor-associated protein DARPP-32 (DA and cAMP-regulated phosphoprotein) is not colocalized with aromatase in the quail brain [2]. This observation thus raises questions concerning the mode of DA action on AA (see Ref. [2] for further discussion).

Many other questions are also unanswered at this point. They relate to the mechanism of DA action but also to the real magnitude of the effects of this catecholamine on AA. Significant decreases of AA have been repeatedly observed after a pharmacological depletion of DA brain concentration but a fairly large amount of residual AA was always present. This could reflect the failure to completely

suppress the DA-dependent stimulation of enzymatic activity but alternatively it could also be the case that DA is only a part of the mechanisms that control aromatase and that other inputs (e.g. vasotocinergic or other peptidergic pathways) also play a significant role. Direct actions of estrogens (e.g. via binding to ER of the beta subtype) that would not be mediated by trans-synaptic actions have also not been completely ruled out. Additional studies are clearly needed on this topic.

### 7. Rapid changes in aromatase activity

The experiments described above indicate that brain aromatase is regulated to some degree by catecholamine action. Given that changes in enzyme activity were detected after several hours or days, it has been assumed that this regulation is mediated via the genome and presumably involves modifications of the enzyme synthesis. These processes are relatively slow by definition. In quail, it has been shown that treatment with T only increases AA after several hours. The enzymatic activity doubles in approximately 8 h but only reaches its maximum after 1 or 2 days [32].

Such relatively slow changes in aromatase concentration and therefore estrogen production are in good agreement with the classical modes of estrogen action. Estrogens are generally viewed as slow-acting messengers that bind to nuclear ER which then act as transcription factors. These effects are also slow and thus consistent with the mechanisms that regulate the local production of estrogens by aromatase in a slow manner (via changes in enzyme concentration). However, recent studies suggest that, in rats, the estrogenic metabolites of T may activate aspects of male sexual behavior rapidly at the membrane level while androgenic metabolites act more slowly via genomic mechanisms [50] (see however Ref. [44] for rapid membrane effects of T). This observation that estrogens in the male brain may act rapidly on cell membranes is consistent with evidence based on the female brain (and peripheral tissues) indicating that effects of estrogens may be quite rapid and mediated by effects on the cell membrane in some cases (e.g. see Refs. [57,61,69,79,80,85,86,101,103]).

Little is known about how AA is physiologically regulated in specific brain areas. However, if estrogen is acting rapidly on a cell membrane one would expect estrogen availability to also be regulated rapidly [23]. Little attention has been paid so far to the idea that AA could be rapidly affected by the neurochemical cellular environment in a manner that would not involve changes in enzyme concentration (see however Ref. [43] for such a study on placental aromatase). During recent studies, we have identified a number of mechanisms that could mediate such rapid changes of the estrogen production in the brain.

### 8. Inhibition of aromatase activity measured in vitro by dopaminergic agonists and antagonists

During experiments that were originally designed to ensure that dopaminergic drugs injected in vivo do not directly affect the measure of AA in brain homogenates, we serendipitously discovered that some of these drugs markedly depress enzyme activity. Because these compounds were added to brain homogenates and were acting almost immediately, a genomic control mechanism had to be excluded and other regulatory modes of action needed to be contemplated.

The possible existence of a direct modulation of aromatase activity by DA was thus systematically investigated with the utilization of in vitro incubations of quail hypothalamic homogenates in which AA was quantified by the production of tritiated water from [ $\beta$ - $^3$ H]-androstenedione [13].

DA ( $10^{-6}$ – $10^{-3}$  M) and several D1 and/or D2 receptor agonists (apomorphine [D1/D2], SKF-38393 [D1] and RU-24213 [D2]) depressed aromatase activity by 40–70% at the  $10^{-3}$  M concentration. One D2 receptor antagonist also produced a major inhibition of aromatase activity (sulpiride) while other antagonists (SCH-23390 [D1], spiperone [D2], pimozide [D2]) either had no significant effect or only produced moderate decreases in aromatase activity (see Fig. 8). NE and prazosin (an  $\alpha_1$ -adrenergic antagonist) had no effect on AA in these conditions.

The inhibitory effect of the agonists was not antagonized by the less active or inactive antagonist [13] which suggests that the inhibitory effects are not mediated through binding to dopamine receptors. This conclusion is also supported by the facts that inhibitions only occur at doses that are several orders of magnitude above the affinity of the D1 or D2 receptors and that inhibitions display no receptor specificity. They are observed after addition of agonists as well as antagonists of the D1 as well as the D2 like type.

It appears likely that these compounds inhibit AA by a direct effect on the enzyme, as suggested by the competitive nature of dopamine and SKF-38393 inhibition of aromatase activity ( $K_i$ 's of 59 and 84  $\mu$ M, respectively [13]). Previous studies have shown that aromatase is a multi-functional enzyme that catalyzes a variety of reactions such as 2 and 6 hydroxylations and *N*-demethylation of various substrates including cocaine [90–92,122]. Dopamine could therefore act as an alternative substrate for aromatase and in this way compete with testosterone and prevent its transformation into estrogens. This mechanism may represent a significant physiological pathway through which neurotransmitters could rapidly affect steroid-dependent processes such as the neural synthesis of estrogens. Such a mechanism would provide a means by which environmental stimuli could affect reproductive behavior and physiology (see also Ref. [26] for additional discussion).

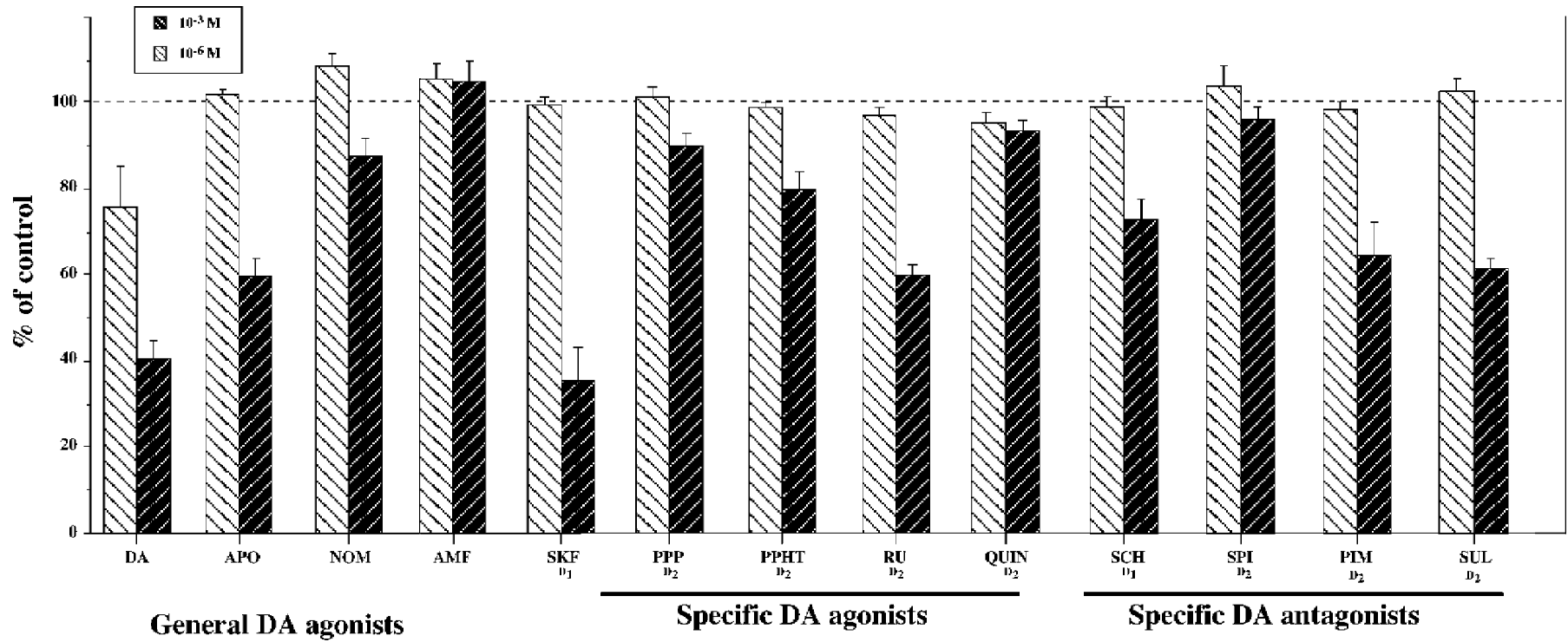


Fig. 8. Effects of dopamine, general dopamine agonists, specific dopamine agonists and antagonists on the aromatase activity in homogenates of the quail preoptic area-hypothalamus. DA, dopamine; APO, apomorphine (general DA agonist); NOM, nomoifensine (indirect DA agonist: reuptake inhibitor); AMF, amfonelic acid (indirect DA agonist: complex mechanisms including enhanced release); SKF, SKF-38393; PPP, 3-PPP; RU, RU-24213; QUIN, Quinpirole; SCH, SCH-23390; SPI, spiperone; PIM, pimoizide; and SUL, sulpiride. The specificity for the D1 or D2-like receptor subtypes is indicated below the name of each compound on the graph. All assays were performed in triplicate, error bars represent the S.D. of the measures that are expressed as percentage of the respective controls (line at 100%). All compounds were tested at two concentrations (10<sup>-3</sup> and 10<sup>-6</sup> M). Redrawn from data in Ref. [13].

These experiments, however, raise questions concerning the mode of rapid DA action on AA. Anatomical studies describing the cellular and subcellular localization of DA in relation to aromatase have indeed failed to identify ARO-ir cells that also contain DA. No colocalization has been discovered between aromatase and tyrosine hydroxylase, the catecholamine synthesizing enzyme except in a small dopaminergic cell group located at the antero-ventral pole of the hypothalamus (putative homologue of the nucleus AVPV; see above). The DA released from the TH-ir positive terminals in the brain and in particular in the vicinity of ARO-ir cells is thought to act on specific surface receptors that mediate intracellular response in the target neurons via a cascade of second and third messenger systems (e.g. activation of adenylyl cyclase activity, CREB phosphorylation, . . .). The *in vitro* data presented above suggest however that the effects of DA on AA in brain homogenates are not mediated by such membrane receptors. These effects are observed in spatially disrupted cellular systems in which the coupling between second messenger systems activated by membrane receptors and physiological intracellular responses is unlikely to be effective and the responses are not related to the receptor characteristics (lack of D1/D2 specificity, similar effects with agonists and antagonist, range of effective concentrations unrelated to the  $K_m$  of receptors). One could therefore wonder whether these inhibitions of AA observed in brain homogenates have any physiological significance.

### 9. Effects of dopamine on aromatase activity expressed by *in vitro* explants

To approach this question, we investigated whether effects of DA and dopaminergic drugs would still be observed in an *in vitro* explant system in which the anatomical integrity of the aromatase cells would not be disrupted as it was in the homogenates. Paired left and right explants of quail preoptic area-hypothalamus were incubated *in vitro* in oxygenated glucose-saline in the presence of 25 nM [ $1\beta$ - $^3\text{H}$ ]-androstenedione. The incubation medium was aspirated with a syringe every 5 min and replaced by fresh medium containing [ $1\beta$ - $^3\text{H}$ ]-androstenedione. Withdrawn samples were immediately cooled in an ice bath and further processed to isolate the tritiated water produced by aromatization from the remaining radioactive steroids as previously described [13,30]. This provided a continuous measure of AA in the blocks (Fig. 9).

In these conditions, nomifensine, a dopamine reuptake inhibitor that had no effect on AA when added directly in POA-hypothalamic homogenates (see Fig. 8), produced an inhibition of approximately 50% of AA within 5 min after its addition to the incubation medium (Fig. 9). DA similarly inhibited AA within 5 min and both effects were reversible when the compounds were washed out. Similar

inhibitory and reversible effects were observed with other dopaminergic agents such as SKF 38393 (a D1 agonist), SCH 23390 (a D1 antagonist), PPHT (a D2 agonist) and sulpiride (a D2 antagonist) [19]. These effects are very reminiscent of those observed on brain homogenates and in particular they also do not display a clear receptor specificity: similar effects are observed with agonists and antagonists of the D1-like and D2-like receptors. This seems to preclude an action that would be mediated via binding to the D1 or D2 receptors. However, the observation that nomifensine had no effect in homogenates but strongly inhibits AA in explants suggests that the accumulation of dopamine within the synaptic cleft is the cause of the AA inhibition. Indications concerning the mechanism(s) that could be involved in this inhibition of estrogen production by dopaminergic compounds come from studies that have analyzed changes in AA that could be mediated by phosphorylations/dephosphorylations of the enzymatic protein.

### 10. Controls of aromatase activity by protein phosphorylation

The activity of many enzymes, such as tyrosine hydroxylase, the rate limiting enzyme in catecholamine synthesis, is rapidly modified by conformational changes in the enzyme molecule, including phosphorylations, that are produced in the presence of suitable concentrations of ATP and the divalent cation,  $\text{Mg}^{2+}$  [4,51]. These phosphorylations are catalyzed by specific kinases that transfer the terminal phosphate group from ATP to the hydroxyl moiety of amino acid residues (tyrosine, threonine, serine) of the enzymatic protein.  $\text{Mg}^{2+}$  is required for this reaction which makes kinase activity critically dependent on the  $\text{Mg}^{2+}$  intracellular concentration. Given that previous studies have implicated divalent cations in the control of AA ( $\text{Ca}^{2+}$  [67,89];  $\text{Mg}^{2+}$  [121]) and because several consensus sites of phosphorylation are present in the mammalian and avian aromatase sequences [49,58,60,82,83,114], we investigated whether  $\text{Ca}^{2+}$  concentrations or ATP and  $\text{Mg}^{2+}$  concentrations such as those used to obtain maximal changes in tyrosine hydroxylase activity [6] would affect AA in male quail brain homogenates.

In the presence of suitable physiological concentrations of ATP,  $\text{Mg}^{2+}$  and  $\text{Ca}^{2+}$ , a profound (80–90%) inhibition of AA is observed in quail brain homogenates. This inhibition is reinforced by the addition of a phosphatase inhibitor such as sodium orthovanadate but prevented by agents that chelate divalent ions such as EGTA or EDTA [21]. These data indicate that AA is almost completely suppressed in conditions that promote protein phosphorylation. This enzymatic inhibition is actually mediated by phosphorylation processes as indicated by the fact that the inhibitory effects of ATP,  $\text{Mg}^{2+}$  and  $\text{Ca}^{2+}$  are blocked in

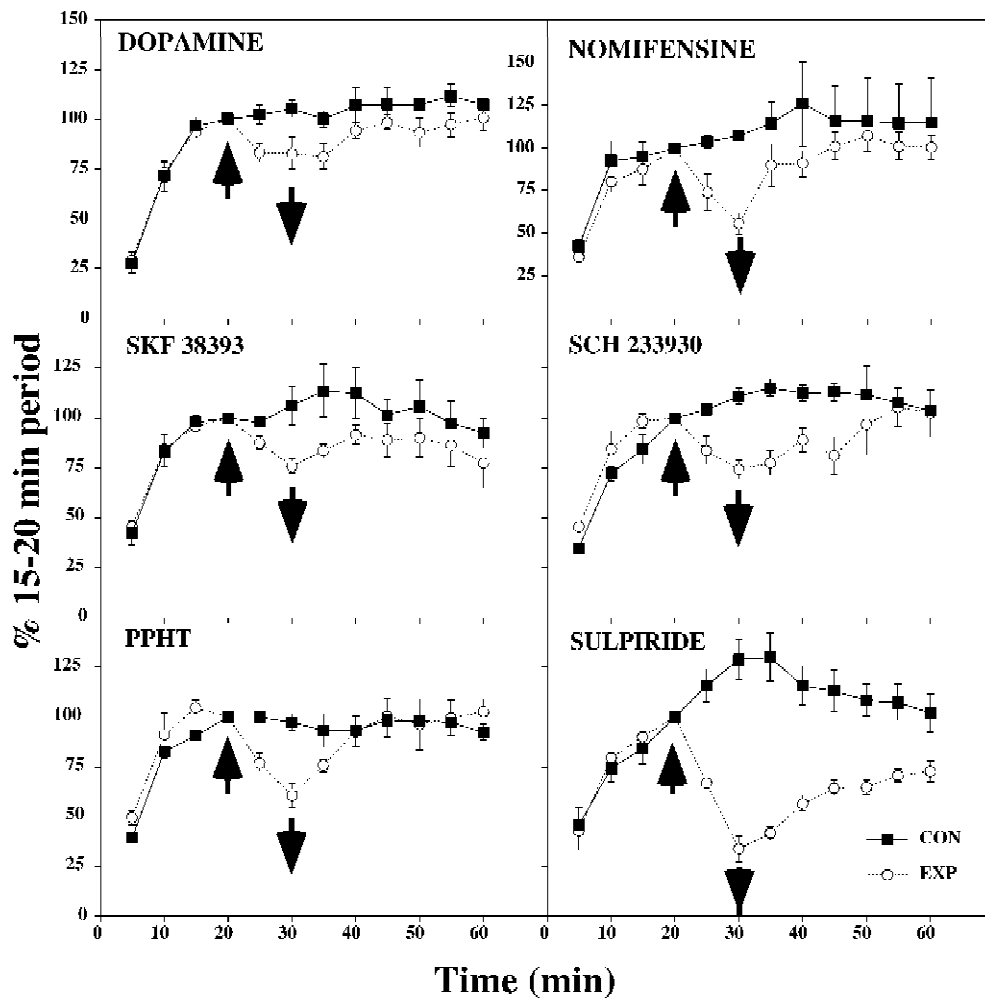


Fig. 9. Effects of dopamine (DA; 1 mM), nomifensine (0.5 mM), SKF 38393 (0.5 mM; a D1 agonist), SCH 23393 (0.5 mM; a D1 antagonist), PPHT (0.5 mM; a D1 agonist) or Sulpiride (0.5 mM; a D2 antagonist) on the aromatase activity (AA) of paired quail POA-hypothalamus explants. All data are means  $\pm$  S.E.M. ( $n=5$ ) of tritiated water production expressed as a percentage of the 15–20 min period. For each brain the experimental treatment was applied to one hemi-explant (EXP) at 20 min (up arrow) and normal saline was restored at 30 min (down arrow), the other hemi-explant served as control (CON). Redrawn from data in Ref. [19].

the presence of inhibitors that block the phosphorylation of Tyrosine and Serine–Threonine residues. This clearly points to the existence of multiple phosphorylation sites that play a significant regulatory role. The pharmacological profile emerging from the studies carried out so far suggests however that a protein kinase C (PKC) and one or several calmodulin (CAM) kinase (possibly MLCK) participate in the control of enzyme activity [20]. Further pharmacological characterization is however desirable.

A rapid (within minutes) and reversible regulation of AA was also observed in hypothalamic explants incubated *in vitro* and exposed to conditions that lead to an increase in the intracellular  $Ca^{2+}$  levels such as a  $K^+$ -induced depolarization, a treatment by thapsigargin or by glutamate agonists (kainate, AMPA or NMDA) [21]. The local production and availability of estrogens in the brain can therefore be rapidly changed by variations in the  $Ca^{2+}$  concentration based on variation in neurotransmitter activi-

ty. It is conceivable that DA could interfere in many ways with these phosphorylation processes although a specific mode of action has not been identified to date.

### 11. The control of aromatase activity by afferent inputs: physiological effects or experimental artifacts?

The studies reviewed here clearly indicate that experimental manipulations of dopaminergic activity have reliable significant effects on AA in the quail brain. *In vivo* studies have demonstrated these types of effects after periods of 1 to a few days which suggest that changes in enzyme activity are mediated by changes in the concentration of the enzymatic protein as a consequence of increased or decreased transcription of the corresponding gene. *In vitro* studies in contrast demonstrate rapid variations (within minutes) of enzymatic activity by DA and

dopaminergic agonists or antagonists that preclude a mode of action involving changes in transcription. These effects appear specific to DA since they are not observed or observed with a different direction after treatments affecting the noradrenergic transmission.

Two types of cellular mechanisms are obviously required to explain the different actions of DA. The *in vivo* genomic effects can easily be understood by an action of DA at the level of its D1-like receptors. Occupation of the receptor would enhance adenylyl cyclase activity leading to an increased phosphorylation of CREB by protein kinase A and finally to a control of aromatase transcription (see Fig. 10; right part). In contrast, the rapid *in vitro* effects of DA do not appear to be receptor-mediated and the underlying mechanisms remain unclear at present.

Several possibilities can be contemplated: (1) Aromatase and DA production could occur within the same cells and

DA could directly interact with the catalytic site of aromatase as suggested by the competitive inhibition produced by dopamine on AA. This however contradicts anatomical data obtained by double label immunocytochemistry that show a lack of colocalization between catecholamine synthesizing enzyme, tyrosine hydroxylase and aromatase except in a few cells located at the rostro-medial pole of the anterior hypothalamus. (2) DA released from catecholaminergic fibers in the vicinity of aromatase cells could enter the cells and interact directly with aromatase located in the cytoplasm. How DA could pass the neuronal membrane is however difficult to conceive. An internalization of DA mediated by its membrane receptor does not appear likely given the lack of specificity of the responses (not D1–D2 or agonist–antagonist specificity) and the differences between concentrations of dopaminergic drugs that affect AA and the affinity of the DA receptors. There

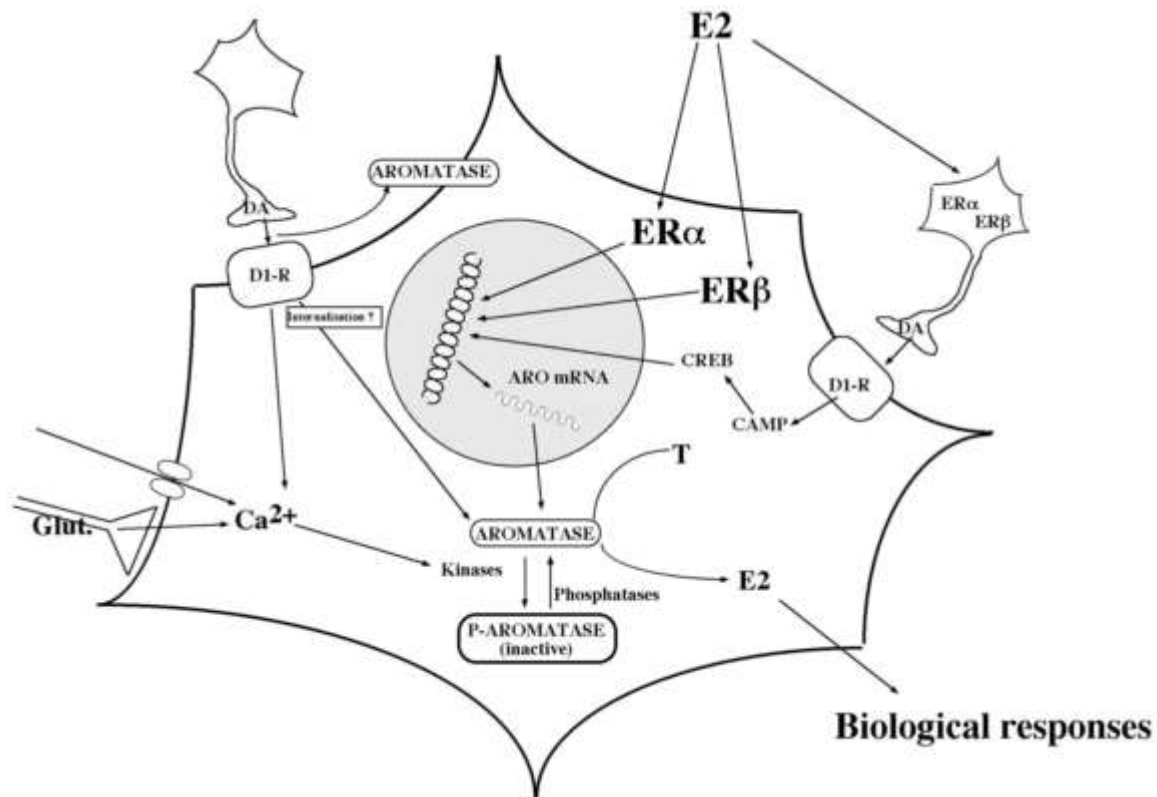


Fig. 10. Diagrammatic representation of the possible mode of action of the neurotransmitter, dopamine (DA) on the activity of aromatase in the brain (ARO). The left and right part of the hypothetical aromatase cell illustrate respectively the slow controls that are presumably mediated by changes in aromatase transcription (right side of figure) and the more rapid effects that do not apparently relate to changes in enzyme concentration (left side of figure). Aromatase concentration, and presumably transcription, can be upregulated by the action of estradiol directly on an aromatase expressing cell via binding to one of the two forms of estrogen receptor (ER $\alpha$  or ER $\beta$ ). Aromatase transcription could also be controlled indirectly by estradiol acting on catecholaminergic estrogen-sensitive neurons. Dopaminergic projections on the aromatase cells could then control the activity of second messenger systems (e.g. cyclic AMP) regulating aromatase transcription as illustrated here for the action of dopamine (DA) via its binding to receptors of the D1 subtype (D1-R). Dopamine could also mediate rapid changes in aromatase activity independent of changes in enzyme concentration. DA could interact directly with intracellular aromatase or with aromatase located at the cell membrane level. How DA could be internalized in aromatase cells is unclear at present. DA could also modify aromatase activity less directly by interfering with the glutamate (Glut.) or Ca<sup>2+</sup>-dependent phosphorylations of the enzyme. Available evidence suggests that changes in activity of a given amount of enzymatic protein are controlled by calcium-dependent phosphorylations of the enzyme (reversible transformation of aromatase in phosphorylated aromatase) that render it inactive. These phosphorylation processes are presumably catalyzed by various kinases. Conversely, phosphatases that catalyze dephosphorylation of the enzymatic protein should enhance enzymatic activity. See also text for additional comments.

is also at present no evidence for the presence of DA receptors on aromatase cells. (3) There is limited evidence for the existence of aromatase in the membrane of certain cell types [5] and DA could then affect the enzyme expressed at the cell surface. However membrane aromatase has not been observed in the brain and examination of ARO-ir cells by confocal microscopy does not reveal specific immunoreactivity on the cell surface (J. Balthazart, unpublished observations). (4) Finally, DA could trigger, by some unidentified mechanism, a change in the intracellular level of calcium in the aromatase-containing cells that would change the phosphorylation state of the enzyme and then its activity. The links between DA and aromatase phosphorylation are however unknown and it is also unclear how such a mechanism could take place both in brain homogenates and in brain explants.

It is clear though that none of these mechanisms can, in isolation, explain all characteristics of the effects of DA on aromatase that have been observed. Additional research will be necessary to identify the cellular mechanisms that mediate the inhibition of AA by DA. Although many questions remain open, it is our contention that these controls observed during *in vivo* and *in vitro* experiments do not represent experimental artifacts but reflect mechanisms that are physiologically relevant. This conclusion is based on the specificity of dopaminergic effects as compared to the effects of compounds that modify the noradrenergic activity and on the converging evidence provided by both pharmacological and anatomical data.

Independent of the underlying mechanisms(s) involved, the studies described above support the notion that rapid changes in the availability of estrogens can take place in the brain and be regulated by neurotransmitter (catecholaminergic) activity. These data suggest that the local bioavailability of estrogens in the brain can change more rapidly than previously assumed and these rapidly changing levels of locally-produced estrogens could control the rapid non-genomic effects that presumably take place at the membrane level. The functional significance of these effects should now be investigated.

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## References

- [1] P. Absil, J. Balthazart, Testosterone effects on neurotensin-immunoreactive cells in the quail preoptic area, *Neuroreport* 5 (1994) 1129–1132.
- [2] P. Absil, A. Foidart, H.C.J. Hemmings, H.W.M. Steinbusch, G.F. Ball, J. Balthazart, Distribution of DARPP-32 immunoreactive structures in the quail brain: anatomical relationship with dopamine and aromatase, *J. Chem. Neuroanat.* 21 (2000) 23–39.
- [3] E.K. Adkins, N.T. Adler, Hormonal control of behavior in the Japanese quail, *J. Comp. Physiol. Psychol.* 81 (1972) 27–36.
- [4] K.A. Albert, E. Helmer-Matyjek, A.A. Nairn, T.H. Müller, J.W. Haycock, L.A. Greene, M. Goldstein, P. Greengard, Calcium/phospholipid-dependent protein kinase (protein kinase C) phosphorylates and activates tyrosine hydroxylase, *Proc. Natl. Acad. Sci. USA* 81 (1984) 7713–7717.
- [5] B.A. Amarnah, E.R. Simpson, Detection of aromatase cytochrome P450, 17 $\alpha$ -hydroxylase cytochrome P450 and NADPH:P450 reductase on the surface of cells in which they are expressed, *Mol. Cell. Endocrinol.* 119 (1996) 69–74.
- [6] M.M. Ames, P. Lerner, W. Lovenberg, Tyrosine hydroxylase: activation by protein phosphorylation and end product inhibition, *J. Biol. Chem.* 253 (1978) 27–31.
- [7] N. Aste, J. Balthazart, P. Absil, R. Grossmann, E. Mühlbauer, C. Viglietti-Panzica, G.C. Panzica, Anatomical and neurochemical definition of the nucleus of the stria terminalis in Japanese quail (*Coturnix japonica*), *J. Comp. Neurol.* 396 (1998) 141–157.
- [8] N. Aste, G.C. Panzica, P. Aimar, C. Viglietti-Panzica, N. Harada, A. Foidart, J. Balthazart, Morphometric studies demonstrate that aromatase-immunoreactive cells are the main target of androgens and estrogens in the quail medial preoptic nucleus, *Exp. Brain Res.* 101 (1994) 241–252.
- [9] N. Aste, G.C. Panzica, C. Viglietti-Panzica, N. Harada, J. Balthazart, Distribution and effects of testosterone on aromatase mRNA in the quail forebrain: A non-radioactive *in situ* hybridization study, *J. Chem. Neuroanat.* 14 (1998) 103–115.
- [10] N. Aste, C. Viglietti-Panzica, J. Balthazart, G.C. Panzica, Testosterone modulation of peptidergic pathways in the septo-preoptic region of male Japanese quail, *Poultry Avian Biol. Rev.* 8 (1997) 77–93.
- [11] T. Bailhache, J. Balthazart, The catecholaminergic system of the quail brain: Immunocytochemical studies of dopamine beta-hydroxylase and tyrosine hydroxylase, *J. Comp. Neurol.* 329 (1993) 230–256.
- [12] T. Bailhache, A. Foidart, C. Surlemont, N. Harada, J. Balthazart, Catecholaminergic innervation of aromatase and estrogen receptor-immunoreactive cells in the quail brain, *Soc. Neurosci. Abstr.* 17 (1991) 269.
- [13] M. Baillien, J. Balthazart, A direct dopaminergic control of aromatase activity in the quail preoptic area, *J. Steroid Biochem. Mol. Biol.* 63 (1997) 99–113.
- [14] M. Baillien, A. Foidart, J. Balthazart, Regional distribution and control of tyrosine hydroxylase activity in the quail brain, *Brain Res. Bull.* 48 (1999) 31–37.
- [15] J. Balthazart, Steroid control and sexual differentiation of brain aromatase, *J. Steroid Biochem. Mol. Biol.* 61 (1997) 323–339.
- [16] J. Balthazart, Steroid metabolism and the activation of social behavior, in: J. Balthazart (Ed.), *Advances in Comparative and Environmental Physiology*, Vol. 3, Springer-Verlag, Berlin, 1989, pp. 105–159.
- [17] J. Balthazart, P. Absil, Identification of catecholaminergic inputs to and outputs from aromatase-containing brain areas of the Japanese quail by tract tracing combined with tyrosine hydroxylase immunocytochemistry, *J. Comp. Neurol.* 382 (1997) 401–428.
- [18] J. Balthazart, P. Absil, C. Viglietti-Panzica, G.C. Panzica, Vasotocinergic innervation of areas containing aromatase-immunoreactive cells in the quail forebrain, *J. Neurobiol.* 33 (1997) 45–60.
- [19] J. Balthazart, M. Baillien, G.F. Ball, Interactions between aromatase (estrogen synthase) and dopamine in the control of male sexual behavior in quail, *Comp. Biochem. Physiol. [B]* (2001) in press.
- [20] J. Balthazart, M. Baillien, G.F. Ball, Phosphorylation processes mediate rapid changes of brain aromatase activity, *J. Steroid Biochem. Mol. Biol.* (2001) in press.

- [21] J. Balthazart, M. Baillien, G.F. Ball, Rapid and reversible inhibition of brain aromatase activity, *J. Neuroendocrinol.* 13 (2001) 61–71.
- [22] J. Balthazart, G.F. Ball, Effects of the noradrenergic neurotoxin DSP-4 on luteinizing hormone levels, catecholamine concentrations, alpha2-adrenergic receptor binding, and aromatase activity in the brain of the Japanese quail, *Brain Res.* 492 (1989) 163–175.
- [23] J. Balthazart, G.F. Ball, Fast regulation of steroid biosynthesis: a further piece in the neurosteroid puzzle, *Trends Neurosci.* 23 (2000) 57–58.
- [24] J. Balthazart, G.F. Ball, Is dopamine interacting with aromatase to control sexual behavior in male quail?, *Poultry Sci. Rev.* 4 (1992) 217–233.
- [25] J. Balthazart, G.F. Ball, The Japanese quail as a model system for the investigation of steroid-catecholamine interactions mediating appetitive and consummatory aspects of male sexual behavior, *Ann. Rev. Sex Res.* 9 (1998) 96–176.
- [26] J. Balthazart, G.F. Ball, New insights into the regulation and function of brain estrogen synthase (aromatase), *Trends Neurosci.* 21 (1998) 243–249.
- [27] J. Balthazart, G.F. Ball, Sexual differentiation of brain and behavior in birds, *Trends Endocrinol. Metab.* 6 (1995) 21–29.
- [28] J. Balthazart, A. Foidart, M. Baillien, N. Harada, and the control of male sexual behavior, *J. Steroid Biochem. Mol. Biol.* 44 (1993) 521–540.
- [29] J. Balthazart, A. Foidart, P. Absil, N. Harada, Effects of testosterone and its metabolites on aromatase-immunoreactive cells in the quail brain: relationship with the activation of male reproductive behavior, *J. Steroid Biochem. Mol. Biol.* 56 (1996) 185–200.
- [30] J. Balthazart, A. Foidart, M. Baillien, N. Harada, G.F. Ball, Anatomical relationships between aromatase and tyrosine hydroxylase in the quail brain: double-label immunocytochemical studies, *J. Comp. Neurol.* 391 (1998) 214–226.
- [31] J. Balthazart, A. Foidart, N. Harada, Immunocytochemical localization of aromatase in the brain, *Brain Res.* 514 (1990) 327–333.
- [32] J. Balthazart, A. Foidart, J.C. Hendrick, The induction by testosterone of aromatase activity in the preoptic area and activation of copulatory behavior, *Physiol. Behav.* 47 (1990) 83–94.
- [33] J. Balthazart, A. Foidart, P. Sante, J.C. Hendrick, Effects of alpha-methyl-para-tyrosine on monoamine levels in the Japanese quail: Sex differences and testosterone effects, *Brain Res. Bull.* 28 (1992) 275–288.
- [34] J. Balthazart, A. Foidart, C. Surlemont, N. Harada, Neuroanatomical specificity in the co-localization of aromatase and estrogen receptors, *J. Neurobiol.* 22 (1991) 143–157.
- [35] J. Balthazart, J.M. Libioulle, P. Sante, Stimulatory effects of the noradrenergic neurotoxin DSP4 on sexual behavior in male quail, *Behav. Proc.* 17 (1988) 27–44.
- [36] J. Balthazart, R. Stoop, A. Foidart, N. Harada, Synergistic control by androgens and estrogens of aromatase in the quail brain, *Neuroreport* 5 (1994) 1729–1732.
- [37] J. Balthazart, C. Surlemont, Androgen and estrogen action in the preoptic area and activation of copulatory behavior in quail, *Physiol. Behav.* 48 (1990) 599–609.
- [38] J. Balthazart, C. Surlemont, Copulatory behavior is controlled by the sexually dimorphic nucleus of the quail POA, *Brain Res. Bull.* 25 (1990) 7–14.
- [39] J. Balthazart, C. Surlemont, N. Harada, Aromatase as a cellular marker of testosterone action in the preoptic area, *Physiol. Behav.* 51 (1992) 395–409.
- [40] J. Balthazart, O. Tlemçani, N. Harada, Localization of testosterone-sensitive and sexually dimorphic aromatase-immunoreactive cells in the quail preoptic area, *J. Chem. Neuroanat.* 11 (1996) 147–171.
- [41] C. Barberis, S. Audigier, T. Durroux, J. Elands, A. Schmidt, S. Jard, Pharmacology of oxytocin and vasopressin receptors in the central and peripheral nervous system, *Ann. N.Y. Acad. Sci.* 652 (1992) 39–45.
- [42] C.A. Barraclough, P.M. Wise, The role of catecholamines in the regulation of pituitary luteinizing hormone and follicle stimulating-hormone secretion, *Endocr. Rev.* 3 (1982) 91–119.
- [43] F.L. Bellino, L. Holben, Placental estrogen synthetase (aromatase): Evidence for phosphatase-dependent inactivation, *Biochem. Biophys. Res. Commun.* 162 (1989) 498–504.
- [44] W.P.M. Benten, M. Lieberherr, O. Stamm, C. Wrehlke, Z.Y. Guo, F. Wunderlich, Testosterone signaling through internalizable surface receptors in androgen receptor-free macrophages, *Mol. Biol. Cell* 10 (1999) 3113–3123.
- [45] J.D. Blaustein, M.J. Tetel, J.M. Meredith, Neurobiological regulation of hormonal response by progesterin and estrogen receptors, in: P.E. Micevych, R.P.J. Hammer (Eds.), *Neurobiological Effects of Sex Steroid Hormones*, Cambridge University Press, Cambridge, 1995, pp. 324–349.
- [46] G.V. Callard, Aromatization is cyclic AMP-dependent in cultured brain cells, *Brain Res.* 204 (1981) 461–464.
- [47] J.A. Canick, S.A. Tobet, M.J. Baum, D.E. Vaccaro, K.J. Ryan, S.E. Leeman, T.O. Fox, Studies on the role of catecholamines in the regulation of the developmental pattern of hypothalamic aromatase, *Steroids* 50 (1987) 510–521.
- [48] D.P. Cardinali, M.N. Ritta, P.V. Gejman, Norepinephrine stimulates testosterone aromatization and inhibits 5 $\alpha$ -reduction via  $\beta$ -adrenoceptors in the rat pineal gland, *Mol. Cell. Endocrinol.* 28 (1982) 199–209.
- [49] C.J. Corbin, S. Graham-Lorence, M. McPhaul, J.I. Mason, C.R. Mendelson, E.R. Simpson, Isolation of a full-length cDNA insert encoding human aromatase system cytochrome P-450 and its expression in nonsteroidogenic cells, *Proc. Natl. Acad. Sci. USA* 85 (1988) 8948–8952.
- [50] E. Cross, C.E. Roselli, 17beta-estradiol rapidly facilitates chemoinvestigation and mounting in castrated male rats, *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 276 (1999) R1346–R1350.
- [51] S.C. Daubner, C. Lauriano, J.W. Haycock, P.F. Fitzpatrick, Site-directed mutagenesis of serine 40 of rat tyrosine hydroxylase, *J. Biol. Chem.* 267 (1992) 12639–12646.
- [52] G.J. de Vries, Sex differences in neurotransmitter systems, *J. Neuroendocrinol.* 2 (1990) 1–13.
- [53] G.J. de Vries, Z. Wang, N.A. Bullock, S. Numan, Sex differences in the effects of testosterone and its metabolites on vasopressin messenger RNA levels in the bed nucleus of the stria terminalis of rats, *J. Neurosci.* 14 (1994) 1789–1794.
- [54] A. Foidart, B. Lakaye, T. Grisar, G.F. Ball, J. Balthazart, Estrogen receptor-beta in quail: Cloning, tissue expression and neuroanatomical distribution, *J. Neurobiol.* 40 (1999) 327–342.
- [55] R. Fürbass, C. Kalbe, J. Vanselow, Tissue-specific expression of the bovine aromatase-encoding gene uses multiple transcriptional start sites and alternative first exons, *Endocrinology* 138 (1997) 2813–2819.
- [56] L.D. Grant, W.E. Stumpf, Hormone uptake sites in CNS biogenic amines systems, in: W.E. Stumpf, L.D. Grant (Eds.), *Anatomical Neuroendocrinology*, Karger, Basel, 1975, pp. 445–463.
- [57] Q. Gu, K.S. Korach, R.L. Moss, Rapid action of 17beta-estradiol on kainate-induced currents in hippocampal neurons lacking intracellular estrogen receptors, *Endocrinology* 140 (1999) 660–666.
- [58] N. Harada, Novel properties of human placental aromatase as cytochrome P-450: purification and characterization of a unique form of aromatase, *J. Biochem.* 103 (1988) 106–113.
- [59] N. Harada, S. Abe-Dohmae, R. Loeffen, A. Foidart, J. Balthazart, Synergism between androgens and estrogens in the induction of aromatase and its messenger RNA in the brain, *Brain Res.* 622 (1993) 243–256.
- [60] N. Harada, K. Yamada, A. Foidart, J. Balthazart, Regulation of aromatase cytochrome P-450 (estrogen synthetase) transcripts in the quail brain by testosterone, *Mol. Brain Res.* 15 (1992) 19–26.
- [61] A.E. Herbison, Multimodal influence of estrogen upon gonadotropin-releasing hormone neurons, *Endocr. Rev.* 19 (3) (1998) 302–330.
- [62] A.E. Herbison, J.E. Robinson, D.C. Skinner, Distribution of estrogen receptor-immunoreactive cells in the preoptic area of the ewe:

- Co-localization with glutamic acid decarboxylase but not luteinizing hormone-releasing hormone, *Neuroendocrinology* 57 (1993) 751–759.
- [63] A.E. Herbison, D.T. Theodosios, Localization of oestrogen receptors in preoptic neurons containing neurotensin but not tyrosine hydroxylase, cholecystokinin or luteinizing hormone-releasing hormone in the male and female rat, *Neuroscience* 50 (1992) 283–298.
- [64] A.S. Heritage, W.E. Stumpf, M. Sar, L.D. Grant, Brain stem catecholamine neurons are target sites for sex steroid hormones, *Science* 204 (1980) 1377–1379.
- [65] G.J. Hickey, J.S. Krasnow, W.G. Beattie, J.S. Richards, Aromatase cytochrome P450 in rat ovarian granulosa cells before and after luteinization: Adenosine 3',5'-monophosphate-dependent and independent regulation. Cloning and sequencing of rat aromatase cDNA and 5' genomic DNA, *Mol. Endocrinol.* 4 (1990) 3–12.
- [66] R.A. Hinde, E. Steel, The influence of daylength and male vocalizations on the estrogen-dependent behavior of female canaries and budgerigars, with discussion of data from other species, *Adv. Study Anim. Behav.* 8 (1978) 39–73.
- [67] Z. Hochberg, T. Bick, R. Pelman, J.M. Brandes, D. Barzilai, The dual effect of calcium on aromatization by cultured human trophoblast, *J. Steroid Biochem.* 24 (1986) 1217–1219.
- [68] J.B. Hutchison, T.H. Steimer, Formation of behaviorally effective 17 $\beta$ -estradiol in the dove brain: steroid control of preoptic aromatase, *Endocrinology* 118 (1986) 2180–2187.
- [69] M. Joëls, Steroid hormones and excitability in the mammalian brain, *Front. Neuroendocrinol.* 18 (1997) 2–48.
- [70] H.W. Korf, G.C. Panzica, C. Viglietti-Panzica, A. Oksche, Pattern of peptidergic neurons in the avian brain: clusters–local circuitries–projections, *Bas. Appl. Histochem.* 32 (1988) 55–75.
- [71] W.J. Kuenzel, M. Masson, A Stereotaxic Atlas of the Brain of the Chick (*Gallus domesticus*), The Johns Hopkins University Press, Baltimore, 1988, 1–166, pp. 1–166.
- [72] G.G.J.M. Kuiper, E. Enmark, M. Pelto-Huikko, S. Nilsson, J.Å. Gustafsson, Cloning of a novel estrogen receptor expressed in rat prostate and ovary, *Proc. Natl. Acad. Sci. USA* 93 (1996) 5925–5930.
- [73] G.G.J.M. Kuiper, P.J. Shughrue, I. Merchenthaler, J.-Å. Gustafsson, The estrogen receptor b subtype: a novel mediator of estrogen action in neuroendocrine systems, *Front. Neuroendocrinol.* 19 (1998) 253–286.
- [74] B. Lakaye, A. Foidart, T. Grisar, J. Balthazart, Partial cloning and distribution of estrogen receptor beta in the avian brain, *Neuroreport* 9 (1998) 2743–2748.
- [75] E.D. Lephart, A review of brain aromatase cytochrome P450, *Brain Res. Rev.* 22 (1996) 1–26.
- [76] R.A. Levine, H.B. Pollard, D.M. Kuhn, A rapid and simplified assay method for tyrosine hydroxylase, *Anal. Biochem.* 143 (1984) 205–208.
- [77] D.L. Maney, D.J. Bernard, F.W. Turek, G.F. Ball, Localization of androgen receptor and estrogen receptor alpha mRNA in catecholaminergic cell groups of the canary brain, *Soc. Neurosci. Abstr.* 25 (1999) 860–866.
- [78] B.S. McEwen, Neural gonadal steroid actions, *Science* 211 (1981) 1303–1311.
- [79] B.S. McEwen, Steroid hormone actions on the brain: When is the genome involved?, *Horm. Behav.* 28 (1994) 396–405.
- [80] B.S. McEwen, S.E. Alves, Estrogen actions in the central nervous system, *Endocr. Rev.* 20 (1999) 279–307.
- [81] B.S. McEwen, L.C. Krey, Properties of estrogen-sensitive neurons: aromatization, progesterone receptor induction and neuroendocrine effects, in: F. Celotti, F. Naftolin, L. Martini (Eds.), *Metabolism of Hormonal Steroids in the Neuroendocrine Structures*, Raven Press, New York, 1984, pp. 117–128.
- [82] M.J. McPhaul, J.F. Noble, E.R. Simpson, C.R. Mendelson, J.D. Wilson, The expression of a functional cDNA encoding the chicken cytochrome P-450arom (aromatase) that catalyzes the formation of estrogen from androgen, *J. Biol. Chem.* 263 (1988) 16358–16363.
- [83] G.D. Means, M.S. Mahendroo, C.J. Corbin, J.M. Mathis, F.E. Powell, C.R. Mendelson, E.R. Simpson, Structural analysis of the gene encoding human aromatase cytochrome P-450, the enzyme responsible for estrogen biosynthesis, *J. Biol. Chem.* 264 (1989) 19385–19391.
- [84] C.R. Mendelson, M.E. Smith, W.H. Cleland, E.R. Simpson, Regulation of aromatase activity of cultured adipose stromal cells by catecholamines and adrenocorticotropin, *Mol. Cell. Endocrinol.* 37 (1984) 61–72.
- [85] P.G. Mermelstein, J.B. Becker, D.J. Surmeier, Estradiol reduces calcium currents in rat neostriatal neurons via a membrane receptor, *J. Neurosci.* 16 (1996) 595–604.
- [86] R.L. Moss, Q. Gu, M. Wong, Estrogen: nontranscriptional signaling pathway, *Rec. Prog. Horm. Res.* 52 (1997) 33–69.
- [87] T. Nagatsu, M. Levitt, S. Udenfriend, A rapid and simple radioassay for tyrosine hydroxylase activity, *Anal. Biochem.* 9 (1964) 122–126.
- [88] B. Nock, H.H. Feder, Neurotransmitter modulation of steroid action in target cells that mediate reproduction and reproductive behavior, *Neurosci. Biobehav. Rev.* 5 (1981) 437–447.
- [89] O.M. Onagbesan, M.J. Podie, Calcium-dependent stimulation of estrogen secretion by FSH from theca cells of the domestic hen (*Gallus domesticus*), *Gen. Comp. Endocrinol.* 75 (1989) 177–186.
- [90] Y. Osawa, T. Higashiyama, Y. Toma, C. Yarborough, Diverse function of aromatase and the N-terminal sequence deleted form, *J. Steroid Biochem. Mol. Biol.* 61 (1997) 117–126.
- [91] Y. Osawa, T. Higashiyama, C. Yarborough, Diverse functions of aromatase cytochrome P-450: catecholesterogenesis, cocaine N-demethylation, and other selective drug metabolisms, in: M.C. Lechner (Ed.), *Cytochrome P450*, 8th International Conference, John Libbey Eurotext, Paris, 1994, pp. 893–896.
- [92] Y. Osawa, T. Higashiyama, C. Yarborough, Formation of 6 $\alpha$ -hydroxyestradiol (6 $\alpha$ -OHE2) from E2 by aromatase: evidence for the reverse side interaction of E2 at the active site of aromatase, *Endocr. Soc. 77 Abstr.* (1995) 88.
- [93] M.A. Ottinger, J. Balthazart, Brain monoamines in Japanese quail: effects of castration and steroid replacement therapy, *Behav. Proc.* 14 (1987) 197–216.
- [94] M.A. Ottinger, M. Schumacher, R.N. Clarke, C.S. Duchala, J. Balthazart, Comparison of monoamine concentrations in the brains of adult male and female Japanese quail, *Poultry Sci.* 65 (1986) 1413–1420.
- [95] H.W.T.M. Overes, R. De Leeuw, H.J. Kloosterboer, Regulation of aromatase activity in FSH-primed rat granulosa cells in vitro by follicle-stimulating hormone and various amounts of human chorionic gonadotrophin, *Hum. Reprod.* 7 (1992) 191–196.
- [96] G.C. Panzica, Sex differences in the avian brain, *Sitta* 2 (1988) 77–92.
- [97] G.C. Panzica, E. Garcia-Ojeda, C. Viglietti-Panzica, N.E. Thompson, M.A. Ottinger, Testosterone effects on vasotocinergic innervation of sexually dimorphic medial preoptic nucleus and lateral septum during aging in male quail, *Brain Res.* 712 (1996) 190–198.
- [98] G.C. Panzica, C. Viglietti-Panzica, J. Balthazart, The sexually dimorphic medial preoptic nucleus of quail: a key brain area mediating steroid action on male sexual behavior, *Front. Neuroendocrinol.* 17 (1996) 51–125.
- [99] G.C. Panzica, C. Viglietti-Panzica, M. Calcagni, G.C. Anselmetti, M. Schumacher, J. Balthazart, Sexual differentiation and hormonal control of the sexually dimorphic preoptic medial nucleus in quail, *Brain Res.* 416 (1987) 59–68.
- [100] G.C. Panzica, C. Viglietti-Panzica, F. Sanchez, P. Sante, J. Balthazart, Effects of testosterone on a selected neuronal population within the preoptic sexually dimorphic nucleus of the Japanese quail, *J. Comp. Neurol.* 303 (1991) 443–456.
- [101] C. Pasqualini, V. Olivier, B. Guibert, O. Frain, V. Leviel, Acute stimulatory effect of estradiol on striatal dopamine synthesis, *J. Neurochem.* 65 (1995) 1651–1657.

- [102] B. Ramachandran, B.A. Schlinger, A.P. Arnold, A.T. Campagnoni, Zebra finch aromatase gene expression is regulated in the brain through an alternate promoter, *Gene* 240 (1999) 209–216.
- [103] V.D. Ramirez, J.B. Zheng, K.M. Siddique, Membrane receptors for estrogen, progesterone, and testosterone in the rat brain: Fantasy or reality, *Cell. Mol. Neurobiol.* 16 (1996) 175–198.
- [104] W.J. Raum, M. Marcano, R.S. Swerdloff, Nuclear accumulation of estradiol derived from the aromatization of testosterone is inhibited by hypothalamic beta-receptor stimulation in the neonatal female rat, *Biol. Reprod.* 30 (1984) 388–396.
- [105] W.J. Raum, R.S. Swerdloff, The role of hypothalamic adrenergic receptors in preventing testosterone-induced androgenization in the female rat brain, *Endocrinology* 109 (1981) 273–278.
- [106] C.E. Roselli, L.E. Horton, J.A. Resko, Time-course and steroid specificity of aromatase induction in rat hypothalamus-preoptic area, *Biol. Reprod.* 37 (1987) 628–633.
- [107] C.E. Roselli, J.A. Resko, Androgens regulate brain aromatase activity in adult male rats through a receptor mechanism, *Endocrinology* 114 (1984) 2183–2189.
- [108] C.E. Roselli, J.A. Resko, In vitro assay of aromatase activity in the central nervous system, in: B. Greenstein (Ed.), *Neuroendocrine Research Methods*, Vol. 2, Harwood Academic Publishers, Chur, Switzerland, 1991, pp. 937–951.
- [109] B.D. Sachs, Photoperiodic control of reproductive behavior and physiology of the male Japanese quail (*Coturnix coturnix japonica*), *Horm. Behav.* 1 (1969) 7–24.
- [110] M. Sar, Estradiol is concentrated in tyrosine hydroxylase-containing neurons of the hypothalamus, *Science* 223 (1984) 938–940.
- [111] B.A. Schlinger, G.V. Callard, A comparison of aromatase, 5 $\alpha$ - and 5 $\beta$ -reductase activities in the brain and pituitary of male and female quail (*Coturnix c. Japonica*), *J. Exp. Zool.* 242 (1987) 171–180.
- [112] M. Schumacher, C. Alexandre, J. Balthazart, Interactions des androgènes et des oestrogènes dans le contrôle de la reproduction, *C.R. Acad. Sci. Paris, Série III* 305 (1987) 569–574.
- [113] M. Schumacher, J. Balthazart, Neuroanatomical distribution of testosterone metabolizing enzymes in the Japanese quail, *Brain Res.* 422 (1987) 137–148.
- [114] P. Shen, C.W. Campagnoni, K. Kampf, B.A. Schlinger, A.P. Arnold, A.T. Campagnoni, Isolation and characterization of a zebra finch aromatase cDNA: In situ hybridization reveals high aromatase expression in brain, *Mol. Brain Res.* 24 (1994) 227–237.
- [115] B.D. Shivers, R.E. Harlan, J.I. Morrell, D.W. Pfaff, Absence of oestradiol concentration in cell nuclei of LHRH-immunoreactive neurones, *Nature* 304 (1983) 345–347.
- [116] R.B. Simerly, Hormonal control of the development and regulation of tyrosine hydroxylase expression within a sexually dimorphic population of dopaminergic cells in the hypothalamus, *Mol. Brain Res.* 6 (1989) 297–310.
- [117] R.B. Simerly, L.W. Swanson, The distribution of neurotransmitter-specific cells and fibers in the anteroventral periventricular nucleus: implications for the control of gonadotropin secretion in the rat, *Brain Res.* 400 (1987) 11–34.
- [118] R.B. Simerly, L.W. Swanson, R.A. Gorski, The distribution of monoaminergic cells and fibers in a periventricular preoptic nucleus involved in the control of gonadotropin release: immunohistochemical evidence for a dopaminergic sexual dimorphism, *Brain Res.* 330 (1985) 55–64.
- [119] E.R. Simpson, M.S. Mahendroo, G.D. Means, M.W. Kilgore, M.M. Hinshelwood, S. Graham-Lorence, B. Amarnah, Y. Ito, C.R. Fisher, M.D. Michael, C.R. Mendelson, S.E. Bulun, Aromatase cytochrome P450, the enzyme responsible for estrogen biosynthesis, *Endocr. Rev.* 15 (1994) 342–355.
- [120] E.R. Simpson, M.D. Michael, V.R. Agarwal, M.M. Hinshelwood, S.E. Bulun, Y. Zhao, Expression of the CYP19 (aromatase) gene: An unusual case of alternative promoter usage, *FASEB J.* 11 (1997) 29–36.
- [121] T. Steimer, J.B. Hutchison, Micromethods for the in vitro study of steroid metabolism in the brain using radiolabelled tracers, in: B. Greenstein (Ed.), *Neuroendocrine Research Methods*, Vol. 2, Harwood Academic Publishers, Chur, Switzerland, 1991, pp. 875–919.
- [122] Y. Toma, T. Higashiyama, C. Yarborough, Y. Osawa, Diverse functions of aromatase: O-deethylation of 7-ethoxycoumarin, *Endocrinology* 137 (1996) 3791–3796.
- [123] Y. Tsuruo, K. Ishimura, S. Hayashi, Y. Osawa, Immunohistochemical localization of estrogen receptors within aromatase-immunoreactive neurons in the fetal and neonatal rat brain, *Anat. Embryol. (Berl)* 193 (1996) 115–121.
- [124] Y. Tsuruo, K. Ishimura, Y. Osawa, Presence of estrogen receptors in aromatase-immunoreactive neurons in the mouse brain, *Neurosci. Lett.* 195 (1995) 49–52.
- [125] G. Verhoeven, Effect of neurotransmitters and follicle-stimulating hormone on the aromatization of androgens and the production of 3',5'-monophosphate by cultured testicular cells, *J. Steroid Biochem.* 12 (1980) 315–322.
- [126] G. Verhoeven, J. Cailleau, Testicular peritubular cells secrete a protein under androgen control that inhibits induction of aromatase activity in Sertoli cells, *Endocrinology* 123 (1988) 2100–2110.
- [127] G.P. Verhoeven, P. Dierckx, P. de Moor, Stimulation effect of neurotransmitters on the aromatization of testosterone by Sertoli cell-enriched cultures, *Mol. Cell. Endocrinol.* 13 (1979) 241–253.
- [128] C. Viglietti-Panzica, J. Balthazart, L. Plumari, S. Fratesi, P. Absil, G.C. Panzica, Estradiol mediates effects of testosterone on vasotocin-immunoreactivity in the adult quail brain, *Horm. Behav.* (2001) in press.
- [129] C. Viglietti-Panzica, G.C. Anselmetti, J. Balthazart, N. Aste, G.C. Panzica, Vasotocinergic innervation of the septal region in the Japanese quail: Sexual differences and the influence of testosterone, *Cell Tissue Res.* 267 (1992) 261–265.
- [130] C. Viglietti-Panzica, N. Aste, J. Balthazart, G.C. Panzica, Vasotocinergic innervation of sexually dimorphic medial preoptic nucleus of the male Japanese quail: Influence of testosterone, *Brain Res.* 657 (1994) 171–184.
- [131] C. Viglietti-Panzica, G.C. Panzica, Peptidergic neurons in the avian brain, *Ann. Sci. Nat., Zool.*, Paris 12 (1991) 137–155.
- [132] C. Viglietti-Panzica, G.C. Panzica, M.G. Fiori, M. Calcagni, G.C. Anselmetti, J. Balthazart, A sexually dimorphic nucleus in the quail preoptic area, *Neurosci. Lett.* 64 (1986) 129–134.
- [133] N. Weiner, A critical assessment of methods for the determination of monoamine synthesis turnover rates in vivo, *Adv. Biochem. Pharmacol.* 12 (1974) 143–159.
- [134] R.I. Weiner, P.R. Findell, C. Kordon, Role of classic and peptide neuromediators in the neuroendocrine regulation of LH and prolactin, in: E. Knobil, J. Neill et al. (Eds.), *The Physiology of Reproduction*, Raven Press, New York, 1988, pp. 1235–1281.
- [135] J.-P. Weniger, A. Zeis, Stimulation of aromatase activity in the fetal rat gonads by cAMP and FSH, *Acta Endocrinol. (Copenhagen)* 119 (1988) 381–385.