HORMONAL INFLUENCE ON SONG STRUCTURE AND ORGANIZATION: THE ROLE OF ESTROGEN

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Abstract—The development of song in songbirds is a complex phenomenon that involves memory and learning, sensorimotor integration, and neural and muscular maturation. Gonadal hormones are involved in each of these steps, as they influence the differentiation of the neural song system, the incorporation and survival of neurones, and the development of muscles used for song production. In young males the development of song, therefore, is closely linked to the secretion of testosterone by the testicles. Castration results in the development of incomplete or unstable songs, and hormone replacement leads to the development of crystallized or stable song. However, testosterone does not act solely as an androgen. The brain of songbirds contains high concentrations of the enzyme aromatase, which converts testosterone into estradiol. Estradiol then binds to estrogen receptors, which in the song system are found only in the nucleus HVC. This forebrain nucleus, also called the "master nucleus," codes for the syntactic structure of song, i.e. for the particular combination of simple elements—syllables— that characterize the song of an individual. In this paper, we will review our studies on the role of estrogen in guiding the organization of song in canaries. © 2005 Published by Elsevier Ltd on behalf of IBRO.

Key words: song, song system, songbird, estrogen, androgen, aromatase.

Songbirds (Passeriformes: Oscine) have a specialized network of brain nuclei, the neural song system, for learning and production of song. Soon after the song system was first described in the canary (*Serinus canaria*) (Nottebohm et al., 1976), it was shown that several nuclei of the system accumulate steroids (Arnold et al., 1976). Subsequent studies in different species confirmed that the secretion of androgen hormones such as testosterone (T) by the testes is necessary for the organization of stable adult song (Heid et al., 1985; Marler et al., 1988; Bottjer and Hewer, 1992). In addition, while several studies had highlighted a critical role of estrogen in the sexual differentiation of the song system (Gurney and Konishi, 1980; Konishi and Akutagawa, 1988; Simpson and Vicario, 1990), others described

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Abbreviations: AR, androgen receptor; AROM, aromatase; BDNF, brain-derived neurotrophic factor; ER, estrogen receptor; FAD, fadrozole; NC, caudal neostriatum; NCM, caudo-medial neostriatum; T, testosterone.

effects of estrogen on the structure of song (DeVoogd, 1986; Weichel et al., 1986, 1989; Marler et al., 1987, 1988; Harding et al., 1988; Walters et al., 1991). Interestingly, only one nucleus of the song system, the HVC, was known to contain estrogen receptors (ER) (Gahr et al., 1987, 1993; Nordeen et al., 1987). This forebrain nucleus, also called the "master nucleus" because of its alleged role as the song pattern generator (Vu et al., 1994; Yu and Margoliash, 1996; Hahnloser et al., 2002; Solis and Perkel, 2005), codes for the syntactic structure of song, i.e. for the particular combination of simple elements—syllables—that characterize the song of an individual. Because only lesions or electrical disturbances of the song system affect the song structure (Nottebohm et al., 1976; Vu et al., 1994; Yu and Margoliash, 1996; Margoliash, 1997; Wild, 1997) and HVC was the only nucleus of the song system known to contain ER, we argued that any estrogen-mediated influence on song structure should be localized in HVC. In this paper, we will review a series of studies that we conducted in the domestic canary to understand how estrogen can influence song structure. We addressed the following questions: What are the sites of estrogen production and action? How are estrogen formation and action regulated? How does estrogen affect song organization?

What are the sites of estrogen production and action in the song system?

The source of the estrogen acting on the song system was unclear since the levels of circulating estrogen are very low in both juvenile and adult males (Hutchison et al., 1984; Adkins-Regan et al., 1990; Schlinger and Arnold, 1992a). However, several brain areas in birds are capable of converting T into the estrogen 17β-estradiol via the enzyme aromatase (AROM) (Callard et al., 1978; Steimer and Hutchison, 1980). Previous work showed high levels of AROM enzymatic activity, receptor protein concentration, and mRNA expression in various telencephalic areas in the zebra finch (*Taeniopygia guttata*) (Vockel et al., 1990a,b; Schlinger and Arnold, 1992a; Shen et al., 1995; Balthazart et al., 1996). In fact, in songbirds brain AROM can produce substantial levels of circulating estrogen (Schlinger and Arnold, 1991, 1992b), and several authors had suggested that the action of T on the song system and song could be mediated by its conversion into estradiol (reviewed by Bottjer and Johnson, 1997; Schlinger, 1997; reviewed by Gahr, 1998). A surprising finding was the lack of AROM expression in the HVC, which has ER, or other song control nuclei of the zebra finch (Shen et al., 1994, 1995). However, the distribution of AROM and ER had been described in separate series of studies, therefore the

possibility of an overlap between AROM and ER could not be ruled out. Thus, by means of *in situ* hybridization of their mRNA we studied the relative distribution of AROM and ERα, and of androgen receptor (AR) in the brain of the canary and of other six species representing six avian orders: the Passeriformes, Psittaciformes, Columbiformes, Apodiformes, Strigiformes, and Galliformes (Metzdorf et al., 1999). At the time of our study ERβ had not been described in birds yet (see later in this section). We included AR in the study because in birds AROM was known to be regulated by androgen and/or estrogen in a tissue-specific fashion (Steimer and Hutchison, 1981; Hutchison and Steimer, 1985; Schumacher and Balthazart, 1987; Balthazart et al., 1991, 1994), thus the pattern of co-localization of AR, ERα, and AROM, might provide useful indications about steroid regulation of the enzyme in the songbird brain. Our study was the first to examine the distribution of AR, ERα, and AROM in adjacent sections of the same brains.

The study provided three main results: 1) there was little if any AROM in the song system of all songbirds examined; 2) AROM expression in the neostriatum (nido-pallium according to the new nomenclature, (Reiner et al., 2004) was specific to songbirds; 3) there was little if any overlap between areas of AROM and ERα expression in the telencephalon; 4) there was considerable overlap between AROM and AR in this same area (Metzdorf et al., 1999). In the caudal neostriatum (NC; the brain part that contains the highest levels of AROM and of ERα in the canary) most ERα were located in the HVC but the HVC contained no or very few AROM expressing cells (Fig. 1). At the level of the most medial extension of the HVC, the field of AROM surrounded the field of ERα and one could localize the HVC, i.e. the ERα-expressing area, as the area of the caudal forebrain lacking AROM expression. The highest concentration of AR in the caudal forebrain

Fig. 1. A schematic drawing of a coronal section of the canary brain illustrating the distribution of AR, ERα, and AROM-mRNA. The dotted line shows the border of HVC. AHP, area parahippocampalis. Medial is to the right, ventral to the bottom. Adapted from Metzdorf et al., 1999.

was in the HVC, which contained no or very few AROM expressing cells. However, a large area of AR expressing cells ventral to HVC showed a considerable overlap with the area of high AROM expression (Fig. 1). The presence of ERα but not of AROM in HVC, and of AROM but not ERα in the caudo-medial neostriatum (NCM) surrounding HVC, suggests that the latter is the source of estrogen acting in HVC. As HVC and NCM have little if any synaptic connections (cfr. Saldanha et al., 2000), this hypothesis is still waiting to be tested, however several observations support it. The mutually exclusive expression of AROM and ERα in the caudal forebrain is a songbird-specific pattern and suggests that AROM expression around HVC has evolved together with the expression of ERα in HVC. The short distance between the HVC and the NCM makes the diffusion of estrogen into the HVC possible, whereas the levels of circulating estrogen in adult male canaries are very low. Later work has shown that ERβ is not expressed within the song system (Bernard et al., 1999). Thus, HVC remains the only site of ER-mediated action within the song system. Having described the sites of estrogen production (expressing AROM) and estrogen action (ERα), the next step was to understand how these processes are regulated.

How are estrogen formation and action regulated?

The fact that AR, and not ERα, are co-expressed with AROM in NCM suggested that androgen could be one of the factors regulating the availability of estrogen for the HVC. Our first approach was to use a natural phenomenon, that is the substantial increase in circulating levels of androgen that male songbirds of temperate zones experience from autumn to spring, when breeding starts. It had been know for a long time that the spring rise in testicular androgen induces the intense singing typical of the breeding season (Nottebohm et al., 1987; Rost, 1990; Smith et al., 1997). There are seasonal changes in the expression level of AR- and ERα-mRNA in the HVC of the canary (Gahr and Metzdorf, 1997), and seasonal differences in AR immunoreactivity were described for the HVC of the Gambel's white-crowned sparrow (*Zonotrichia leucophrys gambelii*) (Soma et al., 1999b). Similarly, seasonal changes in AROM activity had been reported in the diencephalon of the ring dove (Hutchison et al., 1986; Fusani, 1994) and the red grouse (*Lagopus lagopus scoticus*) (Sharp et al., 1986), and in the NCM of the Lapland longspur (*Calcarius lapponicus*) (Soma et al., 1999a). These seasonal differences could be regulated by circulating levels of androgens and estrogen. AR and ERα are autoregulated by their ligands in an area-specific manner in the brain of mammalian (Lauber et al., 1991) and avian species (Nastiuk and Clayton, 1995). In the ring dove and the Japanese quail, the AROM activity in the preoptic-hypothalamic areas is regulated by a synergistic effect of T and its estrogenic metabolites (Hutchison and Steimer, 1984; reviewed by Panzica et al., 1996). However, there are differences between brain regions or between species. For example, AROM activity changes seasonally in the diencephalon but not in NCM in the pied flycatcher (*Ficedula hypoleuca*) (Foidart et al.,
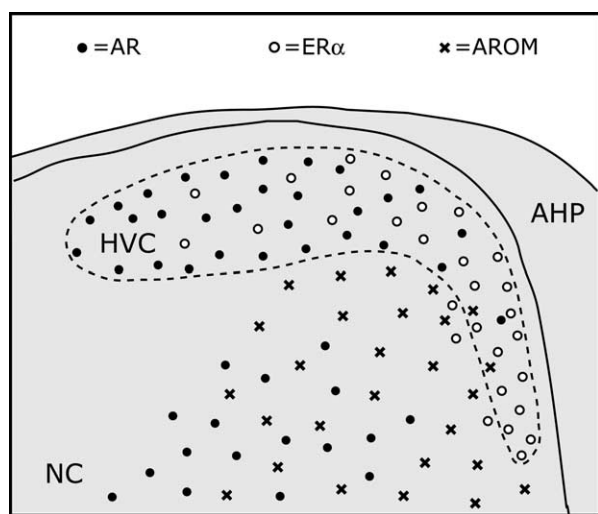
Table 1. Seasonal differences in hormone levels, hormone receptor levels in HVC, and AROM in NCM of male canaries

	Spring vs. autumn
T	↑ ↑ ↑
Estradiol	↑
AR-mRNA (HVC)	–
ERa-mRNA (HVC)	↓
AROM-mRNA (NCM)	↑ ↑
AROM activity (NCM)	–

See text for details. Adapted from Fusani et al., 2000.

1998), the chaffinch (*Fringilla coelebs*), the willow warbler (*Phylloscopus trochilus*), and the great tit (*Parus major*) (Silverin et al., 2000). Thus, we studied the differences in the plasma levels of androgens and estrogen, AROM activity, and expression level of AR-, ERα-, and AROM-mRNA in the NC of male canaries between two seasons of high singing activity, the autumnal singing period (November) and the breeding season (April) (Fusani et al., 2000). This was, to our knowledge, the first study in which seasonal differences in AR-, ERα-, and AROM-mRNA expression, AROM activity, and circulating levels of gonadal steroids were studied in the same songbird individuals.

The main results of the study are summarized in Table 1. As expected, we found that plasma levels of androgen and estrogen were higher in April than in November (Fusani et al., 2000). These differences were similar to those later described for wild canaries in Madeira (Leitner et al., 2001). There was a seasonal effect on the expression of AROM-mRNA in NCM and ERα-mRNA in HVC, but in opposite directions: AROM expression was higher in spring than in autumn, whereas ERα expression was higher in autumn than in spring. Surprisingly, we found no seasonal differences in the expression of AR-mRNA in HVC and in brain AROM activity.

These results supported our hypothesis that in the canary NCM AROM-mRNA expression is regulated by androgen, since both AR and AROM, but not ER, are expressed in this region (Bernard et al., 1999; Metzdorf et al., 1999). The lower expression of ERα in HVC in spring, together the fact that in this season circulating levels of estrogen are higher, suggested that ERα expression in HVC is down-regulated by estrogen (although seasonal changes in ERα-immunoreactivity are less pronounced and might suggest a seasonal regulation in the receptor turnover) (Gahr, 1990a). There are few studies on the regulation of ER in birds, however estrogen down-regulates ERα and ERα-mRNA levels in several brain areas of the rat and the guinea-pig (Lauber et al., 1991; Lisciotto and Morrel, 1993). The down-regulation of ERα in spring could also depend on a higher production of estrogen locally, because both AROM-mRNA expression in NCM and circulating T was higher in this season.

The absence of seasonal differences in AROM activity in NCM is apparently in contrast with the clear seasonal pattern of AROM-mRNA expression in this area. However, this disagreement could be methodological, i.e. it could be accounted for by differences in the anatomical resolution of the two measurements. Samples dissected for the measurement of the AROM activity included the rostral part of NCM, where AROM-mRNA expression is low. Thus, it is possible that seasonal differences in the levels of AROM activity in NCM were masked by the inclusion in the samples of a substantial amount of tissue with low AROM activity. Alternatively, the regulation of AROM in the canary NCM could differ from that described for the quail brain, where the activity of the enzyme seems to be regulated mainly at the transcriptional level (Harada et al., 1993; Panzica et al., 1996). A lack of correlation between seasonal changes in AROM activity and number of AROM-immunoreactive cells in the posterior hypothalamus has been described in the pied flycatcher (Foidart et al., 1998).

The lack of differences in AR-mRNA expression between seasons was unexpected, because previous work had shown androgen-dependent regulation of AR in the song system (Nastiuk and Clayton, 1995), and in our study androgen levels were 50 times higher in spring than in autumn. However, Gahr and Metzdorf (1997) had already shown that the expression level of AR-mRNA in HVC is reduced during the summer and the early autumn (see also Soma et al., 1999b) but increases to spring levels in late autumn, when canaries have resumed singing. Taken together, these studies show that AR expression is low in late summer–early autumn, when the birds are photorefractory and have low plasma levels of androgens, and increases to spring levels in late autumn, when the birds have regained photosensitivity but *before* the vernal increase in plasma androgen levels. Therefore, in the canary HVC seasonal changes in AR expression are, at least in part, independent of gonadal hormones.

In summary, in the canary brain AR-, ERα-, and AROM-mRNA have distinct seasonal patterns of expression. On one hand, the lack of correlation between AR expression and circulating androgens shows that steroid-independent seasonal factors control AR expression. On the other hand, seasonal changes in ERα- and AROM-mRNA expression indicate that the action of estrogen on the song system is seasonally regulated. Androgen appears to be an important regulatory factor for AROM-mRNA expression in NCM, where AR- and AROM-mRNA are co-expressed. Seasonal changes in AROM-mRNA expression (and possibly AROM activity) in NCM may in turn regulate local estrogen production and thus ERα expression in the adjacent HVC. A puzzling result of our study was the lack of correlation between AROM-mRNA expression and AROM activity. In contrast to the number of studies on AROM regulation in the preoptic area and the hypothalamus, where the enzyme is regulated synergistically by androgen and estrogen (Hutchison and Steimer, 1984; reviewed by Panzica et al., 1996), we knew little about the regulation of the enzyme in the telencephalon because in this brain region high concentrations of AROM had been described only in songbirds (Vockel et al., 1990a; Shen et al., 1995).

In a subsequent study, we investigated whether androgen regulates AROM-mRNA expression and AROM activity in the canary NCM (Fusani et al., 2001). In songbirds
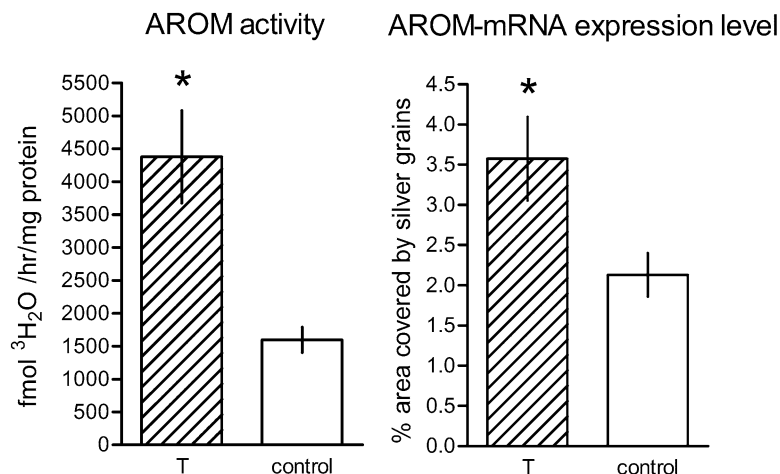
AROM activity   AROM-mRNA expression level

Fig. 2. The levels of AROM activity (left) and AROM-mRNA expression (right) in the NC were significantly increased in T-treated compared with untreated female canaries. Adapted from Fusani et al., 2001.

castration can be a difficult task and in some species may result in high, variable levels of circulating androgen and estrogen that originate from extragonadal sources (Marler et al., 1987; Adkins-Regan et al., 1990). Therefore, as an alternative approach we used intact female canaries, which have low circulating levels of androgen (Weichel et al., 1986). Female canaries were implanted with T pellets and killed 4 weeks afterward. Brains were halved sagittally. In one half of the brain we measured AROM-mRNA expression in the NCM and in the rostral neostriatum with *in situ* hybridization, and in the other half AROM activity in NCM and in the cerebellum (as control area) with a radioenzymatic assay (Fusani et al., 2001). In T-treated females, both AROM activity and mRNA expression in NCM were significantly increased compared with untreated females (Fig. 2). In addition, AROM-mRNA expression was individually correlated with plasma T levels (Fusani et al., 2001). These results showed that in the canary NCM, AROM is regulated by T. It is likely that the increase in AROM activity and mRNA expression is due to T acting on AR rather than to its conversion to estradiol for two reasons: 1) AROM and AR, but not ERα, are co-expressed in NCM (Metzdorf et al., 1999); 2) the increase in AROM expression induced by T is not blocked by simultaneous treatment with an AROM inhibitor (Fusani et al., 1996). Nevertheless, the hypothesis that androgen and not estrogen regulates AROM in NCM has not been tested yet.

At this stage, we had a considerable amount of information about the distribution and the regulation of AR, ERα, and AROM in the songbird brain. The next and most important task was to understand how all this is related to song organization.

How does estrogen affect song organization?

Once again, we profited from a peculiarity of canaries (and other songbirds): When adult females, which usually produce little if any song, are treated with T, they develop a robust, male-like song within 3–4 weeks (Leonard, 1939). This behavioral development is accompanied by the mas-

culinization of the song system (Nottebohm, 1980; De-Voogd et al., 1985; Heid et al., 1985). The T-induction of song in female canaries is a good model to study the influence of hormones on song organization (Nottebohm, 1980; DeVoogd et al., 1985; Fusani et al., 2001). We compared the songs of females implanted with T alone or with T and the AROM inhibitor fadrozole (FAD). To correlate the behavioral effects with estrogen action in the song system, we studied in the same birds the expression of two estrogen-sensitive genes, ERα and brain-derived neurotrophic factor (BDNF) in HVC. BDNF expression is regulated by estrogen in the zebra finch HVC (Dittrich et al., 1999). Finally, to monitor AR-mediated or unspecific effects of the treatment on gene expression, we studied the expression of AR and of ATP-synthase, an indicator of cell activity (Guerra et al., 1987; Pierce et al., 1992).

Females canaries treated simultaneously with T and FAD developed male-like song that, in its gross structure, did not differ from those of T-treated canaries (Fusani et al., 2003). However, a detailed song analysis highlighted some important differences between the songs of the two groups. First, T+FAD females sang more "tours" (i.e. repetition of syllables—song units) of the same type in a same song (Fig. 3). Because the organization of songs in stable sequences of different tours is a marker of the transition from plastic to full song in canaries (Güttinger, 1979), these results suggest that development of full song may depend on the aromatization of T. Secondly, songs of T+FAD females had a lower proportion of tours with high syllable repetition rate (Fig. 3). In female canaries that sing spontaneously the syllable repetition rate is lower than in males and increases after T-treatment (Pesch and Güttinger, 1985) (Gahr, unpublished observations). Therefore, songs of T+FAD females appear to be less masculinized than those of T females. Interestingly, sexually receptive female canaries show higher sexual responses to songs with higher syllable repetition rate, no matter if the songs are recorded from males (Vallet and Kreutzer, 1995) or T-treated females (Vallet et al., 1996). Thus, one of the sexually attractive
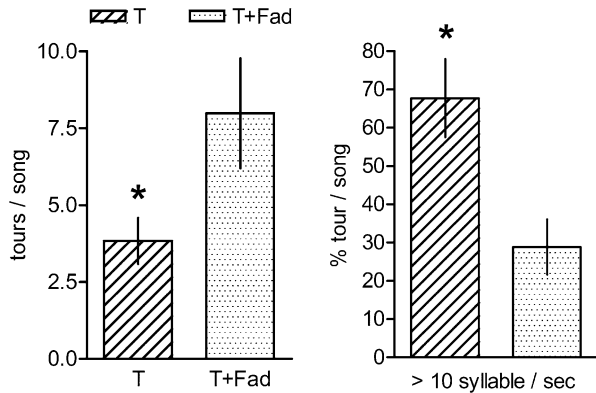
Fig. 3. Comparison of the songs of female canaries treated with T or T+FAD. The number of tours (i.e. repetition of syllables) per song was higher in T+FAD females. In this group, there was a lower proportion of tours with a syllable repetition rate higher than 10 syllables/s. Adapted from Fusani et al., 2003.

parameters of canary song, the syllable repetition rate, seems to depend on estrogen. Recently, similar results on the syllable repetition rate were obtained by treating male canaries with FAD and T or T only (Rybak and Gahr, 2004), confirming the general validity of the results from female canaries (Fusani et al., 2003).

Several data suggest that these behavioral effects of AROM inhibition are due to estrogen-dependent mechanisms. FAD induced an increase in the expression level of ERα (Fig. 4), which is in line with our previous interpretation (Fusani et al., 2000) that estrogen down-regulates ERα. On the other side, the estrogen-dependent expression of BDNF was higher in T females compared with T+FAD and untreated females (Fig. 4). Contrary to the modulation of ERα and BDNF, the expression level of ATP-synthase and of AR did not differ between T-females and T+FAD females (Fusani et al., 2003). Thus, we found differences between groups in the expression of estrogen-sensitive genes (ERα and BDNF) and not in the expression of androgen-sensitive (AR) or genes that indicate general cell activity (ATP-synthase). Therefore, the behavioral effects of FAD appear to be associated to a reduction in estrogen formation. Other observations suggest, moreover, that the effects are linked to changes in estrogen action in HVC. First, HVC is the only nucleus of the song system that contains ERs (Gahr et al., 1993; Gahr and Metzdorf, 1997; Bernard et al., 1999). There are brain regions outside the song system such as the medial preoptic area which express ER (Gahr et al., 1993; Bernard et al., 1999; Metzdorf et al., 1999) and modulate singing activity (Riters and Ball, 1999). Similarly, there are regions that contain ER and project to the song system (Maney et al., 2001). However, no study so far has shown that areas outside the song system can control the *structure* of song, which can be affected only by lesions or electrical disturbances of the forebrain and descending vocal control areas (Nottebohm et al., 1976; Vu et al., 1994; Yu and Margoliash, 1996; Margoliash, 1997; Wild, 1997).

CONCLUSIONS

In summary, with our studies on canaries we have made substantial progresses in understanding the role of estrogen in song organization. We have described where estrogen is produced in the songbird brain and where it may act by binding to ERα. We know that estrogen formation and action change seasonally in relation to the seasonal cycle of T production by the testes, and T can regulate the expression and activity of AROM. Finally, we know that the degree of masculinization of the song and the syllable repetition rate, which is sexually selected in canaries, depend on estrogen action. One of the open questions is how estrogen influences the song structure. It is likely that one action of estrogen is to influence neuronal morphology. For example, estrogen contributes to induce synaptogenesis and dendritic growth in canary HVC neurons (De-Voogd and Nottebohm, 1981; DeVoogd et al., 1985). The T-dependent increase in the number of soma–somatic gap-junctions in the HVC neuronal clusters of canaries (Gahr and Garcia-Segura, 1996) might depend on the estrogenic metabolites of T (e.g. see Garcia-Segura, 1994). In addition, estrogen-dependent factors such as BDNF could modify electrical properties of HVC neurons or of afferent and efferent areas of HVC after anterograde or retrograde transport (cfr. Akutagawa and Konishi, 1998; Johnson et al., 2000). Finally, estrogen might regulate the recruitment and/or survival of newborn neurons in HVC (Hidalgo et al., 1995; Louissaint Jr. et al., 2002) although these neurons do not express ERα (Gahr, 1990b; Hidalgo et al., 1995). Independently of the mechanisms through which estrogen can influence the song structure, our studies have provided an explanation for the presence of ER in the song system of adult songbird. We think that these data can help us to understand what happens during song development. For example, in zebra finches brain levels of estrogen (Holloway and Clayton, 2001), and the expression of both BDNF (Dittrich et al., 1999) and its receptor
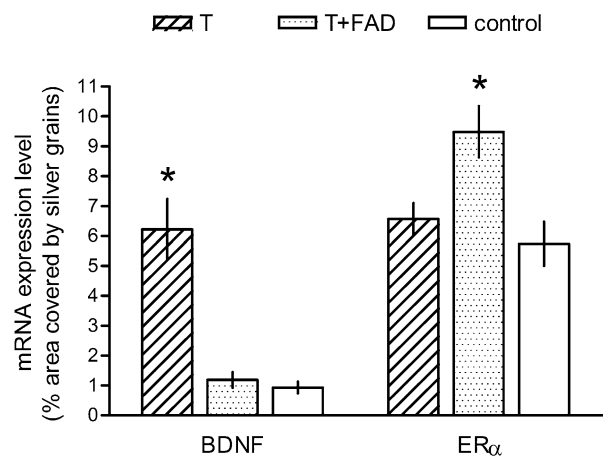

Fig. 4. Expression levels of BDNF and ERα in the HVC of female canaries treated with T only, T+ FAD, and untreated. T treatment induced an increase in BDNF expression which was suppressed by simultaneous treatment with FAD. The expression of ERα was increased by FAD treatment. Adapted from Fusani et al., 2003.

tyrosine kinase receptor B (trkB) (Chen et al., 2005) in HVC are higher in males than in females during the differentiation of the song system. Thus, the processes underlying song organization in T-treated females could illustrate at least in some aspects the mechanisms of song organization during development.

In the last years, a number of studies have highlighted the importance of estrogen for the activation and plasticity of song and the song system. In canaries and white-crowned sparrows, estrogen is important for the seasonal activation of song and the seasonal neuroplasticity of the song system (Tramontin et al., 2003; Soma et al., 2004; Sartor et al., 2005). In addition, estrogen can influence song behavior by acting on areas outside the song system: ERα are expressed in the preoptic area (Gahr et al., 1987), and lesions of this region affect overall singing activity (Riters and Ball, 1999). ERα are also expressed in catecholaminergic regions of the midbrain that send inputs to the song system such as the central gray and the area ventralis of Tsai (Maney et al., 2001). Taken together, our studies and those from other laboratories show that estrogen has multiple functions in the control of singing, song development, song organization, and song seasonal plasticity, and underline the need for further studies on estrogen action in the song system and other brain areas.

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