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Sex differences and similarities in the neural circuit regulating song and other reproductive behaviors in songbirds

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Abstract

In the 1970s, Nottebohm and Arnold reported marked male-biased sex differences in the volume of three song control nuclei in songbirds. Subsequently a series of studies on several songbird species suggested that there is a positive correlation between the degree to which there is a sex difference in the volume of these song control nuclei and in song behavior. This correlation has been questioned in recent years. Furthermore, it has become clear that the song circuit is fully integrated into a more comprehensive neural circuit that regulates multiple courtship and reproductive behaviors including song. Sex differences in songbirds should be evaluated in the context of the full complement of behaviors produced by both sexes in relation to reproduction and based on the entire circuit in order to understand the functional significance of variation between males and females in brain and behavior. Variation in brain and behavior exhibited among living songbird species provides an excellent opportunity to understand the functional significance of sex differences related to social behaviors.

Keywords

HVC (abbreviation used as the common name); Area X of the medial striatum; Robust nucleus of the arcopallium (RA); Canaries; Testosterone; Steroid hormones; Song control system

1. Introduction: Historical Perspective on Sex Differences in Brain Areas Controlling Song

One of the most pathbreaking discoveries in the history of the study of sex differences in the brain occurred in 1976 when Fernando Nottebohm and Art Arnold reported dramatic differences in the volume of key forebrain structures that are part of the avian song control system (Nottebohm and Arnold, 1976) in zebra finches (*Taeniopygia guttata*) and canaries (*Serinus canaria*; Figure 1). This system had only recently been discovered by Nottebohm

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based on studies in canaries (Nottebohm et al., 1976). It was clear even then that the song system represented a clear example of a brain adaptive specialization (*sensu* Krebs, 1990) related to vocal learning and production (Nottebohm, 1980a). A neural specialization is a neural structure that can be clearly linked to an adaptive functional outcome (Krebs, 1990). What became clear in the 1970s and 1980s is that songbirds (members of the suborder passeri or oscines of the order passeriformes) had a well-defined set of brain structures that could be specifically linked to the learning and production of song and that other avian orders and even non-oscine members of the songbird order did not seem to have these specializations (Kroodsma and Konishi, 1991; Mello and Jarvis, 2008). It later became clear that other avian taxa that have evolved the ability to learn their vocalizations exhibit similar specializations (Mello and Jarvis, 2008).

After the song system was discovered, a series of studies based on the measurement of of key forebrain nuclei in the song system revealed several positive correlations among songbird species between variation in song behavior and the volume of these forebrain regions (reviewed in Nottebohm, 1980a). Within males, song repertoire size seemed to vary with nucleus volume, brain nucleus volume changed seasonally and correlated to some extent with seasonal changes in song behavior and finally as already mentioned sex differences were discovered in brain nucleus volume (Nottebohm, 1980a, 1981). These sex differences in volume seemed especially compelling over time given the variety of correlations between song nucleus volume and song behavior that had been described.

Previous to work on the song system, the search for neural sex differences in vertebrate species had focused on relatively subtle differences that required detailed histology studies and careful quantification. For example, the relative distribution of synapses on dendritic shafts and spines in the preoptic area was found to be different in male and female rats (Raisman and Field, 1971). After the discovery of these essentially qualitative sex differences in the zebra finch brain (Nottebohm and Arnold, 1976), studies of neural tissue led to the discovery of a sexually dimorphic nucleus of the preoptic area in rats (Arnold and Gorski, 1984; Gorski et al., 1978) and subsequently in other mammalian species including humans (Allen et al., 1989; Swaab and Fliers, 1985; Tobet and Fox, 1992).

Following the initial discovery in 1976, a series of studies were conducted on a variety of avian species that suggested that species variation in the degree to which there was a sex difference in song behavior was positively correlated with species variation in the degree to which there was a sex difference in the volume of the song control nuclei (Arnold et al., 1986; Brenowitz et al., 1985). Of the over 10,000 species of extant birds on the planet nearly 50% are songbirds (Jarvis et al., 2014). These songbirds have evolved to live in a wide variety of ecological niches from the tropics to the arctic and the song behavior produced by males and females varies greatly. For example, in relatively well-studied temperate zone birds many species are known to breed seasonally and migrate to tropical areas in winter (Ball, 1999; Rappole, 1995; Wingfield and Farner, 1993). This life history pattern is associated with males returning to the breeding grounds first and establishing territories with song and also in many species using song to attract females (Catchpole and Slater, 2008). In the tropics many species are sedentary and though they breed periodically they do not breed as synchronously at a given time in the season and the breeding period can be very long

(Stutchbury and Morton, 2000). In these species, males and females may defend a territory together and both sexes may sing in some cases producing complex duets that involve the close coordination of song types (Farabaugh, 1982). This diversity in the roles played by the sexes and in the patterns of song behavior among males and females provided a very rich opportunity for comparative studies.

2. What do we know today about the function of sex differences in volume among songbird species?

Species variation in the magnitude of sex differences in brain and behavior were apparent in the initial publication (Nottebohm and Arnold, 1976): in zebra finches where females do not sing at all the degree of differences in the volume of key forebrain nuclei such as HVC (acronym used as the proper name), RA (robust nucleus of the arcopallium) and area X of the medial striatum is more extreme than in canaries (Figure 1). Subsequently, studies were performed on a wider range of songbird species including those with nearly equal song behaviors in both sexes such as duetting wren species from central America (Arnold et al., 1986; Brenowitz and Arnold, 1988; Brenowitz et al., 1985) and those with an intermediate degrees of sex differences in brain and behavior such as European starlings (*Sturnus vulgaris*; (Bernard et al., 1993); Figure 2).

Analyses conducted in the late 1990s and into the 2000s (Ball and MacDougall-Shackleton, 2001; Ball et al., 2008; Brenowitz, 1997; MacDougall-Shackleton and Ball, 1999) indicated that, sex differences in the volume of song nuclei and other cellular measures were positively correlated with measures of song behavior across species (Figure 3A–B).

These positive correlations were not perfect but were statistically significant (MacDougall-Shackleton and Ball, 1999). This study by MacDougall-Shackelton and Ball (1999) controlled for phylogenetic relationships but suffered from a relatively few number of species that have been investigated. New genomic-based classifications of birds provide new opportunities for comprehensive comparisons (Brusatte et al., 2015). However it is still relatively rare to comprehensively study sex differences in brain and vocal behavior in a comparative context. Studies of the song system and song behavior have emerged as one of the few examples where easily measurable neural sex differences appeared to have a clear function. However, there were always concerns raised about how robust the correlation actually was (e.g., Gahr et al., 1998). Measures of brain nucleus volume though easy to collect are obviously a rather crude measure of brain function: the cellular basis of variation in volume is not always clear and the cellular changes responsible for a change in nucleus volume vary among the song nuclei (Smith et al., 1997; Tramontin et al., 1998). These volumes may also depend on the markers used to delineate the nucleus boundaries (Gahr, 1997) with some sex differences being observed only with specific neurochemical markers. For example, the larger volume of the nucleus interface in males compared to females can only be reliably detected in sections stained by immunohistochemistry for the vasoactive intestinal peptide (VIP) or for methionine enkephalin (ENK) (Ball et al., 1995). However, many of the reported sex differences in the volume of HVC, RA and area X are consistently found based on a variety of different neurochemical markers (e.g., (Ball et al., 1995; Ball et al., 1994).

A variety of more recent studies have continued to measure song nucleus volume but have focused on species with interesting patterns of sex differences in song behavior. In these studies, it has become clear that there are exceptions to the hypothesized rule that sex differences in song behavior vary linearly with sex differences in song control nuclei volume (Ball, 2016; Hall et al., 2010). These exceptions have been reviewed in detail so we will only highlight a few examples here. Several species of wrens, including the Bay wrens (*Thryothorus nigricapillus*) and the Rufous-and-white wrens (*Thryothorus rufalbus*) have been shown to sing in duets where males and females essentially produce the same amount of vocalizations but nevertheless males have larger song control nuclei including HVC than females (Figure 3C–D) (Brenowitz and Arnold, 1988). Forest weavers (*Ploceus bicolor*) in Africa duet and produce a striking unison song. Despite this remarkable similarity in song, measures of song control nuclei volume and neuron number are 1.5 times larger in males than in females (Gahr et al., 2008)(Figure 3E). The authors point out that females express higher levels of synapsin-related proteins than males, which may have evolved as a compensation for the male biased volume and neuron number (Gahr et al., 2008). In many neotropical icterid species such as the Streak-backed oriole, females sing songs equally complex as male song and at very high rates (Hall et al., 2010). Again, there is a pattern of larger male-biased volumes in HVC and area X even though females have a significantly higher rate of song output (Figure 3F). The authors argue that a review of sex differences in the song system reveals an almost universal pattern of male-biased volumes suggesting that the song system may be involved more broadly in other behaviors than just song. Are there cell types or subset of cells specific to song that should be the focus of studies on the neural basis of sex differences in song? The effects of lesions and the observation of immediate early gene expression that are very specific to song in the song nuclei has led to a focus on the song system as playing a strong role primarily in song production, but is this the case? For example, it is known that HVC is also is involved in the perception of conspecific vs. heterospecific song (Brenowitz, 1991) in addition to its role in song production. The functional organization of HVC is complex indicating that motor as well as sensory motor cells types are important (Fee and Long, 2013; Raksin et al., 2012). In addition, the song control system or at least part of its components is implicated in the production of calls (Simpson and Vicario, 1990; Ter Maat et al., 2014). This additional function might thus obscure the correlations between sex differences in song production and in volume of the song control nuclei.

Males and females may therefore have cellular properties in common in select song nuclei that may relate more to perception than production. For example, electrophysiological studies of duetting species do exhibit the value of asking more precise questions about how males and females perceive and process songs. In songbird species such as the zebra finch where only males sing it was discovered that several nuclei in the song system exhibit cells that only fire in response to the bird’s own song (Margoliash and Fortune, 1992; Reviewed in Prather and Mooney, 2008). Other conspecific songs or reverse songs do not elicit responses (e.g., Doupe and Konishi, 1991).

One obvious question to ask of any duetting species is whether HVC contains cells that are tuned to the song types that males or females produce or to the joint song or both. Plain-tailed wrens (*Thryothorus euophrys*) in Ecuador produce remarkable duets (Mann et al., 2006). Recordings made from neurons in HVC of male and female wrens provided a clear answer. Neurons were tuned to the jointly produce duetting song as compared to the male portion or female portion of the song (Fortune et al., 2011). These findings tell us something about how the duet is perceived and coded by males and females in a cooperative manner but how is the duet produced in a coordinate fashion? Recordings from free-living wild white-browed sparrow weavers (*Plocepasser mahali*) found a remarkable coordination between males and females in vocal premotor activity (Hoffmann et al., 2019). Neural activity during singing in nucleus HVC is in part premotor as has been established in many songbird species but it also integrates auditory inputs and therefore can be considered as a complex association area. In the sparrow weavers auditory signals produced by the duet partner alter the temporal parameters of activity in HVC in the duet-initiating bird. This mechanism allows them to alternate their vocalizations (Hoffmann et al., 2019). Thus, both the coding of the duet song and the ability to produce require a remarkable integration of auditory information between the two partners. These electrophysiological studies illustrate how studies of physiology at the cell level can elucidate how females and males can work in union to produce communicative signals.

Studies of corvids (crows, jays, ravens and magpies, etc.), a songbird family with over 120 species, illustrate very clearly the challenge of elucidating relationships between brain variation and variation in vocal behavior. Corvids are well known for their overall high level of many aspects of cognition such as superb memories and outstanding problem solving abilities (Clayton and Emery, 2005). Many species exhibit complex vocal behaviors so one might think that, with their outstanding memory capability, they might have the most complex songs in the songbird order. However, in many corvid species it is hard to define what is song as compared to calls (Clayton and Emery, 2005). It many species it appears that song-like vocalizations are produced by males and female and focus more on promoting social interactions in an intra-specific social bonding context rather than in territorial defense or mate attraction (Brown, 1985; Wang et al., 2009). Despite the lack of a clearly delineated song in some species, in the few corvid species that have been studied there is clear evidence for the presence of a neural song control system similar to what is observed in other oscine species (e.g., (Deng et al., 2001; Sen et al., 2019; Zeng et al., 2007) including the appropriate connections among the nuclei (e.g., (Deng et al., 2001; Wang et al., 2009). In some cases such as in the long billed crow (*Corvus macrorhynchos*) prominent sex differences in the volume of the key forebrain song control nuclei have been described though it is not clear which vocalizations that are being labeled as song function in a way similar to traditional song (Wang et al., 2009). Australian magpies (*Gymnorhina tibicem*) have multiple males and females who breed together communally and have a complex vocal repertoire involving vocalizations that are parts of communal displays produced by both males and females to defend their territory and promote group cohesion (Brown and Farabaugh, 1991). Both sexes have well defined song control systems that do not appear markedly different at least in volume between males and females (Deng et al., 2001).

Curiously, it was reported that a major projection among song nuclei (HVC to X) is missing in this species but that will require replication.

The message from this consideration of corvid species is that solving the puzzle of the exact function of sex differences in the vocal control system will benefit from species such as these corvids that challenge traditional views of song function. In a similar vein what can we gain from studies of other avian orders that learn complex vocalizations and have neural specializations mediating them like songbirds? In the order psittaciformes (parrots, parakeets, macaws, etc.) males and females in many species are well known to produce complex vocalization that are modified throughout adulthood. A neural circuit has been identified that mediates these vocalizations in parrots. This circuit bears many similarities to the oscine song system though there are clear distinctions (Ball, 1994; Jarvis and Mello, 2000; Striedter, 1994). Male biased sex differences in volume in some of these key forebrain nuclei have been identified and hypothesized as being linked to enhanced vocal plasticity in males as compared to females (Brauth et al., 2005). The case of looking at budgerigars is even more interesting in that recent molecular analyses of avian phylogeny suggest that the song system of psittaciforms and oscine songbirds is based on a derivation from a common ancestor rather than a case of convergent evolution (Brusatte et al., 2015). Studying homologous systems that both exhibit sex differences in brain and vocal behavior is a future opportunity for additional insights. Finally, hummingbirds are an avian order that exhibits vocal learning but in this case this trait seems to have evolved independently (Brusatte et al., 2015). However, this taxon exhibits neural specializations associated with vocal learning and projections between these nuclei that appears to be analogous to what is seen in songbirds and parrots (Brusatte et al., 2015). Sex differences in brain and behavior related to vocalizations in this taxon have not been investigated in detail.

3. The avian song system is part of a larger neural circuit regulating appetitive and consummatory sexual behaviors in male and female songbirds.

The set of interconnected brain nuclei initially identified in the lab of Fernando Nottebohm that control song learning and song production, usually referred to as the song control system, has in many cases been studied almost as a self-contained system considered separately from the rest of the brain (Brainard and Doupe, 2002; Nottebohm et al., 1976). This network is made of two separate pathways that have specific functions. The vocal motor pathway connects HVC (previously high vocal center, now used as a proper name) directly to the premotor nucleus RA (robustus nucleus of the arcopallium) and then directly to the nucleus of the 12th (hypoglossus) nerve, tracheosyngeal part (nXIIts). This pathway is critical for song production and any lesion to one of its nuclei will essentially block all learned vocal production, which is primarily the song. The second pathway called the anterior forebrain pathway (AFP) also connects HVC to RA but through an indirect route via Area X from the basal ganglia, the nucleus dorsolateralis, pars medialis of the thalamus (DLM) and LMAN (lateral magnocellular nucleus of the anterior nidopallium). This pathway is critical for the auditory feedback essential for song learning and maintenance of song stability.

However, song is a hormonally mediated courtship behavior (Ball, 1999; Schlinger and Brenowitz, 2002). One of its key functions is in the context of reproduction and its occurrence is closely related to reproduction. This link to reproduction takes two forms one is related to mate attraction and mate choice and the other is related to territory defense. Both of these functions are closely related to successful reproduction *per se* and could thus be linked to sexual rather than natural selection. Therefore, singing, especially as it is related to mate choice can be considered as a part of the appetitive phase of sexual behavior and it could therefore be expected to be controlled at least in part by the circuit that regulates sexual behavior in males and in females (e.g., Ball and Balthazart, 2004). The preoptic area for example has in a wide range of vertebrate species been linked to the activation of male appetitive and consummatory sexual behavior (Balthazart and Ball, 2007). In this section we will discuss how it is becoming increasingly clear that the avian song control circuit is part of the much larger circuit regulating appetitive and consummatory reproductive behaviors in general. It is also important to note that this larger circuit is present and plays a critical function in both males and females (e.g., Perkes et al., 2019).

Sexual behavior is well known to include some displays and behavioral patterns that are sex-typical. Therefore, portions of the circuit are expected to exhibit differences between the sexes but it is important to note that many aspects of this circuit are common in males and females. We will focus on work in canaries, much of it coming from our labs, to illustrate how studies of sex differences in song behavior will benefit from a consideration of this integrated circuit regulating appetitive and consummatory behavior in males and females. It is also important to note that the regulation of the perception of signals related to mating and the processing of these signals so that they may facilitate an appropriate response is a fundamental component of this circuit (e.g., Perkes et al., 2019).

Several lines of evidence have actually linked song and the song control circuit to the preoptic area. It has first been shown that the medial preoptic nucleus (POM) has to be intact for males to produce courtship song. Lesions to the POM that are well known to suppress sexual behaviors in males also markedly inhibit courtship song in European starlings (*Sturnus vulgaris*) (Alger et al., 2009; Alger and Riters, 2006; Riters and Ball, 1999). Additionally, our recent work in canaries has shown that implantation of testosterone into the POM of castrated males will stimulate song production even if the songs that are produced have a very low quality and low amplitude (Alward et al., 2013; Alward et al., 2016c)(see also next section).

It is well established in other vertebrate species including non-songbird species such as Japanese quail that they exhibit male-biased sex differences in the volume of nuclei such as the POM (Ball and Balthazart, 2010a; Panzica et al., 1996). Sex differences outside the song system have not been studied in detail in songbirds but by comparing data from two independent experiments analyzing male and female canaries of the same breed studied in similar conditions we showed that the POM volume as defined by the dense cluster of aromatase-immunoreactive neurons is larger in male than in female canaries after similar treatment with exogenous testosterone (Shevchouk et al., 2019). As stated previously, similar differences in the volumes of other structures implicated in the control of reproductive behaviors are likely to exist in songbirds based on the abundant literature on this topic in a variety of mammalian and avian species (for review see: Tobet and Fox, 1992), but no data are available at present to evaluate this possibility in songbirds:

The neuroanatomical and physiological basis underlying the ability of testosterone to act in the POM to enhance singing activity is however not fully characterized at this stage. However, the most likely pathway supporting this action of preoptic testosterone on singing behavior involves projections from POM to the periaqueductal gray (PAG) and then from PAG to the song control nuclei (see Figure 4).

Functional studies indeed indicate that neurons in PAG are activated when birds sing, as assessed by an increased expression of immediate early genes (Maney and Ball, 2003) and gene expression in this area is decreased in starlings with lesions of the POM (Alger et al., 2009). In addition, tract-tracing studies have demonstrated the existence of bidirectional connections between POM and PAG (Riters and Alger, 2004) and PAG then sends dopaminergic projections to the song control nuclei HVC and RA (Appeltants et al., 2000; Appeltants et al., 2002). Finally, recent data demonstrate that inactivating PAG by infusions of the GABA agonist muscimol temporarily inhibits song production (Haakenson et al., 2019). Together these data indicate that a re-consideration of the song control system in a broader perspective is in order. The song control system is really part of the extended brain circuitry controlling reproductive behavior, not only singing activity.

This concept is well illustrated by recent work on female canaries (Wild and Botelho, 2015). In male canaries it is well known that midbrain structures such as the dorsomedial nucleus (DM) of the intercollicular complex (ICo) send projections to a nucleus such as nXIIts in the hindbrain that in turn directly innervate the muscles of the vocal production organ, the syrinx (Wild, 1994, 1997; Wild et al., 1997)(see Figure 5). In parallel projections from the midbrain innervate nucleus RAm in the hindbrain that in turn project to cells in the spinal cord that innervate respiratory muscles needed to coordinate breathing with song production (Wild, 1994, 1997; Wild et al., 2009)(see figure 5). In addition to these projections female canaries also have projections from RAm in the hindbrain that in turn project to cells in the spinal cord that innervate muscles needed for the copulatory solicitation display (Wild et al., 2009) (see figure 5) which is a key behavioral element for successful consummatory sexual behavior (i.e. copulation) in female canaries (Amy et al., 2015; Vallet and Kreutzer, 1995) and other songbirds (e.g., Searcy and Marler, 1981). Thus, it is now established that structures involved in the motivation to engage in sexual behavior (i.e. the POM) are also involved in the motivation to sing and outputs involved in the motor projection of song are also involved in the motor production of copulation solicitation displays in females. A missing piece is whether outputs related to song production in males are also involved in stimulating male-typical copulatory behaviors in addition to female copulatory behaviors. Although we are advocating for investigating this entire circuit as an integrated whole, the few previous studies that have investigated aspects of the reproductive behavior circuit and the song circuit simultaneously by assessing gene expression in different seasonal states have revealed distinct patterns of gene expression specific to HVC and the POM for example (Stevenson et al., 2012). This is not surprising given the specific functions of these brain areas but it does stress that a series of nodes with separate functions are working in concert for the production of an organized, multifaceted behavioral response.

4. This comprehensive reproductive behavior circuit is hormonally modulated at many different nodes.

An important dimension of this comprehensive circuit controlling reproductive behavior is that it is modulated in significant ways by steroid hormones. Successful reproduction requires the coordination of a suite of traits related to morphology, physiology and behavior and steroids provide a wide-ranging comprehensive signal that can coordinate this multitude of traits (McEwen and Milner, 2017; McKenna, 2015). In this section we will review the multifaceted ways that steroids can act on this sexual behavior circuit to regulate a broad range of reproductive behaviors in birds (see also Ball et al., 2020).

One critical idea that underlies the analysis of steroid hormones on a circuit is to reduce the continuous flow of behavior into components and try to identify whether they are differentially regulated by steroids. We have applied this approach to the analysis of appetitive and consummatory sexual behavior in Japanese quail and in songbirds (Ball and Balthazart, 2010b; Ball et al., 2020; Balthazart and Ball, 2007; Balthazart et al., 2009). As far as the study of the hormonal regulation of birdsong goes, one of the most important insights was that the many effects of testosterone and its metabolites on song behavior and related sexual behaviors could be parsed out by analyzing steroid action in the many brain sites that express receptors for androgens and estrogens (e.g., Arnold, 1981; Ball et al., 2002). One of the first ideas tested was that motivational effects of steroids on sexual behavior could be separated out from effects on performance. We first developed this distinction related to the neuroendocrine control of these two aspects of sexual behavior based on studies of Japanese quail (e.g., Balthazart et al., 1998; Cornil et al., 2018) and then we extended this analysis to birdsong.

In the case of birdsong, the idea that singing activity might be functionally disconnected from the control of song quality *sensu stricto* was suggested in some of the initial studies of the neural control of song. In particular after male canaries receive HVC lesions they still try to sing: they go through all the beak and respiratory muscles movements normally associated with song but fail to produce any audible songs (Nottebohm et al., 1976). There is now converging evidence coming from lesion, electrophysiological, tract-tracing and gene expression studies indicating that HVC is essential for song production (e.g., Fee and Scharff, 2010; Mooney, 2009; Prather and Mooney, 2008) but this initial lesion study from the Nottebohm laboratory demonstrated that the motivation to sing must be controlled elsewhere in the brain because canaries with HVC lesions clearly try to sing based on their production of other song related movements. Therefore, as explained in the previous section, singing functions at least in part as a courtship behavior and our attention then turned to the preoptic area (see Figure 6).

As previously observed in quail (Charlier et al., 2008), systemic treatment with testosterone was shown to rapidly increase the volume of the medial preoptic nucleus (POM) in both male and female canaries even before the steroid had any effect on the volume of HVC and on the production of song (Shevchouk et al., 2019; Shevchouk et al., 2017). We then tested the idea that testosterone controls singing motivation via its action in the POM specifically by implanting a cannula filled with crystalline testosterone directly into the POM of castrated male canaries. These implants activated within 7 days an intense singing activity that was absent in castrated males that had received an empty implant or a testosterone-filled implant that had missed its target and ended-up elsewhere in the brain (Alward et al., 2013). Another group of control castrates who had received subcutaneous Silastic implants filled with testosterone also started displaying intense singing activity but these birds started singing more frequently after 3 days of treatment and their songs were louder and less variable in structure than the birds that received testosterone treatment only in the POM.

It was therefore concluded that testosterone action was indeed sufficient to activate the motivation to sing, but it was also clear that the steroid had to act elsewhere in the brain to enhance the activation of other features of song that are observed in fully mature male canaries experiencing high concentrations of testosterone in the blood during the reproductive period. In a second series of experiments, castrated male canaries were implanted again with testosterone in the POM but, in addition, some of them received a second testosterone implant in HVC (Alward et al., 2016c). As we did in the previous experiment, another group of castrated males was systemically treated with testosterone via subcutaneous Silastic implants. Replicating the previous experiment, the birds with testosterone in POM started singing actively after a few days similar to birds with systemic testosterone. Again, birds with testosterone in POM sang only poorly structured, more variable and less powerful (lower energy) songs than birds with systemic testosterone. However, the addition of testosterone in HVC corrected many of these deficits in song quality, notably with the exception of song energy. Our conclusion is that birds with testosterone implanted in both POM and HVC sang songs similar to birds with systemic testosterone except that their songs had a lower energy.

We also investigated the actions of testosterone directly at the level of the syringeal muscles that are known to express androgen receptors (Lieberburg and Nottebohm, 1979; Luine et al., 1980; Veney and Wade, 2004). Theories of sexual selection predicted that testosterone action on peripheral effector organs might be especially important in regulating traits related to male-male competition or female choice (Folstad and Karter, 1992; Hillgarth and Wingfield, 1997; Witschi, 1961). We tested the role of testosterone on syringeal function by systemically treating sexually mature male canaries with bicalutamide, an androgen receptor antagonist that does not cross the blood-brain barrier (Fuxjager et al., 2014; Fuxjager et al., 2013). This treatment did not affect the number of songs produced but it reduced their complexity and reduced the performance of rapidly produced special trills (Alward et al., 2016b) that are known to be especially attractive to females (Catchpole and Slater, 2008; Vallet et al., 1998; Vallet and Kreutzer, 1995). However, the song energy was not decreased in bicalutamide-treated males. Testosterone effects on song energy may well be regulated by testosterone action in nucleus RAm that is known to express androgen receptors (Gahr and Wild, 1997) (and as reviewed previously project to cells in the spinal cord that innervate respiratory muscles needed to coordinate breathing with song production (Wild, 1994, 1997; Wild et al., 2009)and could thus mediate effects on amplitude.

In a final set of experiments, we tried to determine whether testosterone has neuroanatomically specific effects on song structure when acting in HVC versus RA.

Because studies in another songbird species had indicated that testosterone implantation in or near HVC or RA does not stimulate singing by itself (Brenowitz and Lent, 2002) and because combining such implants with another implant in POM is technically challenging, we addressed this question by implanting the androgen receptor antagonist flutamide near these two song control nuclei in sexually mature males spontaneously exhibiting a high singing activity (Alward et al., 2017). Flutamide bilaterally implanted near HVC induced marked changes in the use of various syllable types and in the sequences in which different syllables were used but had no effect on the syllable or trill bandwidth stereotypy. In contrast, birds with flutamide implanted near RA on both sides of the brain displayed an increased variability in syllable and trill bandwidth but showed no change in syllable usage.

Taken together, all these results clearly indicate that testosterone regulates multiple aspects of song production in a non-redundant manner by acting in different brain and peripheral sites. Testosterone is also acting at other sites in this reproductive behavior circuit to facilitate and coordinate related behaviors needed for successful reproduction including copulation. These effects of testosterone are usually assumed to be mediated by long-term changes in transcription resulting from the binding to DNA of occupied androgen receptors. Part of the actions of testosterone on singing and on neural plasticity in the song control system are however also known to be produced by estrogenic metabolites of testosterone derived from its local aromatization (Fusani and Gahr, 2000, 2006; Fusani et al., 2003), although this obviously does not concern those aspects of song that are blocked by androgen receptors antagonists, as just described. Estrogens like other steroid hormones, can act via two broad modes of action especially in the brain: they can act in a relatively slow manner via their binding to nuclear estrogen receptors and induction of transcriptional changes but they can also act in a relatively fast manner (seconds to minutes) through signaling initiated at the cell membrane (Balthazart and Ball, 2006; Cornil et al., 2015). Interestingly, this last mode of action has been particularly well characterized in the auditory system of songbirds where hearing conspecific songs induces a rapid release and probably synthesis of estrogens that rapidly modifies auditory sensitivity and selectivity, focusing the subject on features of the conspecific song (Remage-Healey et al., 2010; Remage-Healey et al., 2012; Remage-Healey et al., 2008). Rapid changes in auditory processing, initially identified in zebra finches by electrophysiological methods and confirmed by behavioral outputs, were recently confirmed in starlings by functional magnetic resonance imaging (fMRI). Changes in brain activity in response to conspecific songs were detected within 10–45 min after an acute inhibition of brain aromatase activity induced by the injection of an aromatase inhibitor. These changes were in starlings, as is the case in zebra finches, specifically located in pallial areas that are now considered homologous to the secondary auditory cortex (De Groof et al., 2017).

We thus considered whether rapid changes in estrogen availability would also affect song production. Male canaries normally sing within minutes following light onset at the beginning of the day. Some males were injected within 2–5 min after lights on with the potent aromatase inhibitor Fadrozole or on other days with the vehicle and their singing activity was then continuously recorded for 4 hours in the morning, another 3 hours in the afternoon and again for 2.5 hours on the next day (Alward et al., 2016a). The experiment was repeated 3 days later but treatments were reversed this time so that birds could be used as their own control. In birds treated with Fadrozole, we observed a significant increase of the latency to resume singing and a decrease in the time spent singing. When these Fadrozole-treated birds sang, they sang shorter songs with more variable bandwidth coefficient of variation than when they had been injected with the vehicle. All these effects had vanished on the next day. This study thus indicates that estrogens might also act in a rapid fashion on singing motivation and on song structure but the anatomical localization of these effects remains unknown at present. However, effects on song motivation are likely mediated in the preoptic area that is known to express high levels of aromatase in songbirds (Balthazart et al., 1996; Foidart et al., 1998; Vockel et al., 1990) as is the case in other vertebrate species as well (e.g., Roselli, 2013).

5. There are similarities and differences in hormone action in this circuit in males and females.

We have just reviewed the fact that the integrated circuit that regulates courtship and copulatory behavior in male and female songbirds has many nodes that are clearly modulated by steroid hormones. One of the well-known hallmarks of neural systems regulated by steroids such as the androgens and estrogens is that there are sex differences in the action of the steroids (McEwen and Milner, 2017). Even though many of the basics of the circuit may be the same the ability of steroids to act in particular brain areas and modulate the circuit can vary (Marrocco and McEwen, 2016). These sex differences in steroid action can be tied to sex differences in plasma concentrations of the key signaling hormone but are more often tied to differences in the properties of proteins related to steroid hormone action as expressed in the target neural tissue (e.g., Ball and Balthazart, 2008).

As reviewed previously the song control circuit is a target of androgen and estrogen action. One early discovery about the properties of neural responses to steroids was that there are substantial differences among species in the adult actions of steroids (Arnold and Breedlove, 1985). For example, in zebra finches, which exhibit some of the most extreme sex differences in the song system based on volume measurements, the treatment in adulthood of females with concentrations of androgens in the male range does not induce male-typical singing or result in the song control system looking more male-like (Adkins-Regan et al., 1994; Arnold, 1997) (Balthazart et al., 2017). Manipulations early in ontogeny were required to reverse the development of these sex differences (Gurney and Konishi, 1980; Pohl-Apel, 1985; Simpson and Vicario, 1991a, b) presumably indicating organizational actions of steroids regulating these sex differences rather than activational actions in adulthood. In contrast in canaries it was known for many years that treating a female with testosterone resulted in her producing a more male-like song (Herrick and Harris, 1957; Leonard, 1939; Shoemaker, 1939). Furthermore, Nottebohm demonstrated that treatment with testosterone in adult female canaries in addition to stimulating more male-like song could result in a song system that was much also more male-like again based on volume measurement though not fully masculinized (Nottebohm, 1980b). However, more recent studies have systematically administered steroids to male and female canaries and have still not been able to reverse completely observed sex differences in brain and behavior (e.g., Madison et al., 2015). Testosterone action in the adult female HVC induces changes in the expression of a variety of genes related to the incorporation of new neurons such as BDNF (Dittrich et al., 2014; Rasika et al., 1999) and VEGF (Louissaint et al., 2002) but, to our knowledge, a quantitative comparison of the efficacy of testosterone in regulating these genes in the HVC of males and females has not been tested. Thus, female canaries exhibit a remarkable degree of adult neuroplasticity in response to adult steroid hormone treatment but adult steroid hormone action based on studies completed to date does not seem to be effective in producing a complete sex reversal of song and the volume and other attributes of song nuclei.

Findings like these in canaries and zebra finches suggest that sex differences in the response to androgens and estrogens in adulthood are the result of organizational actions of steroid hormones early in ontogeny to set up enduring sex differences in the response to steroid hormones in adulthood. Most studies of sexual differentiation of species-typical patterns of brain and behavior in songbirds have been conducted on zebra finches but there is in addition a substantial number of studies investigating the sex differences in copulatory behavior in other species (Balthazart et al., 2017). The display of sex-typical copulatory behavior is indeed sexually differentiated. Based on studies of Japanese quail it has been discovered that embryos of both sexes are initially bipotential in that, given proper endocrine conditions during development, they can engage as adults in male and female-typical behaviors. However females are demasculinzed by the action of their endogenous estrogen during a critical period that ends on embryonic day 12 in quail. This is in contrast to mammals such as rodents where estrogens act to masculinize males so that they will engage when adults in male-typical behaviors. This contrast between birds and mammals is associated with the fact that males are the homogametic sex in birds (ZZ) while females are the heterogametic (ZW) which is opposite from the pattern in mammals. The causal link between these two phenomena remains however unclear. Sex-typical copulatory behavior in zebra finches is probably differentiated in a similar manner (Balthazart et al., 2017): treatment of males with estrogens during the first week post hatching demasculinizes them and they fail to mount females as adults (Adkins-Regan and Ascenzi, 1987; Adkins-Regan et al., 1994). However treatment of females with an aromatase inhibitor changed their sexual partner preference (they spent more time with females instead of males than control females) but did not apparently block the demasculinization of their male-typical copulatory behavior and they very rarely displayed male-typical mounting behavior as adults (Adkins-Regan et al., 1996).

The song system though is another matter. Hormone manipulations early in ontogeny are usually not effective in reversing the sex differences in the song system and song behavior. Treating nestling zebra finches with estradiol does not demasculinize males but rather partially masculinizes the song system and song behavior of females (Gurney and Konishi, 1980; Pohl-Apel, 1985; Simpson and Vicario, 1991a, b). The source of estrogens for these effects in physiological conditions does not appear to be of gonadal origin since no consistent sex difference in circulating estradiol concentrations was reported (see (Balthazart and Ball, 1995) for review) and estrogens synthesized in the brain may be critical (Holloway and Clayton, 2001). Together these data suggested that the sexual differentiation of the song system is based on another mechanism (Arnold, 1997). It seemed indeed impossible to reconcile the facts that early estrogens treatment masculinizes the song system of females but demasculinizes male mounting behavior. In addition, multiple experiments failed to establish a female song control system in males by blocking estrogens action (with antiestrogens) or production (with aromatase inhibitors) and even very early treatments with aromatase inhibitors that induced genetic females to develop functional testicular tissue failed to masculinize their song control system (reviewed in Arnold, 1997).

The study of a gynandromorphic zebra finch that was male on one side and female on the other side initially pointed to the possibility the sex chromosome genes (males ZZ; females ZW) may play roles in the sexual differentiation of the song system (Agate et al., 2003). One recent hypothesis is that genes specific to the Z chromosome, contribute to sexual differentiation of the song system (e.g., Beach et al., 2016; Beach and Wade, 2015). Although we still do not have a definitive answer, it is clear that the origins of sex differences related to copulatory behavior and sex differences related to song are very distinct.

Detailed studies of the effects of the implantation of steroids in different brain nuclei related to sexual and courtship behaviors comparing actions in males and females have not been completed. However, in canaries, as reviewed previously, implantation of testosterone into the preoptic area (POA) of males clearly increases singing rate though the quality of song produced is not as good as what is produced when the birds receive a peripheral testosterone injection and experience testosterone throughout the body (Alward et al., 2013). Recent studies have also implanted testosterone into the POA of female canaries and have shown that as in males testosterone implants just in the POA increase the rate of singing though the songs are of short duration and poor quality (Vandries et al., 2019). Thus, the connection between the POA and the song control system that seems to underlie the hormonal regulation of the motivation to sing is present in males and in females. There is also evidence that naturally occurring female aggression in songbirds that is observed in a reproductive context similar to male aggression involves testosterone action and related endocrine mechanisms that are common in males and females to some degree (Rosvall, 2013). However, there are very few studies of the hormone regulation of female song via the investigation of direct actions in the female song control system. This is due in part to the lack of a good model species with substantial female song that will exhibit these behaviors in a laboratory setting. However, there has recently been an increased interest in female song as it has become clear that its occurrence is taxonomically widespread within songbirds and that it appears to be the ancestral pattern among songbirds (Odom et al., 2014). This movement to study female song may result in the identification of species amenable to such mechanistic studies in captivity. It is clear, though, that steroids act in specific nuclei in the comprehensive reproductive behavior circuit to activate female-typical behaviors involved in courtship and copulation. For example, stereotaxic implantation of estradiol into the preoptic–hypothalamic regions in ovariectomized female ring doves demonstrated that estradiol stimulates female-typical courtship behaviors when implanted in the avian homologue of the ventromedial nucleus of the hypothalamus (VMN). Implants more rostral in and around the POA or caudal were not effective (Gibson and Cheng, 1979). The female nest coo, a call involved in courtship with the male, could be elicited in ovariectomized doves after the implantation of estradiol in the midbrain nucleus intercollicularis (Cohen and Cheng, 1981, 1982).

Steroid hormones are also known to modulate selective responses to conspecific song in addition to their effects on song production. The most work has been conducted in female zebra finches and white-throated sparrows (reviewed in Maney and Pinaud, 2011). Identical experiments have not been performed in males although it is clearly established that neuroestrogens modulate the neural response to conspecific song in the secondary auditory areas (e.g., (Remage-Healey et al., 2010; Remage-Healey and Joshi, 2012). There is evidence that there are sex differences in the electrophysiological responses to conspecific song in the HVC of canaries (Del Negro and Edeline, 2001, 2002) but whether these differences are changed by adult testosterone treatment has not been investigated.

6. Conclusion

Studies of sex differences in the song circuit initially focused on a select group of interconnected forebrain nuclei in isolation from other structures in the circuit and from other parts of the brain needed to regulate song production especially when it is produced in the context of courtship and mating. It is now clear that the song circuit is a critical part of a comprehensive neural circuit that mediates appetitive and consummatory reproductive behaviors. Given that aspects of these behaviors are often sex-typical, presumably due to sexual selection in many cases, it is not surprising that attributes of this circuit differ in males and females. The investigation of sex differences in multiple parts of this circuit will put the question in the broader context of what roles males and females play for successful reproduction and may help us understand the function of patterns of sex differences in the song circuit. What is clear is that there is a basic circuit encompassing areas of the telencephalon, diencephalon, mesencephalon as well as the brain stem and the spinal cord that is common in many respects in male and female songbirds. Starting with this premise will be helpful to provide a focus on the functional significance of differences and similarities between the sexes related to more behaviors than just song per se. This notion of a common male and female circuit is very consistent with the hypothesis that female song in oscine songbirds is an ancestral feature and a critical question therefore is why female song has been dropped by many species, especially those in the temperate zone (Odom et al., 2014; Riebel, 2016). It also underscores how both signal production and perception should be studied in males and females (e.g., Ball et al., 2006) from a sex-typical perspective (Ball et al., 2006; Perkes et al., 2019). A new generation of studies that attacks sex differences equally from the male and female perspective could provide a model as to how investigate functional sex differences in brain and behavior relevant to a critical social behavior.

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Highlights

- The song control system has many attributes that exhibit male biased sex differences
- These anatomical differences correlate roughly with sex differences in song
- The song control system is part of a larger network controlling reproductive behavior
- Males and females share most features of this network
- There are sex differences in anatomy and physiology throughout this network
- Testosterone acts on brain sites in a sex specific manner to activate and modulate song

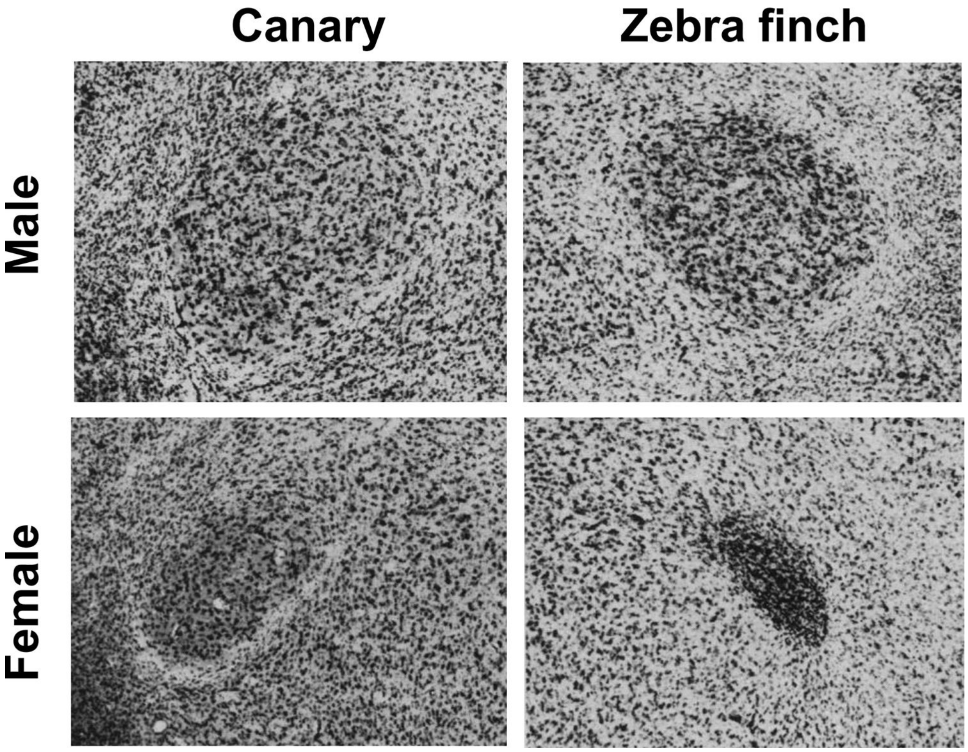

Figure 1.

Photomicrographs of Nissl-stained sections illustrating the larger sex difference in RA volumes observed in zebra finches as compared to canaries. Figure based on material in (Nottebohm and Arnold, 1976).

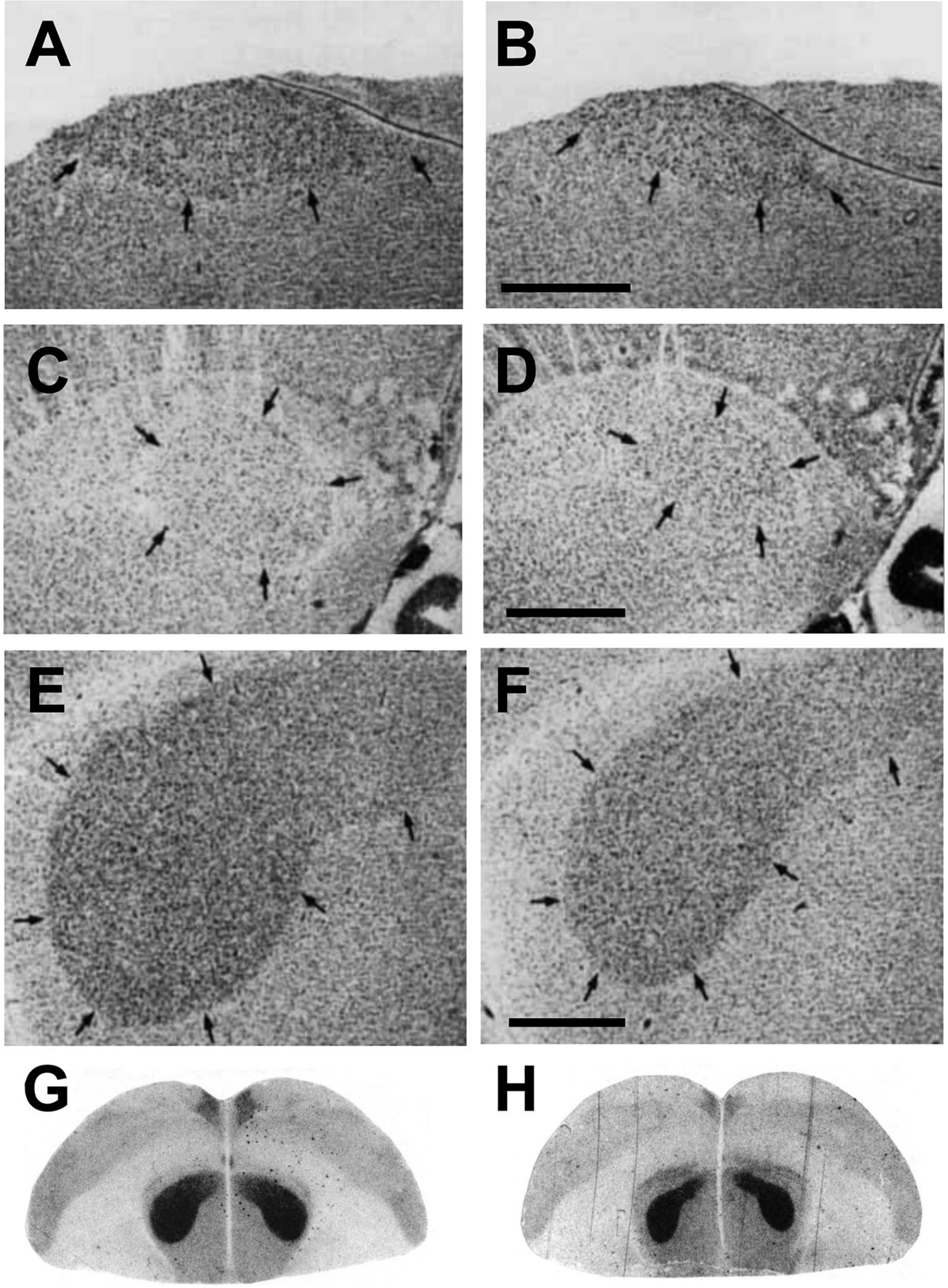

Figure 2.

Photomicrographs of Nissl-stained sections (A-F) and of autoradiograms based onin vitro labeling for alpha2-adrenergic receptors density (G-H) illustrating the sex differences in the volume of the song control nuclei in European starlings. All photomicrographs in the left columns are from males (A,C,E,G) and in the right column from females (B,D,F,H). The boundaries of HVC (A-B), RA (C-D) and Area X (E-F) in Nissl-stained sections are indicated by arrows. The larger Area X in males compared to females is confirmed by a larger volume based on the density of alpha2-adrenergic binding (G-H). Magnification bars= 1 mm. Figure based on material in (Bernard et al., 1993) and (Ball et al., 1993).

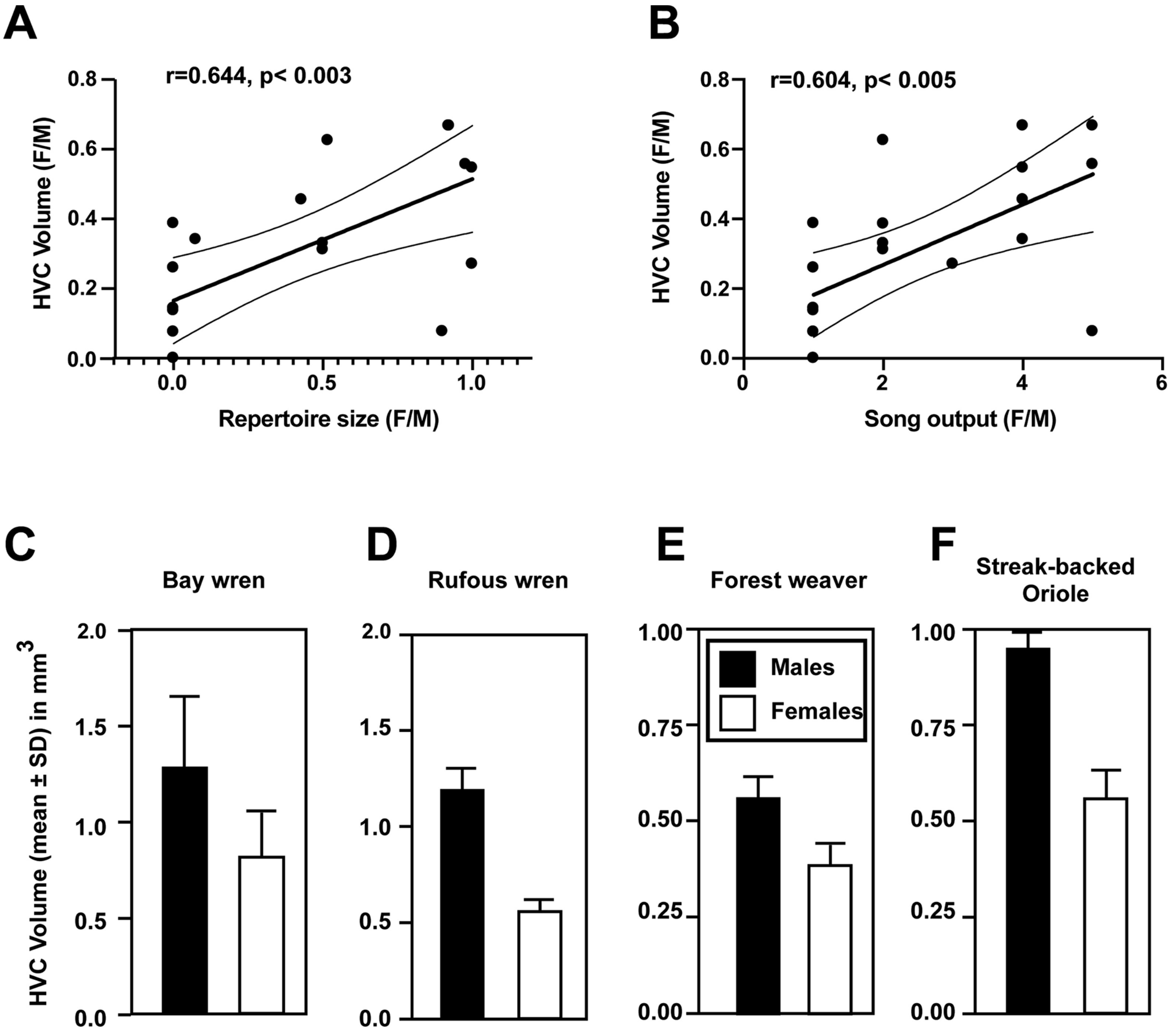

Figure 3.

Relationships between sex difference in singing behavior and sex differences in volume of the song control nucleus HVC. Panels A and B illustrate the relationship across multiple species belonging to different families of songbirds between the ratio of HVC volume in females versus males and the relative repertoire size (A) or the relative amount of songs produced (B) in the two sexes. Song output in B was qualitatively estimated as follows 1: females never sing, 2: females rarely sing, 3: females sing but less often than males, 4: females commonly sing and 5: females and males duet. Panel C to F illustrate the relative volume of HVC in males and females of 4 species in which either males and females are duetting and thus producing approximately similar amounts of songs of similar quality (C-E) or in which females sing more than males but songs have a similar quality in both sexes (F). Redrawn from data in (MacDougall-Shackleton and Ball, 1999),(Brenowitz and Arnold, 1988), (Gahr et al., 2008), (Hall et al., 2010)

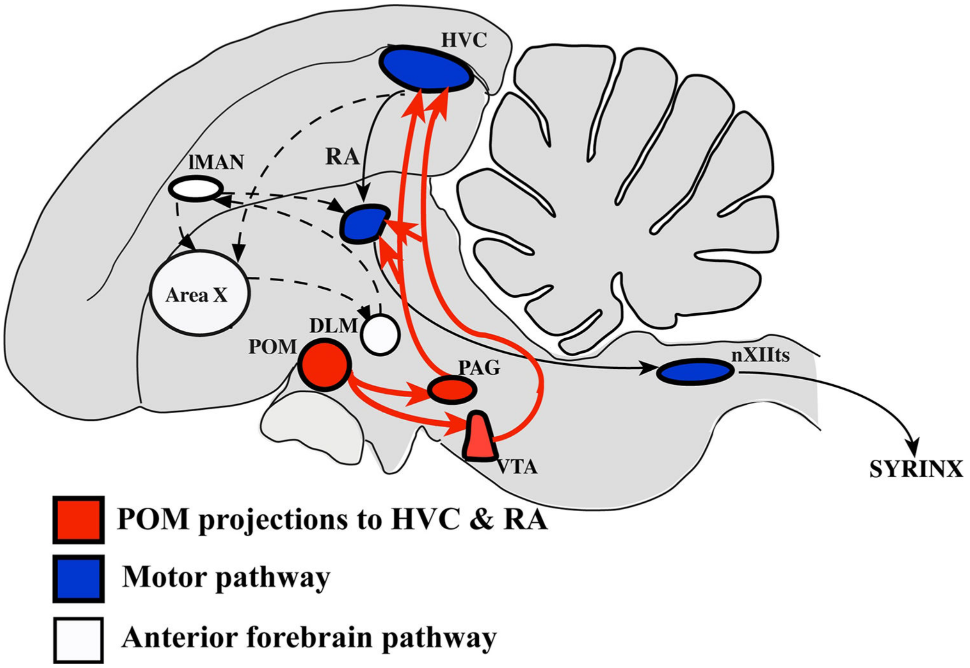

Figure 4.

Schematic representation of the song control system and its putative relationships with the medial preoptic nucleus POM via the periaqueductal gray (PAG) and the ventral tegmental area (VTA) illustrated by red arrows.

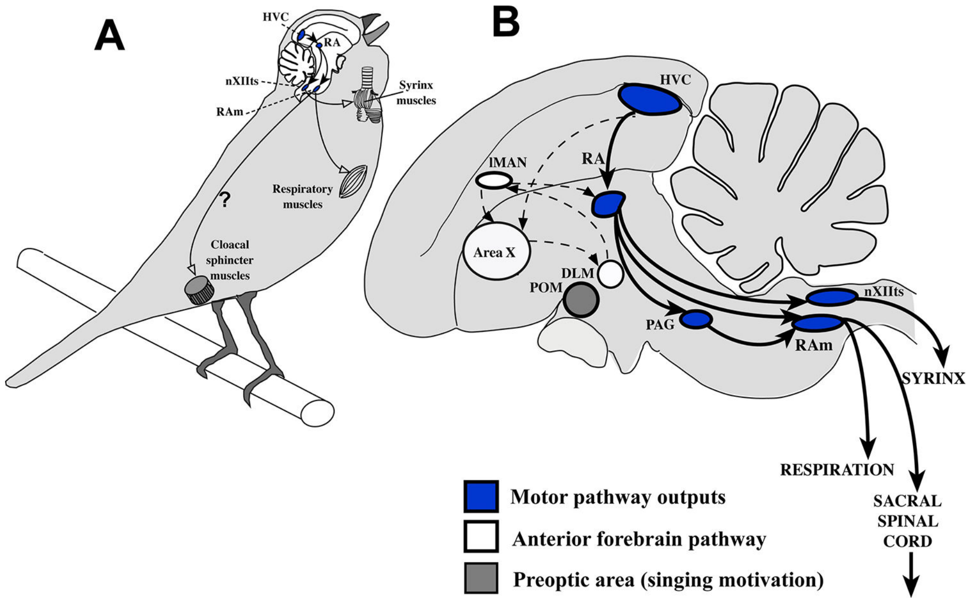

Figure 5.

Schematic representation of the song control system and its downstream projections to the syrinx (required for the production of song), the respiratory muscles (required for the synchronization of singing with respiration) and to the sacral spinal cord neurons that control cloacal contractions and also presumably some of the muscles implicated in the control of the copulatory solicitation display (female sexual receptivity). Whether a similar pathway controls male sexual behavior remains unexplored at this time. Panel A illustrates these connections in the entire body while panel B presents more detail of the implicated brain pathways.

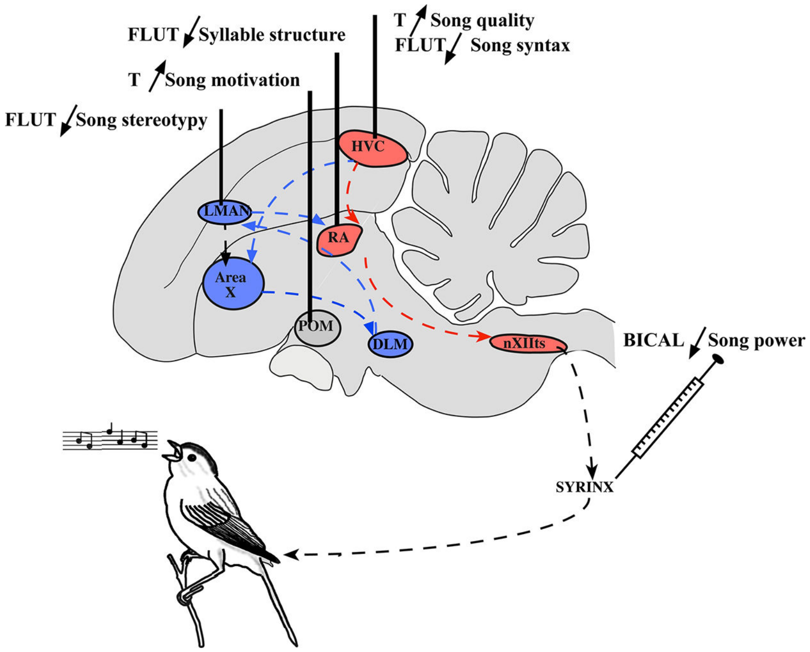

Figure 6.

Summary of the experiments investigating the action of testosterone at multiple brain sites in the context of the activation of singing behavior. Up and down arrows represent increases or decreases of the behavioral trait considered.