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Male song quality modulates c-Fos expression in the auditory forebrain of the female canary

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Abstract

In canaries, specific phrases of male song (sexy songs, SS) that are difficult to produce are especially attractive for females. Females exposed to SS produce more copulation displays and deposit more testosterone into their eggs than females exposed to non-sexy songs (NS). Increased expression of the immediate early genes *c-Fos* or *zenk* (a.k.a. *egr-1*) has been observed in the auditory forebrain of female songbirds hearing attractive songs. C-Fos immunoreactive (Fos-ir) cell numbers were quantified here in the brain of female canaries that had been collected 30 min after they had been exposed for 60 min to the playback of SS or NS or control white noise. Fos-ir cell numbers increased in the caudomedial mesopallium (CMM) and caudomedial nidopallium (NCM) of SS birds as compared to controls. Song playback (pooled SS and NS) also tended to increase average Fos-ir cell numbers in the mediobasal hypothalamus (MBH) but this effect did not reach full statistical significance. At the individual level, Fos expression in CMM was correlated with its expression in NCM and in MBH but also with the frequency of calls that females produced in response to the playbacks. These data thus indicate that male songs of different qualities induce a differential metabolic activation of NCM and CMM. The correlation between activation of auditory regions and of the MBH might reflect the link between auditory stimulation and changes in behavior and reproductive physiology.

Keywords

Immediate early genes; canary song; auditory processing; caudomedial mesopallium; caudomedial nidopallium

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1. Introduction

A key function of birdsong is to attract and stimulate potential mates [1]. In female songbirds, hearing conspecific male song enhances ovarian follicle growth [2, 3] and secretion of luteinizing hormone [4]. In canaries, certain song phrases produced by males have been identified as particularly attractive for females. They are known as “A phrases” or “sexy songs”. Such phrases are quite complex, including a combination of features that are difficult to produce, such as a wide range of frequencies, high repetition rate, and multiple-note syllables [5, 6]. When exposed to such attractive phrases, females show more copulation solicitation displays (CSD) and deposit more testosterone in the eggs that are subsequently laid [7, 8]. However, the neural pathway underlying the detection of sexy song and the translation of this signal into changes in behavior and physiology is not yet understood.

Within the auditory forebrain, the caudomedial mesopallium (CMM) and the caudomedial nidopallium (NCM), two regions analogous to secondary auditory cortices in mammals, have been implicated in the recognition and differentiation of socially relevant auditory cues [9, 10]. Increased expression of the immediate early gene (IEG) *zenk* (also known as *Egr-1* or *Zif268*) [11] has been found in the auditory forebrain of a number of songbird species in response to song. This response has been extensively used to analyze both the types of sounds that activate these auditory forebrain regions as well as the endocrine conditions that are conducive to this activation [12-16]. There is extensive evidence, based on quantification of the expression of *zenk* in CMM and NCM or on behavioral criteria, that females are able to discriminate and respond differentially to particularly attractive song stimuli such as songs of long duration in European starlings [17, 18], dialect complexity in white-throated sparrows [19], local vs. foreign dialect type in white-crowned sparrows [12] or song produced by the female’s mate in zebra finches [20] (reviewed in [14]).

In one experiment performed on female canaries, expression of the *zenk* mRNA was shown to be higher in females that heard sexy (aka “A” phrases; [6, 21]) as compared to non-sexy syllables but no such difference was found in NCM and no difference was observed in the expression of another immediate early gene called *Arc* [6, 21]. The specificity of this genomic response (*zenk* vs. *Arc* mRNA) is a first sight surprising but might simply reflect the classification of these two genes into different classes of IEGs. *Zenk* is indeed belonging to IEGs that function as transcription regulators whereas *Arc* is an IEG of the effector protein class that is able to directly modulate cell function (see [21] for additional discussion). We therefore wondered whether another IEG, specifically *c-Fos*, that is known to regulate transcription and has been widely used to study functional activation of the preoptic area, hypothalamus and limbic regions in a variety of avian and mammalian species [22] would allow us to discriminate activation by sexy vs. non-sexy songs of auditory brain areas in canaries. We also decided to study the induction of *c-Fos* expression at the protein level by immunohistochemistry rather than its mRNA by *in situ* hybridization in order to assess IEG induction in a different time window. Leitner and colleagues indeed observed a higher induction of *Zenk* by sexy songs in CMM but not in NCM (although a trend was present in the latter nucleus). Because brains were collected 30-40 min after song playback in order to quantify mRNA, it might be argued that the latency between the signal and

mRNA expression was not adequate to reveal activation in both auditory brain regions. IEG expression is usually studied at the protein level after longer delays in the range between 90 and 120 min [22] and this potentially provides a broader window to successfully pick up significant brain activation in different brain regions that might react after slightly different latencies.

An additional goal of this study was to collect information on the neural pathways linking the differential auditory inputs provided by sexy vs. non-sexy songs to changes in female behavior or testosterone deposition in the egg yolk. It had been demonstrated in white-throated sparrows that hearing conspecific songs induces an increase in plasma concentrations of luteinizing hormone and that this effect is associated to an activation of the medio-basal hypothalamus as identified by enhanced *zenk* or *c-Fos* expression [4, 14]. However, no information was available concerning brain pathways potentially mediating differential responses to songs of different quality.

We began to address these questions here by exposing female canaries to either sexy song, or non-sexy song or white noise as a control for 60 min and collecting their brain 30 min later. We then quantified by immunohistochemistry *c-Fos* expression in the auditory brain regions and in hypothalamic regions that are potentially implicated in the control of sexual behavior and ovarian activity [23]. This experiment demonstrated the presence of a differential activation of the secondary auditory brain regions of the female brain after exposure to the playback of sexy song. Activation in CMM also correlated with activation in the medio-basal hypothalamus. These results provide some new information on the neural mechanisms mediating effects of songs on reproductive physiology and behavior but still leave open many questions concerning these relationships.

2. Methods

2.1. General protocol

The experiment described here was carried out on adult female canaries that had been kept in groups in short-day conditions (8h light: 16 h of dark [8L:16D] per day) for 4 months and then transferred to long-day conditions (14h light per day; 14L:10D) for 10 days before the beginning of the experimental treatments. This photoperiod was chosen in order to raise circulating estradiol concentration enough to ensure that females would respond to the song stimuli by showing the appropriate behavioral and brain responses [15, 16, 24]. The sex of all subjects had been confirmed by molecular sexing before the beginning of the experiments. Throughout the study, birds were provided with seeds and water *ad libitum*, fresh fruits or vegetables were provided twice a week. All experimental procedures were in agreement with the laws on animal experimentation in France: experimental authorizations were delivered by the French Ministry for Agriculture and Fisheries (LECC: B 92-050-01, G. L., authorization no. 92-230).

On the 9th day of exposure to the 14L:10D photoperiod, 27 females were transferred to individual cages (37 × 28 × 22 cm) located in sound-attenuated chambers (interior dimensions; 36.5 × 47 × 36.5 cm) under the same photoperiod and allowed to adjust to their new environment. On the next day, they were exposed to 60 minutes of playback by a

Marantz Professional PMD670 portable solid-state recorder of either “sexy” song (SS, with a preponderance of “A” phrases, $n = 12$), or “non-sexy” song (NS, lacking “A” phrases, $n = 8$), or white noise (WN, to control for general auditory activation, $n = 7$; see below for detail on these stimuli).

During the first 15 minutes of this playback period, one observer continuously recorded the number of copulation solicitation displays (CSD) produced by the females, a behavior that has been shown to be produced in response to “sexy” songs and which indicates the sexual receptivity of the female [6, 25]. The observer also noted the number of calls emitted.

Thirty minutes later, females were sacrificed by decapitation. Their brain was rapidly dissected out of the skull and immediately fixed for 150 min in a 5% solution of acrolein in 0.01 M phosphate-buffered saline (pH 7.4, PBS). The abdominal cavity was then opened to assess the ovarian development of each subject. A photo was first taken of the ovary with a ruler graduated in mm placed in the same frame to indicate scale and the diameter of the 4 largest follicles was later measured on these photos. The volume of each follicle was then estimated as a sphere ($\frac{4}{3} \pi R^3$) with the corresponding radius. The ovary and the oviduct were then dissected and immediately weighed to the nearest mg.

2.2. Song stimuli

We constructed one-hour tapes of stimuli for each female (to avoid pseudo-replication) using SASLab Pro by Avisoft Bioacoustics. Stimuli were made of a variable number of constructed songs. All song elements that were used were obtained from the laboratory bank of recorded songs and had never been heard by the experimental subjects before.

Attractive (“sexy” songs, SS) and non attractive (“non-sexy”, NS) song tapes were not equivalent in length, number, diversity, and attractiveness of phrases and thus in the length of silences between song bouts and all these characteristics were superior in the “sexy” songs rendering them attractive for females. However, the total duration (in seconds) of songs during the one hour tape was the same for both groups.

Songs were built following the classic canary song model that contains three parts (Figure 1): 1) one non-stimulating phrase (about 1 sec) composed of three introduction notes, 2) a variable number of diverse phrases making up the body of the song (stimulating phrase for SS and non stimulating phrases for NS), and 3) one non-stimulating phrase (1.5 sec) composed of three conclusion notes. The introduction and conclusion phrases of SS and NS songs were equivalent. The two song types differed only in the body (part 2) of the song. All frequencies between 1 and 7 KHz were covered at least at one point in all songs.

The body (part 2) of attractive songs was composed of 5 stimulating phrases that were composed of complex syllables made up of two notes emitted at a repetition rate of 20 syllables per second. Three of these stimulating phrases were “A” phrases (4 KHz frequency bandwidth) and two were “A-” phrases (2 KHz frequency bandwidth). Previous experiments showed that female domestic canaries generally preferred phrases with a larger bandwidth [26]. Each “A” and “A-” phrase was 1.5 seconds long. Total song bouts including the introduction notes, body and conclusion notes lasted 10 seconds.

The body of unattractive songs was composed of four 1 second long non-stimulating phrases made up of single notes with either frequency bandwidths inferior to 2 KHz or repetition rate inferior to 15 syllables per second or both. Total song bouts (introduction, body and conclusion) lasted 6.5 seconds.

Because song length varied between the two groups, we standardized the total duration of song in each stimulus by decreasing the duration of silence periods between unattractive songs. As a result, for every attractive song there were approximately two non-attractive songs in the tapes. Females in the NS groups thus heard twice as many songs but the total duration they were exposed to songs was the same as in the SS group.

The white noise control stimulation was produced using the “Insert White Noise” command in AviSoft Bioacoustic software, with specified set duration of two hours.

2.3. Tissue collection and processing and c-Fos immunohistochemical staining

After the acrolein fixation, brains were rinsed three times for 20 min in 0.01 M PBS and transferred to a 30% sucrose cryoprotective solution at 4°C until they sank. They were then frozen on dry ice and stored at -80°C until sectioning. Each brain was sliced coronally on a cryostat at -20°C into four series of 30 µm-thick sections.

One series of sections (120 µm between any two successive sections) was immunohistochemically stained for the protein product of the *c-fos* gene with a rabbit polyclonal antibody raised against an avian c-Fos protein sequence [27] prepared and validated by D’Hondt and colleagues [28]. Sections were stained in batches of matched series including subjects from all experimental groups to exclude differences between groups that would relate to minor technical differences.

Immunohistochemical labeling was carried out via the avidin-biotin technique on free-floating sections. Briefly, sections were rinsed in PBS and then placed for 15 min in 0.1% sodium borohydride. They were then incubated sequentially in 0.6% hydrogen peroxide for 20 min to block endogenous peroxidases, followed by 5% normal goat serum for 30 min to decrease nonspecific binding. Sections were then incubated overnight at 4°C in the primary rabbit anti-c-Fos antibody diluted 1:1000. The antibody-antigen complex was localized using the avidin-biotin complex method, with a commercial kit used as per the manufacturer’s directions (ABC Vectastain Elite Kit PK-6100, Vector Laboratories PLC, Cambridge, UK). All reagents were diluted in 0.01M PBS containing 0.1% Triton X-100 (PBS-T) and sections were rinsed several times in PBS between staining steps. The peroxidase was finally visualized by incubation in a 0.04% solution of 3,3-diaminobenzidine tetrahydrochloride (DAB) in PBS with 0.003% hydrogen peroxide. Sections were rinsed several times in PBS, mounted on microscope slides in a gelatin-based medium and coverslipped.

2.4. c-Fos quantification

c-Fos expression was quantified in nine brain regions that were previously demonstrated to contain large numbers of c-Fos-immunoreactive (Fos-ir) cells in several avian species, focusing on regions involved in audition, auditory processing, and sexual behavior or

reproductive endocrinology namely, the nucleus ovoidalis (OV), the dorsolateral nucleus of the mesencephalon (MLd), the caudomedial mesopallium (CMM), caudomedial nidopallium (NCM), HVC (used as a proper name; [29]), the medial preoptic nucleus (POM), an area immediately dorsal to the POM (dPOM), the medio-basal hypothalamus (MBH; ventral part of the hypothalamic region just caudal to the anterior commissure), and a pretectal area where we unexpectedly detected a dense cluster of Fos-ir cells and identified as the visual medial spiriform nucleus (SPM). We reasoned that this nucleus could serve as a control region showing *c-Fos* expression that would not be modulated by auditory stimuli.

For all nuclei showing clear boundaries such as OV, MLd, HVC and SPM, the counting frame was placed in the center of the area of interest. In other cases it was placed in a standardized manner based on external landmarks. NCM was counted in the same section as HVC in an area within the nidopallium located ventromedially with respect to HVC. CMM was counted at a similar location but in more rostral sections containing the mesopallium where HVC was no longer present. POM and dPOM were counted in two adjacent counting frames adjacent to the 3rd ventricle in the section where the anterior commissure reaches its maximum extension and finally MBH was counted in the section immediately caudal to the POM section (120 μm more caudally) in a counting frame that was adjacent to the wall of the 3rd ventricle and to the base of the brain. The counting frame thus overlapped with the ventromedial nucleus of the hypothalamus but was clearly larger than this nucleus.

The number of cells displaying *c-Fos* staining denser than background was counted in a 0.04 mm^2 square area within each region of interest, except for the MBH, where counts were made in a 0.08 mm^2 rectangular area (200 μm wide, 400 μm high).

Fos-ir cells in a given nucleus were counted in every section where this nucleus was visible. To account for slight variations between individuals in the sectioning angle and in the number of sections through each region, total cell counts for each experimental subject were converted to densities per unit area by dividing the total number of Fos-ir cells in the different sections containing nucleus by the total area counted (i.e., number of slices containing the region of interest multiplied by 0.04 or 0.08 mm^2 , as appropriate).

Counting was performed in a semi-automated manner using the free software ImageJ (NIH). Briefly, the section of interest was photographed with a CCD camera attached to the microscope and the region of interest was cropped out of the original photomicrograph. The image was then converted to an 8-bit greyscale. In most images, the pale background was then removed from the image with the 'Subtract Background' command in ImageJ while allowing a rolling ball radius of 10 pixels. For images without strong positive staining or with heavy background staining this process was done manually using the 'Threshold' adjustment option to avoid introducing artifacts. The resulting image was converted to binary (i.e. maximized contrast) and the number of objects covering at least 5 μm^2 (i.e. positively-labeled cell nuclei) in the area of interest was then counted using the 'Analyze Particles' function. We previously demonstrated that this method of semi-automatic counting correlates almost perfectly with results obtained by manual counting [30].

2.5. Statistical Analyses

All data were analyzed by t-test, one-way or two-way analyses of variance (ANOVAs) as appropriate with the Prism 5.0 (GraphPad Software, LaJolla CA) and SuperANOVA (Abacus Concepts, Berkeley CA) softwares running on Macintosh computers. Significant effects of treatments on numbers of Fos-ir cells were further analyzed with the Newman-Keuls post-hoc test. Behavioral data were analyzed by the non parametric Kruskal-Wallis Analysis of variance and presence/absence of behavior by the Fisher exact probability test. Correlations were computed with the non parametric Spearman rank correlation coefficient since data included behavioral results that had an obviously non normal distribution. Effects and differences were considered significant for $p < 0.05$.

3. Results

3.1. Behavioral and morphological results

As could be expected based on previous work, female canaries reacted to the broadcast of conspecific male songs by producing short calls and occasionally displaying copulation solicitation displays (CSD). As an average, calls and CSD were more frequent in NS than in WN females and even more in SS than in NS birds (Figure 2, top row). However, overall these differences did not reach statistical significance (non parametric Kruskal Wallis ANOVA; calls: $H=4.538$, $p=0.1034$; CSD: $H=3.761$, $p=0.1525$). This lack of significance obviously relates to the very large range of call frequencies within each group (WN: 0-103; NS: 0-310; SS: 0-510; one or two completely silent females in each group). Only a fraction of females showed at least one CSD in response to the broadcast of male songs (0/6 in WN; 4/8 in NS and 5/12 in SS). These proportions are associated with statistical tendencies but are not significantly different (WN vs. NS: $p=0.069$; WN vs. SS: $p=0.092$; all by Fisher Exact probability test). In general more females hearing male songs (NS+SS) tended to produce CSD than females hearing WN (9/20 vs. 0/6, $p=0.054$).

Measures taken at autopsy indicated that the degree of sexual maturation of the females assigned to the three experimental groups was slightly variable within groups but in average similar between groups. Ovarian weight and oviduct weight (Figure 2, middle row), two markers of estrogens production and action were indeed very far from being different across groups (Ovary: $F_{2,21}=0.5237$, $p=0.599$; Oviduct: $F_{2,24}=0.2619$, $p=0.7718$). The mean values were slightly decreasing from the WN to the NS to the SS group suggesting that if anything the reactivity to male song should decrease in parallel [24, 31]. A similar pattern was revealed by measures of the largest 4 ovarian follicles (F1 to F4; Figure 2, bottom). Analysis of these data by two-way ANOVA (groups as independent and successive follicles as repeated factor) identified no group effect ($F_{2,24}=0.4359$, $p=0.6517$) and no interaction between groups follicle hierarchy ($F_{3,72}=0.8152$, $p=0.5617$) but of course a very significant effect of the follicle hierarchy ($F_{6,72}=5.6921$, $p=0.0015$).

3.2. Fos-ir cells

Due to technical problems in tissue processing (poor fixation, sections tearing), quantitative data were lost in a few subjects and could only be reliably obtained from 21 subjects (7 WN, 6 NS and 8 SS). Average numbers of Fos-ir cells per mm^2 in 9 different brain nuclei are

displayed in figure 3. Representative photomicrographs of the immunohistochemical signal are presented in figure 4

Auditory ascending pathway to HVC—The density of Fos-ir cells was nearly identical in the three experimental groups in two nuclei of the ascending auditory pathway, MLd ($F_{2,18}=0.7643$, $p=0.4802$) and OV ($F_{2,18}=0.1958$, $p=0.8239$). In contrast, a differential brain activation was observed in the two secondary auditory brain nuclei, CMM ($F_{2,18}=5.605$, $p=0.0128$) and NCM ($F_{2,18}=7.441$, $p=0.004$) (Figure 4). In these two nuclei, post-hoc tests showed that Fos-ir cell density was significantly higher in the SS than in the WN group. In addition, cell density was also significantly higher in SS than in NS females in NCM. Despite the fact that HVC receives projections from both CMM and NCM, there was no differential expression of *c-Fos* in this song control nucleus after exposure to the three types of auditory stimuli ($F_{2,18}=0.2206$, $p=0.8042$).

Diencephalic nuclei—The ANOVA of Fos-ir cell density in the POM indicated a significant difference between groups ($F_{2,18}=3.602$, $p=0.0483$). This effect seemed to result from a decreased average expression in the SS group but the post hoc Newman Keuls tests failed to identify any significant group difference when groups were compared two by two. No overall effect was observed in the dPOM ($F_{2,18}=0.7078$, $p=0.5059$) nor in the MBH ($F_{2,17}=1.958$, $p=0.1717$) although there was in this brain region an average numerical increase in *c-Fos* expression in the two groups that had been exposed to male songs (NS and SS) as compared to females exposed to WN. However, even the specific difference between the pooled groups exposed to song (NS+SS) with the WN group was not fully significant ($t=2.034$, $df=18$, $p=0.0569$). Finally, in the SPM the average number of Fos-ir cells decreased from the WN to the NS to the SS group. Overall these differences were significant ($F_{2,17}=4.317$, $p=0.0305$) and post hoc tests confirmed the existence of a significant difference between the WN and SS groups.

3.3. Correlations between Fos-ir cells in different nuclei and behavior

We finally examined whether *c-Fos* expression observed in different brain areas was inter-correlated and whether this expression was related to the behaviors expressed by the females i.e. the calls and CSD produced. Only brain regions where significant changes in *c-Fos* expression had been observed i.e. CMM, NCM, POM, MBH and SPM were considered to limit type I error. The correlation matrix containing the 21 ($7 \times 6/2$) corresponding Spearman correlation coefficients only contained 4 significant coefficient (see Figure 5) relating Fos-ir numbers in CMM and in NCM ($r_s=0.591$, $p=0.005$), in CMM and MBH ($r_s=0.640$, $p=0.002$) and in CMM and SPM ($r_s=-0.513$, $p=0.021$) and relating Fos-ir cells in CMM and numbers of calls ($r_s=0.527$, $p=0.017$). All other coefficients were not significant ($p>0.05$). Only one of these correlations remains significant after a Bonferroni correction, namely the correlation between Fos-ir cells in CMM and MBH ($p=0.002 \times 21=0.042$) but it would not if all brain areas considered were included in the correction. As can be observed in figure 5, these significant correlations resulted partly from the group differences described before and partly from inter-individual differences within groups. However, the low number of subjects in each group did not provide enough statistical power to demonstrate these within group

correlations: they were all non significant ($p>0.05$) except for the relationship between *c-Fos* in CMM and call numbers in the NS group ($r_s=0.865$, $p=0.024$).

4. Discussion

Although differences between groups did not reach statistical significance, the present behavioral results support the notion that female canaries are able to discriminate between male songs containing or not the so-called “A phrases” also known as “sexy songs” that are difficult to produce and thus potentially represent an indication of male quality. Females of the SS group indeed displayed a larger number of CSD and produced more calls than females of the NS group, which were themselves more active than females exposed to white noise. These differences were not related to a differential endocrine condition as attested by the similar development of the ovary and oviduct in the three groups of females (if anything an opposite pattern of development was observed i.e. $WN>NS>SS$). The behavioral differences between groups did not reach statistical significance due to the lack of response in some subjects although the percentages of active subjects tended to increase from the WN to the NS to the SS group ($0.05<p<0.010$). The lack of response in some subjects is probably explained by the fact that female canaries have been found to be more discriminative towards their mate’s songs during the last 3 days preceding the laying of the first egg when sexual motivation is high [32]; in this experiment, many females probably did not reach this acme of sexual responsiveness. Therefore these data are in agreement with and tend to confirm the discrimination capacity of these females and also the fact that the number of calls can probably be used as a behavioral index of this discrimination (see [25, 33]).

It has been previously shown that exposure to sexy songs as opposed to non-sexy songs enhances expression of the immediate early gene *zenk* in CMM but not in NCM, although a clear trend in the same direction was present, while the other immediate early gene *Arc* was not differentially expressed in these secondary auditory areas [21]. We demonstrate here that exposure to these two types of songs as opposed to WN significantly affects the expression of another early gene *c-Fos* in the two secondary auditory areas, CMM and NCM. Results of these two studies are thus highly consistent and the small differences observed probably do not reflect true discrepancies but rather procedural differences. Leitner et al. (2005) only compared sexy to non-sexy songs while we added here a control group exposed to WN. Inclusion of a third experimental group slightly decreased the power of our experiment but at the same time provided a larger contrast to evaluate the effect of sexy songs on IEG induction. This could be why we showed here an effect of sexy songs compared to WN in both CMM and NCM but failed to detect a difference between SS and NS in CMM. It is also important to note the *zenk* induction was studied by Leitner and colleagues at the mRNA levels while we quantified here the *c-Fos* protein. These two types of effects are detected after different latencies (longer latency for protein expression) and this might also justify the slight differences between these two sets of results.

Importantly the present study used *c-Fos* instead of *zenk* induction to quantify activation by song of auditory areas. Although most previous studies on the topic used *zenk* a marker of activation, *c-Fos* was used in a few cases alone or in combination with *zenk* [34-36] and surprisingly important differences between the responses as detected by these two IEGs

were sometimes identified [35, 36]. The pattern of activation of NCM and CMM seen here with *c-Fos* is consistent with the pattern detected by Leitner and colleagues with *zenk* but markedly diverges from the lack of activation that was suggested by studying another IEG *Arc*. [21]. These discrepancies thus draw attention to the well-established but often ignored fact that a negative result in studies involving IEGs does not necessarily mean that the area studied was not activated but simply that the specific IEG under consideration is not implicated in the cellular processes that are concerned [22].

Based on the present and numerous previous studies on canaries and other songbirds (e.g. [12, 24, 34, 35, 37]), it thus seems that the two IEG that act as transcription factors, *zenk* and *c-Fos* are both useful markers of the activation of auditory brain regions by relevant auditory stimuli (but see [35, 36]). *Arc*, another type of IEG that codes for a direct effector protein [21, 22] would in contrast not be implicated in this process or would be implicated with different modalities (responses to different aspects of the stimuli, after different latencies,);

Taken together, these data clearly show that female canaries discriminate between different types of male songs and between these songs and WN. This induction of IEGs in auditory areas is likely to lead to changes in the synthesis of a large number of proteins but how these metabolic changes and/or the associated changes in neurotransmission lead to the expression of female behaviors (CSD, calls) and to an increased deposition of testosterone in the eggs remains mysterious.

We additionally observed here changes in *c-Fos* expression in the medial preoptic nucleus (POM) and the nucleus spiriformis medialis (SPM). The number of Fos-ir cells was numerically lower in the SS than in the other groups in POM and SPM but although these differences were associated with a significant overall ANOVA, the only significant post-hoc test concerned the comparison of the WN and SS groups for SPM. It must be noted that an increase of *zenk* expression by conspecific songs was previously observed in the POM of white-throated sparrows (*Zonotrichia albicollis*) [15]. The significance of these decreases in *c-Fos* expression is not immediately clear. One might just hypothesize that the increased focus on auditory stimuli decreased in counterpart metabolic activity in brain regions involved in the expression of social behavior (POM) or processing visual stimuli (SPM) but there are currently no experimental data allowing to support or reject this hypothesis.

We had anticipated that the metabolic activation of auditory areas would extend to diencephalic brain regions controlling reproductive behavior and pituitary-gonadal activity such as the POM or MBH but no clear experimental confirmation of this idea could be obtained. It must be noted, however, that females exposed to male songs (NS as well as SS) displayed a numerical increase of Fos-ir cells in their MBH. These changes, however, were not statistically significant in the general ANOVA ($p=0.1717$) and only associated with a statistical tendency in a t-test comparing all birds exposed to song (NS+SS) or to WN ($p=0.0569$). In contrast, a *zenk* or *c-Fos* response was previously observed in the MBH of white-throated sparrows exposed to song [4, 14]. These two studies on sparrows however quantified IEG expression in a part of the MBH that is more caudal than the region considered here. These studies focused on the region immediately dorsal to the median

eminence and the pituitary stalk while quantifications were performed here in the rostral part of the MBH overlapping with the ventromedial nucleus of the hypothalamus, the brain region implicated in the control of female sexual behavior [38, 39]. This anatomical difference presumably explains the observed discrepancy. This lack of significant effect in the present study might also just relate to the limited power of its design (Power, $1-\beta = \pm 0.30$ for the analysis by ANOVA of MBH in the 3 groups): given the degree of individual variance observed, the total sample size should be 60 instead of 20 to make the ANOVA significant for a power of 0.80).

Correlation analyses suggested the existence of functional relationships between *c-Fos* expression in CMM and MBH ($r_s = 0.640$, $p = 0.002$ or $p = 0.048$ after Bonferroni correction). The rostral MBH that overlaps with the ventromedial nucleus of the hypothalamus should to be a key center of hormone action in the activation of female sexual behaviors based on mammalian literature and on experimental work in ring doves (see [38, 39]). This brain region may also participate less directly to the control the pituitary-gonadal activity based on anatomical data (e.g. distribution of Gonadotropin releasing hormone-containing neurons; [40]), lesion experiments (e.g., in quail: [41]) and various other functional studies [42, 43]. MBH is however a broad area with functional diversity and additional work will be needed to identify the specific location and neurochemical identity of the activated (*c-Fos*-positive) neurons as well as their connections, which could suggest the functional significance of these activations.

How cellular activation in CMM as identified by *c-Fos* expression is propagated to the MBH as suggested by the observed correlation remains unclear. It was shown that, in ring doves, neurons in the preoptic area and anterior hypothalamus selectively respond (single unit recordings) to the playback of male and even more of female nest coos while increases in plasma luteinizing hormone concentrations are observed in parallel [44]. These responses might represent the physiological bases of the ovarian growth induced in female doves by their production of nest coos in response to the coos of the male, the now well documented self-stimulation of ovarian development [45, 46].

This suggests the existence of more or less direct connections between secondary auditory areas and the MBH but these physical links have not been identified to our knowledge. Tract-tracing and electrophysiological studies have however identified mono-synaptic connections between the MBH and auditory areas in the ascending pathway, the thalamic nucleus ovoidalis [47, 48], and the mesencephalic nucleus intercollicularis, a midbrain vocal control nucleus [49]. It must be noted that auditory signals are classically not supposed to be “interpreted” at the level of ovoidalis (before they reach CMM and NCM) so that, at first sight, it appears unlikely that a differential response to songs vs. other neutral stimuli could originate in this thalamic nucleus. This would also be consistent with the fact that in the present study no differential *c-Fos* expression was detected in OV or MLd after exposure to different sounds. However a recent fMRI study identified in zebra finches a stimulus-specific activation (measured by increased oxygen consumption) in MLd an early relay in the auditory ascending pathway positioned even before OV [50] indicating that either differential responses to auditory stimuli appear much earlier than expected in the ascending auditory pathway or that descending projections feedback on the activity of earlier steps in

auditory processing. In support of this notion, previous IEG studies detected a differential induction of *zenk* in the ascending auditory pathway in MLd [13] and possibly OV [24] (conclusion based on the analysis of pooled material from two previous studies) in white-throated sparrows exposed to conspecific songs vs. synthetic tones. Based on these findings, it was proposed that auditory information reaches the diencephalic structures implicated in the control of behavioral and endocrine aspects of reproduction either from the thalamic nucleus OV or even the mesencephalic nucleus MLd [14]. The correlation between *c-Fos* expression in CMM and MBH observed here would then represent the influence of a descending feedback from secondary auditory areas onto the ascending pathway. Why this descending feedback did not induce a differential *c-Fos* activation in MLd and OV remains unexplained in this scenario but as noted above the absence of IEG induction is difficult to interpret and can have multiple causes. The presence of suboptimal concentrations of estrogens in the experimental females could for example be invoked (see [14]) or a differential reaction of *zenk* and *c-Fos* in the ascending auditory pathway [35].

Alternatively the observed correlation between *c-Fos* expression in CMM and MBH could relate to the differential production of calls and CSD by the experimental females. *C-Fos* expression in CMM was indeed positively correlated with the number of calls emitted by the experimental females and was additionally correlated to *c-Fos* expression in the other secondary auditory area NCM. The intuitively most obvious causal chain linking auditory stimuli to *c-Fos* expression in CMM/NCM to *c-Fos* expression in MBH to CSD and call production might actually not be responsible for the observed results. One could alternatively consider a causal chain of events going from the perception of auditory stimuli to the production of calls and CSD that would then be related causally to an increased *c-Fos* expression in CMM/NCM (calls) and in MBH (CSD). In this scenario, part or all of the *c-Fos* induction would be related to a differential self-stimulation of the females by their own behavior in response to the different auditory stimuli, very much like ovarian growth in ring doves exposed to male coos is in fact the result of a self-stimulation by females coos produced in response to the male behavior [46]. This reversed causal chain is however unlikely based on studies in white-throated sparrows showing that in general the vocalizations or the CSD produced by a given subject do not correlate with IEG expression in its own auditory areas [4, 12, 13, 16] although possible exceptions have been reported (correlation between bird's own thrills and *Zenk* expression in CMM: [12]) and there was even a negative correlation in one study between the number of CSD and *zenk* expression in POM [15]. Only additional experimental manipulations could discriminate between these alternative interpretations.

Independent of this interpretation, the present experiment establishes that sexy songs induce a higher metabolic activation of NCM (and possibly CMM) than non-sexy songs and that this activation of auditory regions likely correlates with the activation of the MBH which could represent the link to changes in behavior and in reproductive physiology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Sexy songs of male canaries increase calls and copulation solicitations in females
- Sex songs increase Fos expression in the secondary auditory areas of females
- Fos expression in CMM and in the medio-basal hypothalamus are positively correlated

Figure 1.

Examples of the auditory stimuli that were broadcasted to female canaries during experiments 1 and 2. Panel A illustrates sexy songs (SS) and panel B illustrates non-sexy songs (NS). The figure also illustrates the different components of each song. See text for additional explanations.

Figure 2.

Behavioral and morphological results (Means $\pm$ SEM) from the three experimental groups involved in Experiment 1. Data include the number of calls and of copulation solicitation displays produced by the females (top row), the ovary and oviduct weights at sacrifice (middle row) and the volume of the 4 largest ovarian follicles (bottom row). All data were analyzed by one way ANOVAs that were followed when appropriate by Newman-Keuls post-hoc tests whose results (see text for results).

Figure 3.

Mean ($\pm$ SEM) numbers of Fos-ir cells per mm² counted in 9 brain areas of the three experimental groups during experiment 1. Data for each brain region were analyzed by one way ANOVAs (see text for results) that were followed when appropriate by Newman-Keuls post-hoc tests whose results are indicated above the bars as follows: * = $p < 0.05$ vs WN, # = $p < 0.05$ vs NS. MLd: dorsolateral nucleus of the mesencephalon, OV: nucleus ovoidalis, CMM: caudomedial mesopallium, NCM: caudomedial nidopallium, HVC: used as a proper name, previously High Vocal Center, see [29], POM: medial preoptic nucleus, dPOM: area immediately dorsal to the POM, MBH: medio-basal hypothalamus, SPM/ medial spiriform nucleus.

Figure 4.

Representative photomicrographs illustrating Fos-ir cells for the two secondary auditory area, the caudomedial mesopallium (CMM) and the caudomedial nidopallium (NCM) in the three experimental groups that had been exposed to white noise (WN), non-sexy songs (NS) or sexy songs (SS). The magnification bar is equal to 200 μ m and applies to all panels.

Figure 5.

Significant correlations observed during experiment 1 between *c-Fos* expression in different brain areas and behavior. Correlations were calculated by the non parametric Spearman rank correlation coefficient but the figure also shows the significant regression lines calculated with parametric procedures. Data points from the different experimental groups are represented as follows: WN: open circle, NS: star, SS: closed circle.