Synaptocrine Signaling: Steroid Synthesis and Action at the Synapse

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Sex steroids have long been recognized for their dramatic impact on brain and behavior, including rapid modulation of membrane excitability. It is a widely held perception that these molecules are largely derived from peripheral sources and lack the spatial and temporal specificity ascribed to classical neuromodulatory systems. Neuromodulatory systems, in contrast, are defined by their regulated neuronal presynaptic secretion and by their functional modulation of perisynaptic events. Here we provide evidence for regulated presynaptic estrogen synthesis and functional postsynaptic actions. These results meet all the criteria for a neuromodulatory system and shift our perception of estrogens from that of peripheral signals exclusively to include that of a signaling system intrinsic to the brain itself. We apply the term synaptocrine to describe this form of neuromodulation. **(*Endocrine Reviews* 32: 532–549, 2011)**

I. Introduction: Distance, Range, and Specificity—Fundamental Concepts in Endocrinology

A consideration of a steroidal neuromodulatory system requires a critical rethinking of long-held concepts regarding steroids as endocrine signals. Distance is a core consideration in endocrinology, so we first define current concepts and then place synaptic steroid synthesis within that framework.

From its point of synthesis to its site of action, the distance that a hormone might travel can vary by many orders of magnitude. This distance has served as the basis for defining several forms of hormone signaling (Fig. 1). Classical endocrine systems involve hormone secretion into the vasculature, where the chemical signals are carried to distant target tissues centimeters or even meters away. In other cases, however, the hormonal signal diffuses over a relatively small distance through extracellular fluid to reach target cells, often within the same organ. Signaling in this manner is considered paracrine and typically involves distances of micrometers to millimeters. In the most extreme cases of autocrine/intracrine physiology, a cell is activated by its own hormonal signals, and distance becomes a concept only in the context of cell physiology.

These signaling systems offer their own unique advantages and disadvantages. Long distance endocrine signaling can orchestrate the responses of many tissues simultaneously but has little capacity to achieve targeted responses. Para-

ISSN Print 0021-972X ISSN Online 1945-7197
Printed in U.S.A.

doi: 10.1210/er.2011-0004 Received January 27, 2011. Accepted May 3, 2011.
First Published Online May 26, 2011

* C.J.S. and L.R.-H. contributed equally to the experiments and concepts described in this review.

Abbreviations: aCSF, Artificial CSF; BOS, bird's own song; CNS, central nervous system; CON, conspecific song; CSF, cerebrospinal fluid; DHEA, dehydroepiandrosterone; EM, immunoelectron microscopy; ER, endoplasmic reticulum; FAD, fadrozole; GABA, γ-aminobutyric acid; HP, hippocampus; HPOA, hypothalamic preoptic area; HSD, hydroxysteroid dehydrogenase; NCM, caudomedial nidopallium; NMDA, N-methyl-D-aspartate.

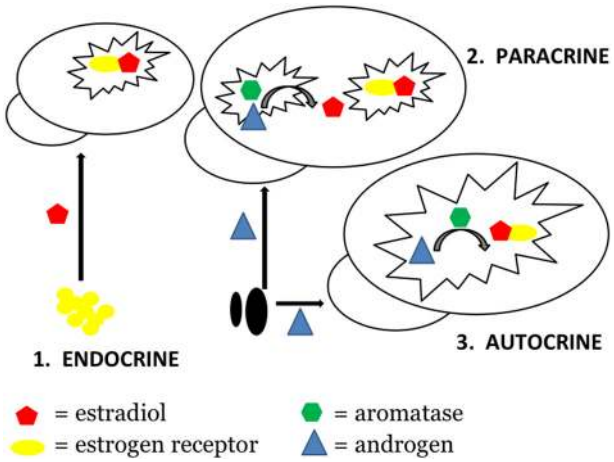

Fig. 1. Classical modes of hormone delivery and action. Endocrine (1): Hormones are made available to the vertebrate brain via peripheral synthesis and passage through the vasculature [in this example, circulating ovarian-produced estradiol (*red pentagons*) modulates neural estrogen receptors (*yellow ovals*)]. Paracrine (2): Local synthesis in neural cells and diffusion across paracellular compartments to adjacent neural targets [in this example, neural aromatization (*green hexagons*) of androgenic substrates (*blue triangles*) produces estradiol that diffuses through the extracellular space with actions on adjacent estrogen receptor-expressing cells]; and autocrine (3), and by synthesis and action within or on the same individual neural cell (in this example, aromatase and estrogen receptor are coexpressed in individual cells, thus the cell is both hormone source and target).

crine secretion achieves more targeted actions, but specificity is dictated primarily by the proximity of hormone-sensitive cells. Autocrine/intracrine actions offer extreme specificity, but cell-to-cell interactions are excluded. Thus, typical hormone signaling pathways would exemplify a trade-off between range and specificity of action. Although hormone synthesis can vary in amount, producing hormone titers that can differ in function, no endocrine cell can dictate the target specificity of its actions. Whereas plasma-binding proteins and the blood-brain barrier may modify the availability of hormone to specific neural targets, these regulatory mechanisms lack spatial specificity. Specificity of hormone action, as we understood it, therefore appears to be a function of the target cell 1) by its proximity to endocrine cells, and 2) by its expression of hormone receptors and conversion enzymes that establish hormone sensitivity and guide function.

II. The Synaptocrine Hypothesis

The synthesis of hormones in presynaptic boutons represents a new frontier in neuroendocrinology. There is abundant evidence that the enzyme aromatase (estrogen synthase) resides in a subset of presynaptic boutons in the vertebrate central nervous system (CNS), having been identified in several avian and mammalian species including humans (1–8). The question of whether estradiol might be considered a neurotransmitter has been raised previously (9). We have labeled this form of communication "synaptocrine" (10, 11), a term that has been adopted by other labs as well (12, 13). Importantly, this mode of hormone provision is conceptually distinct from neurosecretion *per se*. Hormone synthesis by neurons is not new. Neither is hormonal packaging in specialized compartments of neurons such as within the presynaptic bouton. Furthermore, hormonal release upon neuronal depolarization is well established. In fact, all three of the characteristics highlighted above are the traditional definition of the "neuroendocrine cell." The neurohormones under discussion, however, are invariably peptidergic, are synthesized and packaged into dense-core vesicles in a manner similar, if not identical, to the synthesis and packaging of neurotransmitters in clear vesicles, and are secreted into the circulation. Synaptocrine function differs from classical neurosecretion by: 1) the targeted and functional modulation of local hormone concentrations at the synapse; 2) the use of substrates for hormone synthesis from peripheral, central, or intracellular stores; and 3) the synthesis and secretion of molecules that cannot be packaged in vesicles, such as lipophyllic steroids.

In what follows, we provide a conceptual and historical framework for synaptic hormone synthesis, detailing the evidence for the presence of steroidogenic/metabolic enzymes at the synapse. We introduce an avian model that exemplifies many core features of synaptocrine signaling. We conclude by outlining criteria that define accepted neuromodulatory systems and provide evidence that synaptocrine estrogens fulfill the primary conditions of a neuromodulatory signaling system.

A requisite consideration for any synaptocrine signaling system includes the presence of target molecules upon which the synaptocrine signal can act. With respect to synaptocrine estrogen signaling, there is an extensive literature on estrogen receptors both within neurons and on neuronal membranes, including on dendrites, somas, or even presynaptic boutons where synaptically formed hormone may act (14–20). Estrogens can directly modulate neurotransmitter receptors, intracellular signaling, and neuronal gene expression to achieve relatively rapid to relatively slow responses (16, 21–27). Of course, estradiol from any source can potentially act on these receptors. We limit our focus here to synaptic estrogen synthesis and secretion that defines this neuromodulatory system.

III. Evolving Concepts of Neuroendocrine Signaling

The neuroendocrine cell was first formally described in the landmark paper of Scharrer (28) as hypothalamic neurons capable of secreting hormones into the peripheral circulation: 1) directly by terminals located in the posterior

pituitary, and 2) via the pituitary hypophyseal portal system by terminals at the basal lamina interface within the tuberal hypothalamus and adenohypophysis. In both of these cases, the functional consequences of neurohormonal secretion adhered to the classical definition of hormones in that although the source of hormones was novel (neuronal), their mode of action still invoked endocrine pathways. That is, the field considered the neuron as a new cell type capable of hormonal provision, but the mode of delivery was classically endocrine because it relied upon secretion into the vasculature.

Appreciation for these newly identified neuroendocrine cells led to an explosive phase of research into the hormones they produced and the peripheral organ systems they controlled. Two peripheral targets of neurohormones, the gonads and adrenals, produced steroids. Given their lipophyllic nature, they were seen to have unrestricted access back to the brain where they acted to control neuroendocrine cell secretion as well as organizing and activating behavior (29, 30). Although local hormone signaling (paracrine/autocrine/intracrine) had been well described in endocrine glands such as the gonads, pancreas, and adrenals (31–33), the brain was viewed as a mere target of steroids until the estrogen synthetic enzyme aromatase was detected in the male hypothalamus (34, 35). This discovery resolved perplexing evidence that estrogens could induce masculine neural functions: circulating testosterone in males was converted into estrogen in brain and this locally produced estrogen organized and/or activated some masculine neural function. Thus, whereas circulating androgens were readily available throughout the body, via local neural synthesis, estrogens could act upon receptive elements in and around aromatase-expressing neurons in the hypothalamus. Aromatase was soon found to be present in the brains of virtually all vertebrates (36), and evidence began to grow that the aromatase enzyme was present in neurons (37).

These studies prompted additional research that identified multiple steroid metabolic enzymes in brain (38, 39) and led to later identification of the full suite of cholesterol transporters and enzymes required to fully synthesize steroids *de novo* (40–43). Although a detailed discussion of neurosteroidogenesis is beyond the scope of this paper, its presence raises a crucial question that lies at the core of our perspective on steroid neuroendocrinology, namely how and when are steroids made available to the vast array of neural circuits when these lipophyllic molecules have free access to the whole brain, can be produced peripherally or centrally, and can be significantly modified in discrete neural circuits.

We propose that specificity of steroid action in brain is achieved when a steroidogenic cell achieves targeted connections with a steroid target cell. Neurons send projections over varying distances to synapse upon individual target cells. If the synapse can synthesize hormone or can metabolize hormone present in the extracellular space, then it can actively participate in the hormonal regulation of that specific target cell. In this way, one neural circuit gains steroidal control over another. This synaptic regulation of postsynaptic hormonal environments we call synaptocrine actions. We describe here the evidence for this presynaptic expression of the estrogen synthetic enzyme aromatase as an exemplar of synaptocrine actions in the vertebrate CNS.

IV. Presynaptic Localization of the Aromatase Enzyme

The activity of the aromatase enzyme can be directly measured in fresh dissected brain tissue *in vitro* having been studied extensively in all of the major vertebrate lineages (reviewed in Refs. 44–50). Aromatase activity measured in discrete brain macro-areas occurs in a nonuniform distribution in the vertebrate CNS and aligns largely, but not exclusively, with the distribution patterns revealed using *in situ* hybridization analyses of aromatase mRNA expression and with histochemical anatomical methods revealing aromatase protein. Thus, brain estrogen synthesis is a highly conserved property of the vertebrate brain.

Furthermore, there is little doubt that under ordinary circumstances, aromatase in the CNS is largely or exclusively neuronal in homeotherms. When combined with immunocytochemistry with antibodies created against the aromatase protein (Fig. 2), neurons are the only cells immunostained when tissues are collected from normal, uninjured animals (5, 51, 52). Staining is cytoplasmic and is seen over somata and throughout processes (see Ref. 53). Cytoplasmic staining is consistent with the view that aromatase is associated with endoplasmic reticulum (ER) that is widespread throughout the cytoplasm of cells. As is the case for many cytochrome P450 enzymes, aromatase is membrane bound (54, 55). Differential centrifugation of tissue homogenates to produce microsomes (small circular bits of ER) are enriched in aromatase relative to other organelles or by-products of cellular disruption (2).

Under light-field microscopy, some aromatase immunestaining appears punctate (Fig. 2C) and isolated from other immunostained cells (7, 51, 56). These could be terminals, but they are usually not studied further. However, when brain tissue is subject to differential centrifugation as described above, nerve terminals pinch off and reseal. These synaptosomes can be isolated and purified for neurochemical analyses. Indeed, the first evidence for the synaptic localization of steroid-metabolizing enzymes came from the studies of Callard and colleagues (57), who found that the activity of aromatase, the estrogen synthetic enzyme, could be detected in purified synaptosomal preparations prepared from the

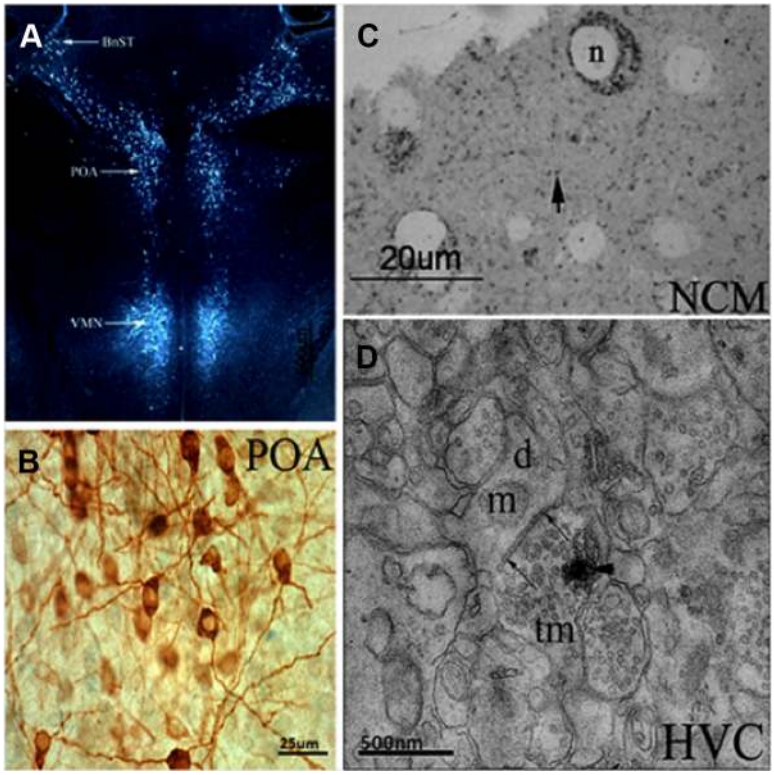

Fig. 2. Aromatase expression in the passerine brain. A, Low-power pseudocolored image of aromatase expression in the ventromedial nucleus of the hypothalamus (VMN), preoptic nucleus (POA), and bed nucleus of the stria terminals (BnST) of an adult male black-capped chickadee (*Poecile atricapillus*). B, High-power photomicrograph of aromatase expression in preoptic neurons of an adult zebra finch. C, Aromatase expression in the NCM of adult zebra finches is detectable in the cytoplasm of cell bodies, but not in the nucleus (n) of neurons and in abundant puncta (*arrows*). D, Immunoelectronmicrograph of aromatase expression (*arrowhead*) in a presynaptic bouton (tm) that innervates (*arrows*) an unstained dendrite (d) containing an unstained mitochondrion (m) in HVC of an adult male zebra finch.

brains of goldfish. Although a recent study raises questions about the neuronal localization of aromatase in teleosts like goldfish (58, 59), a subsequent study found that aromatase activity was enriched in purified synaptosomal preparations of the quail hypothalamus (2). Synaptic localization of aromatase was confirmed and extended by immunoelectron microscopy (EM) studies of the brains of quail, rats, monkeys and humans (5). Here, aromatase-positive synaptic terminals were seen on some aromatase-positive dendrites and somata, but also on aromatase-negative targets. Because these studies focused on the hypothalamus, bed nucleus of the stria terminalis, and amygdala, areas relatively rich with aromatase somata and fibers, the functional implications for aromatase in terminals was difficult to assess. Unfortunately, other brain regions were not examined. Nevertheless, these studies confirmed that aromatase was restricted to sets of individual neurons with an intracellular distribution, whereby estrogens could impact nuclear receptors *in situ*, adjacent estrogen-sensitive cells via local diffusion of estrogen, or by synaptic contact involving aromatase-positive terminals.

In addition to these regions containing an abundance of aromatase positive fibers, with protein in dendrites and putative axons, in many cases, axons were seen to project in directions in which the postsynaptic targets were not obvious (Fig. 2B). Although it was possible that these fibers returned back to cell bodies in the same nucleus, given the lengths of these immunostained axons (sometimes several hundred micrometers long), it was more likely that they synapsed on distal unidentified neurons. If this proved to be the case, then neurons of one circuit could potentially alter the steroid environment of another circuit by projecting afferents and forming synaptic contacts using terminals that contain steroid-metabolic enzymes. It is this concept that forms the basis of the synaptocrine hypothesis. In *Section V*, we will describe evidence that just such a system is operational in the brain of songbirds.

Before moving on, however, it is important to emphasize that aromatase is not the only sex steroid metabolic enzyme to be compartmentalized in synaptic terminals. There is some evidence for a presynaptic localization of the androgen-synthetic enzyme CYP17 in the mammalian hippocampus (HP) (6). In birds, 5β-reductase can be released from synaptic terminals by hyperosmotic lysis (2). 5β-Reductase inactivates testosterone (39), converting it into 5β-dihydrotestosterone, so the synaptic localization of this enzyme could influence postsynaptic androgen actions. This enzyme can also use progesterone as a substrate and participate in the synthesis of 5β-allopregnanolone, a compound that strongly potentiates γ-aminobutyric acid$_A$ (GABA$_A$)-induced postsynaptic hyperpolarization (60). In birds, 5β-allopregnanolone is more potent even than the 5α-reduced isoform (Schlinger, B. A., unpublished; see also Ref. 61). Whether it inactivates androgens or synthesizes neuromodulatory progestins, this cytoplasmic enzyme is expressed widely throughout the brain, so it is likely to be present in cell bodies, processes, and terminals, where it may be positioned to influence the local sex steroid environment in multiple ways.

V. Criteria for a Synaptocrine System and Its Neuromodulatory Influence

Here we list criteria and formal properties of a synaptocrine system. We begin, when appropriate, by delineating predictions of neuromodulatory systems in general and then focus upon the synaptocrine hypothesis in particular. We end with empirical (experimental) evidence from stud-

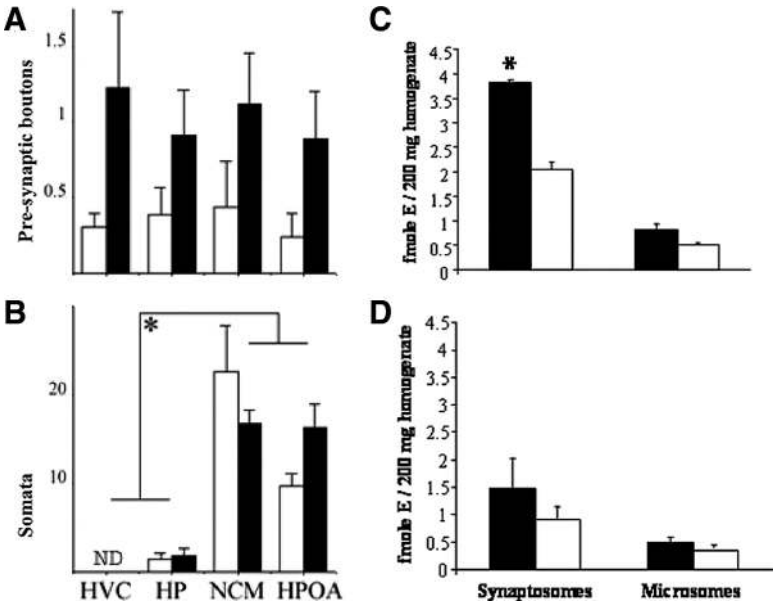

Fig. 3. Aromatase-expressing presynaptic boutons (A) are more frequent in males (*black bars*) relative to females (*white bars*) in several parts of the zebra finch brain such as the HPOA, NCM, and other brain areas where (B) somal expression of this protein is either extremely low, such as in the HP, or is undetectable, such as in HVC (proper name). *, $P < 0.001$. [Adapted from R.S. Peterson et al., Aromatase is presynaptic and sexually dimorphic in the adult zebra finch brain. Proc Biol Sci 272:2089–2096, 2005 (7). © 2005, Proceedings of the Royal Society.] This pattern of data is complemented by measures of aromatase activity where synaptosomes, but not microsomes, from (C) posterior telencephalic homogenates show higher activity in males relative to females. *, $P < 0.05$. No differences in activity are detectable across sexes or ultrastructural fractions (D) from homogenates of the anterior telencephalon. [Adapted from K.N. Rohmann et al., The subcellular compartmentalization of aromatase is sexually dimorphic in the adult zebra finch brain. Dev Neurobiol 67:1–9, 2007 (8), with permission. © 2007, John Wiley & Sons.]

ies of neural estrogen synthesis and action in songbirds that meet these expectations.

The well-described neural circuits of the passerine song circuit have served as an excellent platform for numerous studies on steroid hormone action on cognition, behavior, neuroanatomy, and neuroplasticity (62, 63). The largely cortical and striatal song circuit is rich with intranuclear androgen and estrogen receptors, making it unusually steroid-sensitive, a neuronal property that, in many species, is restricted to a small group of hypothalamic and limbic loci (64). For the remainder of this review, we will focus on a subset of this song circuitry that includes the caudomedial nidopallium (NCM), an auditory processing region, and nucleus HVC (proper name), a key nucleus that receives projections from the auditory system and, in turn, projects to learning and premotor centers required for appropriate song production (63).

VI. Specific Predictions of the Synaptocrine Hypothesis

A. Prediction 1

The secretion of a neuromodulator occurs at the synapse to locally alter the postsynaptic neurochemical environment independent of similar neurochemical changes made elsewhere. In a synaptocrine system involving steroids that are lipophyllic and cannot be stored, synthesis and immediate secretion must be coupled. Therefore changes in the synaptic steroidal environment depend upon synaptic expression of steroidogenic enzymes that are independent of other sources of steroid synthesis.

1. Synaptosomal aromatase is abundant in the songbird brain

Accompanying the elevated steroid sensitivity of the songbird brain is widespread neural expression of the enzyme aromatase (51, 65–79). Aromatase mRNA, protein, and activity are found in traditional neural sites such as the hypothalamus, bed nucleus of the stria terminalis, and the homolog of the mammalian amygdala, but also in especially in large amounts in the telencephalon. As is found in other species, this aromatase is detectable in neurons and is distributed in the soma and dendrites in all areas in which immunoreactivity has been observed (7, 51, 79). In the zebra finch, we were struck further by the large number of punctuate structures present under light microscopy, so we explored aromatase further using EM. To do so, we developed a fixation technique that permitted excellent preservation of antigenicity and unequivocal identification of ultrastructure to visualize aromatase-expressing subcellular compartments in zebra finch brain. These studies showed that aromatase is expressed in presynaptic boutons (Fig. 2D) in every brain area examined (7), including the hypothalamic preoptic area (HPOA), NCM, the HP, and in HVC (Fig. 3, A and B).

These studies were strongly supported by experiments examining the subcellular localization of aromatase activity. As discussed in *Section IV*, in the brains of most vertebrates, biochemical measures of aromatase reveal activity present in synaptosomes but greater enrichment in microsomes (2, 50). In the zebra finch telencephalon, however, aromatase is present in near equal amounts in synaptosomes and microsomes (Fig. 3D) (8). Thus, a substantial portion of telencephalic aromatase (about 50% of total neural aromatase) is present in presynaptic boutons, underscoring the potential importance of synaptic estrogen provision in songbirds and greatly increasing our chances of understanding its physiological relevance.

2. Aromatase-positive somata and synapses in brain are distributed independently

Notably, whereas many somata immunoreactive for aromatase were detected in the HPOA and NCM, the ven-

tral HP contained very low numbers of immunoreactive somata but abundant presynaptic boutons with aromatase immunoproduct (Fig. 3, A and B) (7). This pattern of distribution was even more striking in the HVC, where immunoreactive somata were undetectable but immunoreactive presynaptic boutons were readily detectable. Aromatase activity had been described previously in the zebra finch HVC (66), but there was little or no expression of aromatase mRNA (69). Aromatase present in terminals whose cell bodies lie outside of HVC resolved this apparent paradox. Thus, in the zebra finch, areas with low or even undetectable somal aromatase nonetheless contain high levels of synaptic aromatase. These data suggest the possibility that the major source of estrogen within these areas is synthesized in presynaptic boutons within areas devoid of somal aromatase.

The zebra finch HP bears similarities with HVC in having elevated aromatase activity (80, 81), many aromatase-positive synaptic profiles, and a relatively low number of aromatase-positive somata (Fig. 3, A and B) (7). These somata are spatially restricted to a small population of neurons at the dorsomedial aspect (51). Neurons in the dorsomedial HP project almost exclusively within the HP itself (82), supporting the hypothesis that aromatase-positive neurons in the dorsomedial HP are interneurons that transport aromatase protein down collaterals to terminals within the HP. A comparison of aromatase mRNA expression using *in situ* hybridization (69, 80) with aromatase protein expression using immunocytochemistry (51) shows more cells with mRNA than with protein. This observation raises the possibility that some neurons may transport most or all of their aromatase from somata into terminals. Although this latter possibility remains to be tested, given the overall low somal but elevated presynaptic aromatase immunoreactivity seen in the HP (7), we believe that estrogen provision within the songbird HP is largely synaptic.

These studies confirm predictions of the synaptocrine hypothesis that the steroidogenic enzyme aromatase is present in synapses and these synapses reside where other sources of aromatase are absent.

B. Prediction 2

Regulation of synaptic neuromodulator concentration, independent of its content in other subcellular compartments, can, upon activation, provide differential and targeted signaling needs. In a synaptocrine system involving steroids, steroidogenic enzyme activity must be specifically and actively regulated at the synapse independent of its activity in other neuronal compartments. For purposes of clarity, we offer separate predictions concerning temporal forms of enzyme regulation that are constitutive and/or that occur over a relatively long time scale *vs*. those that occur more rapidly (*Section VI.C. Prediction 3*).

1. Seasonal regulation of aromatase

There is considerable evidence vertebrate-wide that aromatase is subject to seasonal regulation with photoperiod acting through gonadal steroids to modulate activity. In male animals that breed seasonally under the long days of spring and summer, increased photoperiod stimulates testicular steroid secretion that, in turn, up-regulates hypothalamic neuronal aromatase expression (46, 83–85). Elevated aromatase in the HPOA is a requirement for the activation of masculine behaviors in many species (53, 84, 86–89). In adult males, aromatase is present in both microsomal and synaptosomal preparations of the HPOA. In quail, exposure to photostimulatory long days increases microsomal aromatase activity approximately 2.5-fold, but about 6-fold in synaptosomal preparations (2). This differential increase in synaptosomal enzyme could result from an increased number of aromatase-positive synapses, an increase in the concentration of aromatase in each synapse, or seasonal changes in the activity of the enzyme. Because androgen-to-estrogen conversion is necessary for the activation of masculine reproductive behavior in this species and the seasonal increase in HPOA aromatase is required to synthesize sufficient amounts of estrogen (90), seasonal increases in synaptic aromatase may be key to providing the requisite amounts of bioactive estrogen.

2. Sex differences in aromatase

Sex-specific regulation of gene and protein expression is basic to the creation of sexually dimorphic phenotypes. Our data indicate that sexually dimorphic synaptic aromatase is central to sex differences in steroidal signaling in the zebra finch. The zebra finch has long proven an excellent model in behavioral neuroendocrinology, in part due to its dramatic sexual dimorphisms in singing behavior (91) and in brain structure (92). Estrogens are implicated in at least some of the masculine development of the song circuitry (93–95), in the plasticity underlying the masculine learning of song (96), in the seasonal growth of song circuitry in seasonal breeders (97), and in the functional activation of this circuitry for the masculine production of song (Ref. 98). Nevertheless, despite numerous attempts to describe sex differences in estrogen provision in zebra finches that are the basis of these estrogen-dependent phenotypes, including studies of circulating estradiol (77, 99, 100), gonadal estrogen synthesis (65, 77), neural aromatase activity and expression (1, 66, 69, 101), and aromatase immunoproduct (reviewed in Ref. 102), the only masculine-biased feature so far consistently identi-

fied is the sexually dimorphic number of aromatase-positive fibers and terminals (7, 8, 51).

In keeping with measures of aromatase activity (66), immunostaining with a zebra finch-specific antibody showed no sex differences in aromatase-positive somata in several brain regions, including the HPOA and NCM (51). Nevertheless, males possessed significantly higher numbers of aromatase-positive fibers in these brain areas, suggesting that aromatase-expressing neurons were more branched in males or that the aromatase protein was selectively compartmentalized into fibers in the male zebra finch brain (51).

These studies were conducted using conventional light microscopy, preventing unequivocal inferences as to the dendritic and/or axonal nature of the fibers in question. Based on these observations, as part of our ultrastructural analysis of the subcellular localization of aromatase, we counted the number of synaptic terminals containing aromatase immunoreactivity, and we found that aromatase-expressing presynaptic boutons were more plentiful in the male brain relative to females (7). Indeed, both the absolute and the relative (as a function of total synaptic profiles) incidence of aromatase-expressing presynaptic boutons were male biased (Fig. 3, A and B). These studies were key in revealing that the male zebra finch brain may be provided with higher levels of estrogen at various telencephalic areas relative to the female. Notably, somal and dendritic aromatase expression were not dimorphic (7), supporting the concept that synaptocrine, and not paracrine, estrogen provision better explains neural estrogen provision in these brain regions of this species.

To confirm these immunohistochemical observations and to verify that the immunoreactivity we detected was translated into functional protein, we reasoned that subfractionation of zebra finch brain homogenates should parallel the results of EM studies; that is, synaptosomal preparations of brain homogenates should have greater aromatase activity in males than in females, whereas microsomal preparations, presumably better reflecting somal aromatase, should show no sex differences. As predicted (Fig. 3, C and D), synaptosomal preparations of the male telencephalon had significantly higher levels of aromatase activity than did similar preparations of the female telencephalon; microsomal preparations as well as measures of whole homogenates themselves demonstrated no sex differences (8). Given the significant overall enrichment of aromatase in synaptosomal preparations *vs.* microsomal preparations, it is unclear why we do not detect the overall sex difference in whole homogenates. Perhaps proteases and other steroid-metabolic enzymes present in homogenates obscure detection of sex differences. Measures of the activity in purified subfractions, however, empirically match our counts of aromatase-positive profiles at the EM level, providing independent confirmation of our conclusion that estrogenic synaptocrine signaling is elevated in the brain of male songbirds compared with females and forms the basis for sex differences in neuroestrogenic signaling.

3. Synaptic aromatase and song production

As described in *Section IV*, aromatase activity in brain is enriched in subcellular compartments, microsomes, and synaptosomes. In the zebra finch forebrain, approximately half of the total aromatase is localized to the synaptosomal fraction (8), providing complementary evidence for the anatomical localization of aromatase in terminals (7). Synaptosomal aromatase is particularly enriched in the forebrain of adult males relative to females (8), suggesting a link to male-typical behavior and/or neural processing in this species, such as singing or song learning.

Aromatase-positive synaptic boutons enshroud the "song system" nuclei in the caudal forebrain of zebra finches (7, 47, 51). This anatomical localization led to the focus on links between singing and potential synaptocrine regulation of aromatase in the male zebra finch brain. In addition, several recent observations showed that brain aromatase activity might shift during changes in behavioral state in vertebrates (104–106).

To examine the regulation of synaptic aromatase, male zebra finches were briefly exposed to natural stimuli, and brains were collected immediately after each behavioral trial. In the first experiment, adult males were housed singly in sound-attenuation chambers and exposed to females for 30 min. Some males sang to the females ("singers"), whereas others did not sing ("nonsingers"). Brain samples were carefully dissected to separate the posterior telencephalon (containing several important song nuclei enriched in presynaptic aromatase) from the anterior telencephalon (fewer aromatase-positive cells and little to no terminal aromatase). Aromatase activity was elevated more than 2-fold in the singers relative to the nonsingers, and this aromatase up-regulation was specifically localized to the synaptosomal fraction within the posterior telencephalon (107). Playback of zebra finch song *vs.* control white noise to males in chambers did not cause similar differences in synaptosomal aromatase activity, indicating that the association between singing and synaptic estrogen production likely did not depend on auditory self-stimulation and was instead related to the motor or cognitive aspects of song production. Courtship singing therefore involves an up-regulation of aromatase activity specifically in synaptic terminals, although it is unclear whether this depends on rapid phosphorylation events (see *Section*

VI.C, Prediction 3) or longer-term, constitutive up-regulation of terminal aromatase. These experiments provided evidence that the vertebrate CNS has the capacity to regulate aromatase in synaptic terminals independent of somal aromatase in a behaviorally relevant context.

These studies confirm predictions of the synaptocrine hypothesis that the presence and/or activity of the aromatase present in synapses is subject to multiple forms of regulation enabling regionally specific and biologically relevant supplies of estrogen.

C. Prediction 3

Terminal release of neuromodulators is controlled by depolarization events, typically associated with voltage-gated Ca++ channel opening. In synaptocrine systems involving steroids, terminal steroidogenic enzyme activity must be subject to regulation by local voltage-gated and/or Ca++-sensitive mechanisms.

1. Rapid regulation of neuronal aromatase: diencephalon and telencephalon

It is important to emphasize that the actions of estrogens in brain are highly localized and that many steroid-mediated neural events occur much too rapidly to be achieved by long-term (genomic) regulation of gonadal steroid secretion or neurosteroid production (26, 27, 108). Studies of the aromatase enzyme itself in quail and in mice suggest that neural estrogen levels change rapidly by the rapid regulation of the aromatase enzyme itself. Specifically, aromatase in the quail hypothalamus first undergoes Ca++-dependent phosphorylation that can lead in minutes to a decrease in aromatase activity (109, 110). In this model, rapid inhibition of aromatase activity can occur via phosphorylations and/or via an interaction between the Ca++-calmodulin complex and the aromatase molecule (111). Moreover, treatments of hypothalamic explants with K^+ or with glutamate receptor agonists N-methyl-D-aspartate (NMDA), AMPA, and kainate all inhibit aromatase over a similar time frame (109). Such results suggest that neuronal aromatase is phosphorylated upon excitation, locally reducing a neuron's capacity to synthesize estrogen.

If this form of rapid regulation was confined to enzyme located in synaptic terminal in close proximity to estrogen-sensitive postsynaptic membranes, then a mechanism for spatiotemporal neuromodulation would be achieved. Our studies of songbirds approach this question using newly developed microdialysis procedures to measure localized and acute changes in neuroestrogen levels.

2. Measurement of local estrogens in vivo

If aromatase activity changes within synaptic terminals in the songbird forebrain, the synaptocrine hypothesis proposes that estrogenic end-products are immediately released and result in altered levels of estrogens at or near the synapse. In other words, local levels of neuroestrogens near synaptocrine terminals should fluctuate relative to surrounding extracellular cerebrospinal fluid (CSF). To test these ideas, methods were developed recently to directly measure fluctuations in local neurosteroid levels *in vivo*, in awake, behaving songbirds (Fig. 4A) (112). *In vivo* microdialysis has been a powerful tool for the study of neuropeptide and neurotransmitter flux in a variety of experimental settings (113, 114), but the possibility of using this approach for brain steroids like estrogens had not been previously explored in depth (but see Ref. 115). We now describe specifics on the development and findings using *in vivo* microdialysis for neurosteroids.

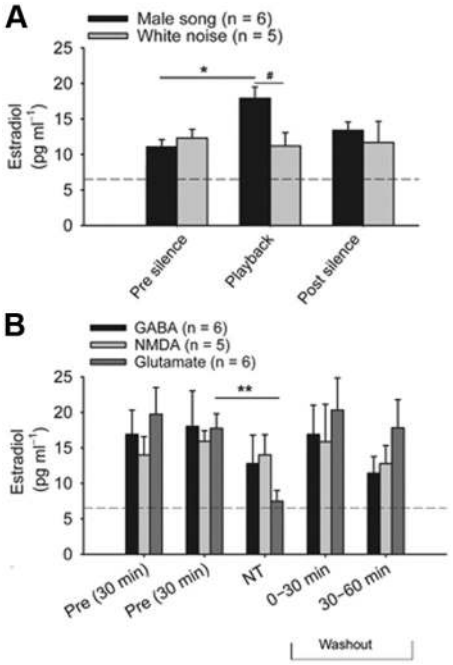

Fig. 4. A, Exposure to male song for 30 min (playback) increases estradiol in an auditory processing region, NCM, in adult male zebra finches compared with 30-min periods of silence (Pre) and (Post). Exposure to white noise had no effect on NCM estradiol levels. *Dashed lines* indicate background steroid concentrations for aCSF alone. Data were analyzed by repeated measures ANOVA and appropriate *post hoc* analyses. *, $P = 0.011$ for playback *vs.* pre-silence in the male song group; #, $P = 0.023$ for male song *vs.* white noise during playback. B, Microdialysate estradiol levels before (two successive 30 min pre treatment periods) or after (1–30 or 30–60 min) retrodialysis of neurotransmitters GABA, NMDA, or glutamate into the NCM region of male zebra finches. Glutamate (10 mM) only caused a significant change (decrease) in estradiol levels.**, $P = 0.008$. [Adapted from Remage-Healey L et al., Forebrain steroid levels fluctuate rapidly during social interactions. Nat Neurosci 11:1327–1334, 2008 (112), with permission. © 2008, Nature Publishing Group.]

Ref. 112). First, *in vitro* experiments verified that radiolabeled estrogens could be captured predictably when CMA-7 microdialysis probes (or similar probes appropriate for sampling from constrained areas in small animals) were immersed in aqueous [artificial CSF (aCSF)] solution. In these experiments, the degree of absorption increased in a log-linear fashion in parallel with increases in the *in vitro* estradiol concentration. Second, the chemical identity of endogenous 17β-estradiol in microdialysate collected from awake animals (as measured by a commercial ELISA) was independently confirmed using gas chromatography/mass spectrometry. Thus, the brain-derived steroid species in question was unequivocally confirmed to be 17β-estradiol. Third, peripheral injections of 17β-estradiol caused significant, concomitant increases above baseline in forebrain 17β-estradiol levels as measured by *in vivo* microdialysis. Fourth, local delivery (reverse dialysis) of the potent aromatase inhibitor fadrozole (FAD) to the forebrain caused rapid suppression of local endogenous 17β-estradiol levels in awake males (112). These findings confirmed that neurosteroids like 17β-estradiol were both detectable and quantifiable using microdialysis. Additional data described in subsequent sections shows that the 17β-estradiol measured in dialysates fluctuates inversely to that of testosterone, can be predictably altered by retrodialysis of pharmacological agents that regulate aromatase, and is only detected in significant amounts from brain regions containing aromatase. These data confirm that the bulk of the 17β-estradiol measured by microdialysis reflects neuroestrogen, is locally synthesized in brain.

The development of neurosteroid microdialysis has allowed unprecedented spatiotemporal precision in determining the steroid modulation of forebrain circuit function *in vivo* in awake animals. Despite these advances, the accurate detection and quantification of neuroestrogen fluctuation at the individual synapse is beyond the scope of current technology. Even so, synaptosomal aromatase accounts for a large quantity of the total aromatase in the forebrain of adult male zebra finches (8). Therefore, acute fluctuations in local estrogens captured using *in vivo* microdialysis likely reflect, in part, the integration of synaptocrine events.

3. Changes in neuroestrogens during neurotransmitter activation

Experiments in both hypothalamic and hippocampal preparations have shown that *in vitro* aromatase activity is sensitive to application of both glutamate agonists and antagonists (116, 117). Specifically, in Japanese quail hypothalamic explants, glutamate agonists rapidly suppress aromatase activity, most likely via a calcium-dependent phosphorylation of the aromatase enzyme (110, 111, 116). Similarly, rapid neurotransmitter-mediated shifts in aromatase activity could account for synaptocrine fluctuation of neuroestrogens in the zebra finch NCM. Exogenous treatment with neurotransmitter agonists/antagonists should therefore result in acute changes in local neuroestrogens *in vivo*, as measured by microdialysis.

This prediction was supported in microdialysis experiments using reverse-delivery (retrodialysis) of glutamate, NMDA, and GABA into the NCM of awake, behaving male zebra finches. Infusion of glutamate caused a rapid suppression of local 17β-estradiol levels within 30 min, whereas treatment with either NMDA or GABA each had no effect (Fig. 4B) (112). Infusion of GABA caused a rapid increase in local testosterone levels within 30 min, whereas NMDA and glutamate each had no effect. Thus, neurotransmitter activation was directly associated with local and acute changes in NCM steroids. Recently, the rapid suppression of local 17β-estradiol by glutamate-evoked excitation has been recapitulated with delivery of aCSF containing high concentrations of K^+ ions (causing increased local depolarization events; Remage-Healey, L., and B. A. Schlinger, unpublished observations). Moreover, changing estradiol levels measured by *in vivo* microdialysis are blocked when the local nucleus is treated with the presynaptic terminal-specific voltage-gated calcium channel blocker omega-conotoxin (Remage-Healey, L., and B. A. Schlinger, unpublished observations), further implicating a synaptocrine source for estrogens detected via this technology.

Collectively, these observations demonstrate that neuroestrogen levels within the NCM are acutely sensitive to neuronal excitation. It is interesting to note that glutamate-dependent aromatase down-regulation (as observed in quail hypothalamus) (116) could be a conserved mechanism for the control of neuroestrogen production in the vertebrate CNS.

These observations raise the question as to whether endogenous activation of neural circuits regulates the synthesis and secretion of local steroidal neuromodulators.

4. Changes in neuroestrogens in vivo during behavior

The validation of *in vivo* microdialysis for neurosteroids has allowed for the examination of acute changes within the forebrain of the zebra finch that might reflect rapid changes in synaptocrine synthesis. The focus of these experiments has been on the NCM, an auditory cortical nucleus that is enriched in synaptosomal aromatase (7, 103). When males are exposed to females and actively courting and singing, microdialysis within NCM reveals that 17β-estradiol levels are acutely elevated during 30 min behavioral trials relative to baseline conditions (Fig. 4A) (112). These changes appear to be specific to NCM;

when probes are targeted to anterior forebrain (containing very little synaptosomal or somal aromatase), there is no significant change observed in local estrogens. A similar acute rise in 17β-estradiol within NCM also occurs when males are exposed to conspecific songs but not control white noise sounds, indicating that auditory processing of song stimuli alone can drive fast changes in brain steroid levels. Importantly, circulating levels of both 17β-estradiol and testosterone are not responsive to these same manipulations. Together, these findings reveal an extremely localized flux in neuroestrogens within a discrete region of auditory cortex that occurs during changes in socially relevant behavior.

These studies confirm predictions of the synaptocrine hypothesis that aromatase present in synapses is subject to relatively rapid regulation, consistent with a role in neuromodulation for these locally formed neuroestrogens.

D. Prediction 4

In a synaptocrine system involving steroids, the source of that substrate could be from endocrine, paracrine, or autocrine sources. If a steroid is synthesized at the synapse, a concomitant local decrease in the concentration of its immediate substrate is predicted.

1. Source of androgen substrate for neuroestrogen synthesis

All active steroidal signaling molecules are derived from cholesterol, belong to one of several families of similar molecular composition, and are produced by a series of enzyme-catalyzed reactions (54, 55). Androgens are derived from progestins, and estrogens from androgens, with aromatase being the last of four reactions required to make estradiol from cholesterol. A complex feature of synaptocrine signaling is that the androgenic precursor for synaptic estrogen synthesis can be derived from one of several sources, including endocrine delivery from the periphery, paracrine delivery from nearby neuronal or glial cells, or autocrine delivery with androgen synthesized in the presynaptic neuron itself. In the case of the zebra finch NCM, it is not yet clear whether aromatase-positive cells synthesize androgens or any of its precursors. However, we and others have documented expression and, in some cases activity, of all of the fundamental enzymes and transporters of the steroidogenic pathway, including the cholesterol transporter proteins steroidogenic acute regulatory protein and translocator protein; the side-chain cleavage, 3β-hydroxysteroid dehydrogenase (3β-HSD), and CYP17 enzymes are all expressed in NCM, making it possible that the androgens required for presynaptic estrogen signaling are produced locally in brain (47, 118–120).

Additional evidence for a role for neurosteroidogenesis functioning coordinately with synaptic aromatase comes from studies of the enzyme 3β-HSD that converts pregnenolone to progesterone or the androgen precursor dehydroepiandrosterone (DHEA) to androstenedione, a substrate for aromatization. This enzyme shows many properties in brain similar to that of aromatase. First, DHEA can be converted into estradiol in the songbird brain, a process that requires the action of 3β-HSD, aromatase, and an isoform of 17β-HSD (121–123). DHEA can activate masculine behavior in songbirds, including during the nonbreeding season when the gonads are regressed and likely producing little or no androgen (124, 125). Importantly, the enzyme 3β-HSD is up-regulated in several brain regions during the nonbreeding season compared with the breeding season (126), and it can be regulated in a sexually dimorphic manner (127). Notably, like our evidence for aromatase, 3β-HSD is up-regulated rapidly (within 30 min) during aggressive encounters (126). Similar to our results using *in vivo* microdialysis (112), these data argue that rapid regulation is a key property of steroidogenic enzymes, providing a mechanism for the rapid local flux of androgenic precursors for synaptic estrogen production.

2. Changes in local steroid substrate

As mentioned previously, reverse dialysis of FAD into the forebrain caused rapid suppression of local endogenous 17β-estradiol levels in awake males. A secondary prediction is that this treatment would produce a simultaneous increase in dialysate levels of testosterone, the immediate substrate that is aromatized into estradiol. Therefore, a modification of the ELISA procedure was introduced to allow measures of testosterone from what remained after estradiol ELISA was performed. The results of these analyses confirmed our expectation: local testosterone levels measured in these same samples were significantly higher during FAD treatment, consistent with a local buildup of androgen precursors after aromatase inhibition (see Ref. 112). Similar inverse changes in dialysate levels of testosterone and estradiol were detected during exposure of male songbirds to specific behavioral contexts or auditory stimuli.

These studies lend support for predictions of the synaptocrine hypothesis that the substrate for synaptic aromatase can be locally produced in brain but might also arise from alternate neural or peripheral sources.

E. Prediction 5

Neuromodulators released from synaptic terminals have pre- or postsynaptic physiological functions. In a synaptocrine system involving steroids, the steroid synthesized at synaptic terminals will exert either autocrine

(presynaptic) or synaptocrine (postsynaptic) actions independent of steroids produced elsewhere.

There are several implications for the behaviorally driven acute changes in local estrogen levels that occur in the brains of songbirds. Chiefly, local release of steroids should have acute actions on neuronal firing patterns within the NCM circuit. We and others have collected evidence in favor of this prediction originating from the observed acute changes in neuroestrogens via *in vivo* microdialysis.

1. Modulation by brain estrogens in vivo

Similar to several other classes of steroid hormones (*e.g.*, Ref. 128), 17β-estradiol has rapid actions on neuronal excitability that can occur within seconds to minutes of treatment. This phenomenon has by now been widely observed throughout the vertebrate CNS, including the hypothalamus, amygdala, striatum, HP, and hindbrain/spinal cord (25, 26, 129–132). The majority of rapid estrogen effects have been described in these "limbic" or motor regions of the vertebrate CNS, and only recently has attention turned toward neuroestrogen actions on sensory processing areas (for longer-term estrogen effects see Refs. 133–135).

2. Locally derived estrogens: electrophysiology and auditory processing

The phenomenon of fluctuating neuroestrogen production within the songbird NCM as revealed by microdialysis suggested that 17β-estradiol could have rapid effects on excitability of neurons involved in auditory processing and/or song memory. Microdialysis technology was recently coupled to extracellular electrophysiology recordings in the NCM. This combined approach allows precise regulation of local neurosteroid levels via reverse microdialysis for drug delivery alongside recordings of neuronal activity. This approach recently showed that neuroestrogen production is indeed responsible for boosting the neuronal auditory responses to song stimuli within 30 min in the zebra finch NCM (136). Brief application of 17β-estradiol directly into the NCM caused 50% increases in the firing rate of auditory neurons during song playback, relative to the previous 30 min of vehicle delivery. Close inspection of the firing patterns revealed that 17β-estradiol caused significant increases in the occurrence of "bursts" of action potentials (Fig. 5). Such a switch to neuronal bursting is consistent with the hypothesis that burst firing enhances the synaptic drive to efferent targets and can possibly aid in sensory discrimination (137),

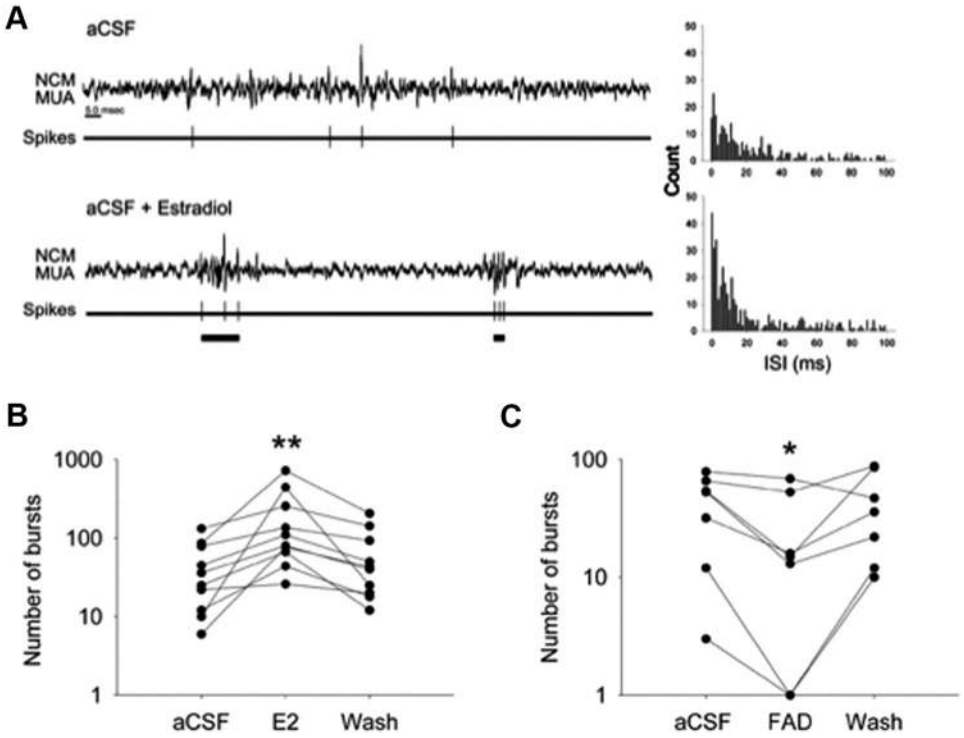

Fig. 5. Estradiol causes acute changes in the occurrence of multiunit bursts. A, Example of NCM spike trains for both aCSF (*upper*) and estradiol (*lower*) conditions. Triggered spike plots are depicted below each trace, and two examples of identified burst-periods (*black bars*) are depicted. *Right*, ISI distribution plots over the entire 30-min treatment periods from the same experiment are shown for aCSF (*upper*) and estradiol (*lower*). An increased number of rapid interspike interval (<5–10 msec) events during estradiol treatment is consistent with a transition from isolated spiking to burst firing. B, Estradiol (E2) increases the number of auditory-evoked single unit bursts in NCM. **, $P = 0.005$; n = 10 U from seven birds. C, By contrast, FAD decreases the number of auditory-evoked single unit bursts in NCM. *, $P = 0.018$; n = 7 U from five birds. [Adapted from L. Remage-Healey et al., Brain estrogens rapidly strengthen auditory encoding and guide song preference in a songbird. Proc Nat Acad Sci USA 107:3852–3857, 2010 (136), with permission. © 2010, The National Academy of Sciences.]

thereby further compounding the simple increases in firing rate caused by 17β-estradiol. Conversely, aromatase blockade with FAD caused a rapid suppression of bursting (Fig. 5), consistent with a role for local, endogenous neuroestrogens in modulating firing properties of auditory neurons.

In a remarkable convergence of findings from an independent laboratory, 17β-estradiol and aromatase inhibitors delivered to NCM via pressure injection (coupled with extracellular recordings) were found to similarly modulate NCM firing patterns in awake, head-restrained zebra finches (138). In follow-up patch recordings *in vitro*, Tremere *et al.* (138) observed that 17β-estradiol exerts rapid suppression of GABAergic currents (miniature inhibitory postsynaptic currents) in NCM slices and that this effect is most likely a presynaptic phenomenon (17β-estradiol inhibits the frequency but not amplitude of miniature inhibitory postsynaptic currents). More recent studies from the same lab directly and quantitatively assessed the extent to which the neural discrimination of auditory signals is enhanced by locally produced estrogen (139). Together, these convergent findings provide convincing evidence for the acute modulation of auditory-evoked activity in NCM neurons by locally produced neuroestrogens.

These studies confirm predictions of the synaptocrine hypothesis that estrogens synthesized locally in brain modulate neuronal membrane excitability on a rapid time scale.

F. Prediction 6

Modulation of neuronal activity by synaptic neurosignal release produces changes in neural function and behavior. In the case of a synaptocrine system involving steroids, active steroids produced at the synapse themselves influence behavior, independent of steroids produced elsewhere.

1. Modulation of behavior

A final, important component of the synaptocrine hypothesis is the implication for behavior. If the synaptocrine synthesis, release, and subsequent acute actions of neuroestrogens on brain function are important, there should be associated changes in behavior. Because *in vivo* microdialysis has been optimized for awake, behaving zebra finches, the technology can therefore be used to study variation in the suite of natural social behaviors that are expressed in laboratory conditions in this species.

One way in which microdialysis has been used to examine neuroestrogens and behavior is the study of song preferences. Adult zebra finch males and females exhibit behavioral preferences for specific song types, typically acoustic playback of their own song (in the case of males)

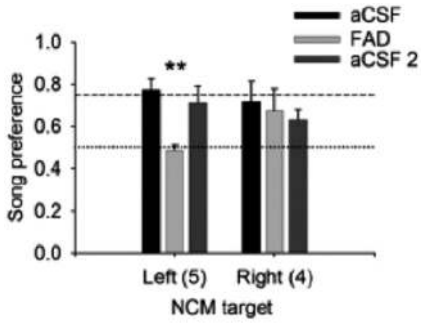

Fig. 6. Discrimination depends on estrogen production in the left hemisphere. In freely-behaving males, discrimination of BOS (1.0 = 100% BOS discrimination preference *vs.* 0% CON) is eliminated during retrodialysis of the aromatase inhibitor FAD into the left hemisphere NCM region (**, $P < 0.007$), but not the right. *Dashed line* represents the species-typical BOS discrimination ratio (~75%). *Dotted line* delineates no discrimination. Sample sizes are in *parentheses*. [Adapted from L. Remage-Healey et al., Brain estrogens rapidly strengthen auditory encoding and guide song preference in a songbird. Prod Nat Acad Sci USA 107:3852–3857, 2010 (136), with permission. © 2010, The National Academy of Sciences.]

or their tutor's song (in the case of both sexes) compared with conspecific or heterospecific songs. This preference can be measured by the time spent choosing to be in physical proximity to a speaker broadcasting songs and/or via an operant task in which birds are trained to peck keys associated with playback of preferred *vs.* nonpreferred songs (see Ref. 140). The physical proximity approach was used in conjunction with *in vivo* microdialysis to test the effects of the potent aromatase inhibitor FAD on song preference in awake, behaving adult males. During 30-min trials in which aCSF was perfused through NCM, males on average spent approximately 75% of their time choosing in close proximity to the speaker playing their own song [bird's own song (BOS)] *vs.* a conspecific song (CON; corrected for side-bias; Fig. 6), a finding comparable to preference scores in other studies in this species (140, 141). By contrast, during 30-min trials in which FAD was delivered to NCM via retrodialysis, the preference score dropped to 50% (Fig. 6), *i.e.*, no preference for either BOS or CON (136). Interestingly, this drop in preference was only observed when FAD was delivered to the left hemisphere NCM (n = 5) and not the right hemisphere NCM (n = 4), indicating a laterality for endogenous neuroestrogen actions in support of song preference and possibly a laterality in function for left *vs.* right NCM. It is unclear from these experiments whether the block of song preference was due to synaptocrine or somal aromatase inhibition (or a combination) because FAD likely does not differentially interfere with aromatase activity in these two cellular compartments. Nonetheless, the rapidity of the behavioral actions (30 min) of FAD in NCM, coupled with the observation of similarly fast FAD-induced suppression of burst firing in NCM neurons, suggests that synaptocrine

neuroestrogen production partially accounts for the boost in both neuronal auditory processing and downstream behavioral preferences. Recent experiments from the Pinaud lab have largely confirmed this original observation (139).

As described in *Section IV*, the songbird forebrain contains abundant expression of the steroidogenic enzyme 5β-reductase (66, 142). Similar to the actions of aromatase, this enzyme converts androgens and progestins into their 5β-reduced metabolite counterparts. Although the rapid effects of estradiol on songbird auditory physiology are clear, 5β-reduced androgens do not produce significant changes in NCM firing properties (136). Therefore, local, likely synaptic, aromatase activity is critical for boosting auditory responsiveness in NCM and guiding song preference (136), whereas the predominant 5β-reduced androgen, 5β-dihydrotestosterone, is naturally synthesized in part via synaptocrine actions but has no effect on the excitability of auditory neurons in NCM (see Ref. 139). These observations suggest that synaptocrine actions of steroids are specialized and perhaps controlled at the synaptic level for local modulatory events. To date, a direct, physiological role for 5β-reductase in synaptic terminals has not yet been identified. 5β-Reduced steroids can allosterically modulate the $GABA_A$ receptor in cultured zebra finch neurons (60). Given their potency and potential for synaptocrine actions, further analysis of 5β-reduced steroid actions on adult songbird neurons will be a fruitful avenue for research.

These studies confirm predictions of the synaptocrine hypothesis that estrogens formed locally in brain exert specific and relatively rapid control over neural events that influence expression of behavior.

G. Prediction 7

To preserve spatiotemporal resolution of neuromodulatory events, pre- and/or postsynaptic mechanisms exist to rapidly terminate the influence of neuromodulators. In the case of a synaptocrine system involving steroids, after synaptic steroid synthesis, release, and local action, a putative sequestration mechanism exists to rapidly quench steroid concentrations within the synaptic cleft.

The preservation of neuromodulatory information content depends on the temporal fidelity of the modulatory signal to match rapidly changing informational states. This fidelity match is achieved in part via mechanisms to rapidly sequester the released pool of neuromodulator, such as reuptake proteins, feedback pathways, and degradation events. Research into the uptake of biogenic amine neurotransmitters in particular has provided a wealth of drug targets for use in depression and anxiety disorders (143). In the case of synaptocrine steroid production, it is presently uncertain which mechanisms serve to locally preserve the spatiotemporal fidelity of steroids produced at the synapse. Several candidate mechanisms include: 1) phase I rapid hydroxylation of steroids by enzymes such as aromatase itself to increase their aqueous solubility and thereby decrease their potency (144); 2) so-called phase II steroid metabolism, which includes sulfate- and glucuronide-conjugation processes that can inactivate steroids within the brain (145); and 3) constitutive sequestration via binding proteins such as sex steroid binding globulin (146) or even α-fetoprotein (147), which could theoretically be located at or near the synaptic cleft. Therefore, the evidence is gathering that synaptic steroid sequestration is possible, but it is unknown whether these mechanisms operate at the acute spatial and temporal scale to provide the rapid sequestration of steroids produced at the neuronal synapse.

Although more work in this area is required, there are a variety of potential neurochemical mechanisms to lo-

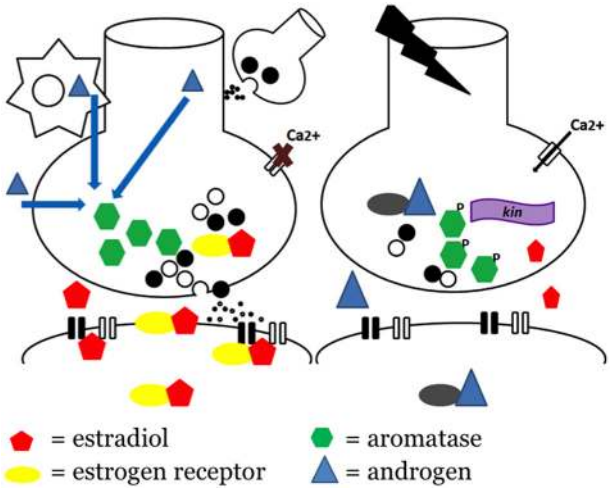

Fig. 7. Synaptocrine signaling. *Left*, Androgenic precursors (*blue triangles*) from the extracellular space, adjacent cells such as astroglia, or within individual neurons are available as substrates for the enzyme aromatase (*green hexagons*) located within presynaptic boutons. Estrogens (*red pentagons*) synthesized within the presynaptic bouton may be available to bind presynaptic ER (*yellow ovals*) and modulate the dynamics of presynaptic neurotransmitter release, diffuse into the synaptic cleft, and thereby interact with postsynaptic inhibitory (*black*) or excitatory (*clear*) receptors, or via interactions with postsynaptic ER, modulate postsynaptic neurotransmitter receptors and have genomic effects on the postsynaptic cell. These scenarios are most likely to occur during inhibition of the synaptocrine neuron by afferent inhibitory modulation (afferent synaptic bouton with dark neurotransmitter vesicles and dark neurotransmitter) and with the exclusion of extracellular calcium (Ca++) from the synaptocrine presynaptic bouton. *Right*, In contrast, depolarization of the synaptic neuron and concomitant Ca++ influx into the presynaptic bouton may activate a calcium-dependent kinase, phosphorylate presynaptic aromatase (P) and thereby decrease synaptocrine estrogen synthesis (*smaller red pentagons*, decreasing estrogenic synaptocrine signaling, but potentially increasing synaptocrine androgenic (*larger blue triangles*) via interaction with presynaptic androgen receptors (*gray ovals*) or after diffusion across the synaptic cleft, by genomic effects via androgen receptors on the postsynaptic neuron.

cally regulate brain estrogens in keeping with predictions of the synaptocrine hypothesis.

VII. Conclusions

Although our focus here is on estrogen signaling in the songbird brain, there is a growing body of evidence showing that synaptocrine signaling is conserved across vertebrates and across brain regions (117, 131, 148). Moreover, whereas neural estrogen actions are widely studied, especially their rapid membrane actions, other neuroactive steroids are likely formed presynaptically (*e.g.*, Refs. 149–151), thereby functioning also as targeted synaptocrine signals. The subcellular localization of enzymes required to synthesize neuroactive steroids merits further investigation.

An alternate hypothesis regarding a role for steroids synthesized in the presynaptic bouton may well be the modulation of presynaptic physiology. Indeed, receptors for both estrogen (15, 19, 20) and androgen (152) have been documented within presynaptic boutons of the rodent brain and in the zebra finch HVC (Saldanha, C. J., unpublished observations). Steroids could interact with their cognate receptors within the presynaptic boutons and thereby affect presynaptic function (81). For example, estrogen can rapidly potentiate the excitability of rodent hippocampal neurons via a presynaptic mechanism (153), perhaps via receptors located on the walls of neurotransmitter vesicles (103). Given these data, we cannot exclude the possibility that estrogens synthesized within the presynaptic bouton may alter the probability of neurotransmitter release, thereby affecting synaptic function.

Irrespective of the pre- or postsynaptic site of action, synthesis and/or metabolism of steroidal molecules at the synapse for targeted neuromodulatory actions represents a conceptually new mechanism of hormone delivery (Fig. 7). Synaptocrinology will be a rewarding path of future neuroendocrine investigation.

Acknowledgments

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This work was supported by the National Institutes of Health (Grants F32NS058009, R01MH061944, RO1NS042767 and K99/R00NS066179).

Disclosure Summary: The authors have nothing to disclose.

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