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Sexually-motivated song is predicted by androgen-and opioid-related gene expression in the medial preoptic nucleus of male European starlings (*Sturnus vulgaris*)

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Abstract

Across vertebrates, communication conveys information about an individual's motivational state, yet little is known about the neuroendocrine regulation of motivational aspects of communication. For seasonally breeding songbirds, increases in testosterone in spring stimulate high rates of sexually-motivated courtship song, though not all birds sing at high rates. It is generally assumed that testosterone or its metabolites act within the medial preoptic nucleus (POM) to stimulate the motivation to sing. In addition to androgen receptors (ARs) and testosterone, opioid neuropeptides in the POM influence sexually-motivated song production, and it has been proposed that testosterone may in part regulate song by modifying opioid systems. To gain insight into a possible role for androgen-opioid interactions in the regulation of communication we examined associations between sexually-motivated song and relative expression of ARs, mu opioid receptors (muORs), and preproenkephalin (PENK) in the POM (and other regions) of male European starlings using qPCR. Both AR and PENK expression in POM correlated positively with singing behavior, whereas muOR in POM correlated negatively with song. Furthermore, the ratio of PENK/muOR expression correlated negatively with AR expression in POM. Finally, in the ventral tegmental area (VTA), PENK expression correlated negatively with singing behavior. Results support the hypothesis that ARs may alter opioid gene expression in POM to fine-tune singing to reflect a male's motivational state. Data also suggest that bidirectional relationships may exist between opioids and ARs in POM and song, and additionally support a role for opioids in the VTA, independent of AR activity in this region.

Keywords

Androgens; Songbird; Courtship; Communication; Motivation; Opioids

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1. Introduction

Across vertebrates, communication conveys information about an individual's motivational state (e.g., readiness to mate or fight) [1]. The neural regulation of vocal production is relatively well understood in a few species, including songbirds [2–4]. Songbirds possess a unique set of brain nuclei known as the song control system (SCS), which controls the acquisition, perception, and performance of song [5]. Lesions to distinct nuclei in the SCS dramatically affect song learning, audible sound output or song structure. However, lesioned birds continue to sing or assume a singing posture even if audible song is not produced [5–7], suggesting that the motivation to sing lies elsewhere, including in areas involved in motivation such as the medial preoptic nucleus (often referred to as POM in birds [8–10]).

In seasonally breeding male songbirds, the factors that motivate a bird to sing depend on season and endocrine state [11–13]. Increases in the steroid hormone testosterone (T) in spring stimulate high rates of sexually-motivated courtship song in response to females [14–21]. However, among males with elevated T, high individual variation in courtship song can be found, suggesting that factors such as steroid receptors in the POM and SCS and other areas involved in sexual motivation may fine-tune changes in behavior to better reflect an individual's current motivational state [22–24]. Androgen receptors (ARs) within brain regions involved in sexual motivation and vocal production increase in association with seasonal increases in courtship singing and mating behavior in several songbird species [25–27]. Furthermore, in European starlings, song bout length (a feature of song attractive to females [17]), is correlated with AR immunoreactivity in distinct SCS nuclei as well as the POM, and T implants within POM increase song rate while peripheral T (acting on POM and the SCS) increases song rate and song quality [22, 28]. It is generally assumed that T or its metabolites act within the SCS to modify song structure and in the POM to stimulate the motivation to sing [28–32], yet the mechanisms by which changes in the androgen system modify vocal behavior are not well established.

In the POM, both T and its metabolite estradiol are implicated in courtship and breeding behaviors [33–35]. With respect to courtship song, both androgens and estrogens are required in zebra finches to fully restore courtship-singing behavior in castrated males [36], suggesting that androgens and/or estrogens in the POM may influence sexually-motivated song production in male starlings. We focus here on ARs because of the correlations between sexually-motivated song and ARs in POM described in the paragraph above and also based on a study in white-crowned sparrows showing that although estrogens are involved in seasonal neuroplasticity, androgens (rather than estrogens) may play a more central role in facilitating courtship song [37].

In addition to ARs and T, opioid neuropeptides in the POM influence sexually-motivated song production. In European starlings, immunolabeled mu opioid receptors (muORs) and met-enkephalin densities in POM were higher in low singing males compared to males singing high rates of song [38, 39], suggesting that opioids in POM may inhibit sexually-motivated song. Consistent with this possibility, blocking opioid receptors directly in the POM with the nonselective opioid receptor antagonist naloxone increases singing behavior in low singing male starlings. However, in high singing males, naloxone in the POM

decreases song, suggesting that some optimal level of opioid activity is necessary to facilitate sexually motivated song [39–41]. It is possible that additional opioid ligands and receptors (e.g., dynorphin, endorphins, kappa and delta receptors) may also play roles in the regulation of courtship song; however, as a starting point we focus here on muORs and enkephalins based on past data implicating them in sexually-motivated male song [32, 42].

Steroid hormones, including T, influence opioid activity [43, 44], and it has been proposed that steroids may in part regulate vocal production by modifying opioid systems (reviewed in [45]). In support of this idea, estradiol has been found to interact with opioid motor neural systems to modify vocal output in plainfin midshipman (*Porichthys notatus*) [46]. Studies in rodents demonstrate that T can both promote and inhibit expression of opioid receptors and ligands, suggesting it as ideal for adjusting opioid activity to optimal levels to promote sexually-motivated singing behavior. Specifically, castration resulted in increased muOR binding in the rat brain (whole brain lysate) [47]; whereas T treatment reduced muOR binding sites (measured as radioactivity in homogenized punches) in the preoptic area and medial basal hypothalamus [48]. Dihydrotestosterone (an AR specific agonist) reduced opioid binding density in the medial preoptic nucleus, while binding was increased in response to flutamide, an AR antagonist [49]. In contrast to opioid receptors, opioid ligand precursors such as preproenkephalin and proopiometanocortin have been found to increase in the brain in response to T and estrogen treatments [50–52]. Considering that the POM contains ARs, muORs, and enkephalin opioids [22, 32, 38, 39, 53], it is plausible that androgens may modify expression of opioid receptors and ligands in the medial POM to promote sexually-motivated singing behavior.

Based on these past studies, we predict that increases in T concentrations and/or ARs in the spring breeding season may act to fine-tune sexually-motivated singing behavior in part by altering both opioid receptor and opioid ligand expression (perhaps in opposite ways based on past studies in rats). To gain insight into this hypothesis, here we examined associations between sexually-motivated communication and relative expression levels of ARs, muORs, and preproenkephalin (PENK) in the POM (as measured by qPCR). As controls, we additionally examined these markers in the medial ventral tegmental area (mVTA), a region in which opioids are implicated in sexually motivated song [38, 54] but does not contain high densities of ARs [22, 55, 56], as well as two SCS regions that contain ARs and opioids but are not implicated in sexual motivation, HVC and Area X [25, 57–60].

2. Methods

2.1. Animals

Male (n=25) and female starlings (n=4) were captured during winter 2009–2010 on a single farm in Madison, Wisconsin using baited fly-in traps. After capture, birds were housed indoors in stainless steel, single sex cages (91 cm × 47cm × 47 cm) in groups of 5 birds per cage in the University of Wisconsin's Department of Zoology animal facilities. Food (Purina Mills Start and Grow Sunfresh Recipe, 61S3-IGH-G) and water were provided ad libitum. Each animal was assigned a numbered band for identification. All procedures and protocols adhered to the guidelines of the *National Institutes of Health Guide for the Care and Use of*

Laboratory Animals and a protocol approved by the University of Wisconsin Institutional Animal Care and Use Committee.

2.2. Housing conditions

Prior to study initiation, birds were housed indoors and placed on artificial photoperiods of 18h light (L):6h dark (D) for 6 weeks, followed by a photoperiod of 8L:16D for an additional 6 weeks. These photoperiod manipulations induce photosensitivity, a condition in which male starlings exposed to day lengths > 11h light per 24h period show increased plasma T concentrations as occurs during the spring breeding season [20]. Immediately prior to releasing males into outdoor aviaries (described below), blood was collected from each bird via alar venipuncture into heparin treated capillary-action centrifuge tubes and centrifuged at 3000 rpm for 20 min at 4°C. Blood plasma (supernatant) was then collected and stored at -80°C until assayed for T. Males were placed randomly into one of five outdoor aviaries (five birds per aviary; aviary dimensions 2.13 m × 2.4 m × 1.98 m) with access to natural light, perches, nesting material, bird bath, food and water ad libitum, and four nest boxes (although not a variable of interest in the present study we provided 4 nest boxes to 5 birds to ensure that we would have some birds without nest boxes as this has been an important variable in past studies [22, 38, 61]. At least one nest box remained unclaimed in each aviary, so any bird that was motivated to take a box would have been able.) Natural day length during this time was approximately 13L:11D. Birds habituated to aviaries for 12 days before the beginning of behavioral observations. (During habituation to the outdoor aviaries 5 birds were removed for a variety of reasons.)

2.3. Behavioral observations

Behavioral observations were collected for analysis on 4 days prior to brain collection for qPCR. Aviaries were observed in a rotating order. Prior to observations a female conspecific was released into an aviary, and fresh nesting material (grass clippings and green leaves) was placed on the floor of the aviary. Four female conspecifics were used to stimulate male courtship song during observation periods on a daily rotating basis. Females were housed in light conditions resembling outdoor photoperiod indoors in a single cage. A single female was used each day, rotating through all five aviaries. A single observer recorded behavior for 20 minutes for each aviary on each observation day. Bouts of gathering nesting material, feeding, preening and drinking were recorded, with a bout defined as a behavior separated by more than two seconds from a previous occurrence. Additionally, the numbers of times males displaced others, entered a nest box, landed on a nest box perch, and looked into a box were recorded continuously. For singing behavior, the number of songs (song rate) and duration of each song (song bout length) in seconds were recorded. After observations, the female conspecific was removed.

2.4. Tissue preparation for qPCR

Immediately following final observations, males were sacrificed by rapid decapitation. A terminal trunk blood sample of approximately 1.5ml was also gathered and supernatant collected and stored as described above for the pretest blood sample. Brains were immediately removed and submerged in Isopentane (Catalog No. 277258; Sigma, St. Louis,

MO) on dry ice for 30 seconds to freeze. Brains were then stored at -80°C until processing. They were sectioned coronally at $200\mu\text{m}$ on a cryostat at -18°C onto gel-coated slides on dry ice and brain nuclei of interest were punched using Stoelting brain punch set (Catalog No. 57401; Stoelting, Wood Dale, IL). We specifically collected samples from the POM, mVTA, Area X and HVC (Figure 1). POM was identified as the portion of the preoptic nucleus caudal to tractus septopalliomesecephalicus and rostral to the anterior commissure. mVTA was defined as the portion of VTA caudal to the appearance of third cranial nerve at the level of substantia nigra (as in [62]). Punches were expelled into centrifuge tubes on dry ice and then stored at -80°C until RNA extraction.

RNA extraction was completed using the Bio-Rad Aurum Total RNA Fatty and Fibrous Tissue Kit (Catalog No. 732-6830; Bio-Rad, Hercules, CA). Briefly, tissue was homogenized at medium speed using a Dremel tool for 25 seconds. RNA was isolated with PureZOL and treated with DNase according to the manufacturer's instructions. RNA was eluted from the columns using 30ul nuclease free water. RNA concentration was measured with a Nanodrop system (Catalog No. ND-2000; Thermo Scientific, Wilmington, DE), and RNA integrity was verified on a subset of samples with Agilent 2100 BioAnalyzer and Agilent RNA 6000 Pico Kit (Catalog Nos. G2940CA and 5067-1513; Agilent Technologies, Santa Clara, CA), according to manufacturer's directions.

Following verification of RNA integrity, conversion to single stranded cDNA was carried out using Invitrogen SuperScript III First-Strand Synthesis System (Catalog No. 18080-051; Life Technologies, Carlsbad, CA) according to manufacturer's instructions with 100ng starting RNA (50ng for mVTA due to low RNA yield from punches) and oligo(dT) (from kit). cDNA concentration was measured with the Nanodrop system. Standard cDNA (from whole hypothalamus) was then diluted to a starting concentration of 500ng/ μl and sample cDNA was diluted to a concentration of 100ng/ μl (400ng/ μl for mVTA) using nuclease free water to guarantee that amplification for these samples would fall on the standard curve. Relative gene expression for AR, muOR, and PENK was quantified via qPCR for HVC, Area X, POM, and mVTA.

2.5. qPCR

qPCR was performed using the BioRad CFX96 Touch Real-Time PCR Detection System (Catalog No. 185-5195; Bio-Rad, Hercules, CA). Briefly, reaction tubes contained SsoFast EvaGreen Supermix (Catalog No. 172-5201; Bio-Rad, Hercules, CA), nuclease free water, forward and reverse primers (prepared by University of Wisconsin, Biotech Center) at $5\mu\text{M}$, and cDNA as prepared above. Each plate contained 5 standards at 1:4 dilution series, beginning at 500ng/ μl and a negative control in which cDNA was replaced by nuclease free water. Standards and samples were plated in triplicate. All runs were run with an initiation step at 95°C for 30s, and then 40 cycles of a dissociation phase of 95°C for 5s, a 30s annealing phase unless otherwise noted below (annealing temperature (T_a) varied based on primer set), and a 20s elongation phase at 72°C . All plates then went through a melt curve from 60°C to 88°C , 0.5 degrees for each 5s step. Plates were read following each elongation step and each melt curve step. Runs had to achieve efficiency between 90–110% and an R^2

of at least 0.990 to be acceptable. Additionally, melt curves required a single peak, displaying specificity of the primers.

Primers were designed based on the zebra finch (*Taeniopygia guttata*) genome. NCBI's Primer-BLAST was used to locate primers and determine specificity. We developed our primers using the zebra finch genome because this is the only songbird genome publicly sequenced. Both our single peak melt curves and the size of the products produced by the primers suggest that these primers were identifying the genes of interest. Netprimer (Premier Biosoft) was used to examine secondary structure of primers to avoid primer products. Two reference genes were used, Beta Actin and Hydroxymethylbilane Synthase (HMBS). Genes of interest were Androgen Receptor, mu Opioid Receptor (primers used previously in zebrafishes [63]), and pre-proenkephalin (PENK). Details of primers and run statistics are described further in Table 1.

2.6. qPCR data transformation

Mean Ct values for each sample (the cycle number at which each sample crosses the amplification threshold, which here was set to 200 RFU), were transformed via the Pfaffl Method to yield individual relative expression level values for each gene of interest for each bird. This method is comparable to the 2 delta-delta Ct method commonly used to analyze qPCR data [64], but additionally allows input of efficiencies for each individual run, instead of assuming 100% efficiency [65]. Two reference genes were used for each nucleus, and the geometric mean of the Ct values for the two reference genes was used to transform the Ct values for each gene of interest. Equations used are as follows, where E represents the efficiency of each run, Ct values are the mean of each sample triplicate and each sample is normalized first to the geometric mean of the reference genes of the same sample and then to the geometric mean of all sample values. Using this method, we were able to obtain individual values to be used in multiple regression analyses to look for relationships between behavior and gene expression.

$$\frac{(\prod_{i=1}^{20} X_i)}{(1+E)^{Ct_{Gene\ of\ Interest}} / \sqrt{(1+E)^{Ct_{Beta\ Actin}} \times (1+E)^{Ct_{HMBS}}}} \quad \text{Where } X_i = \frac{(1+E)^{Ct_{Gene\ of\ Interest}}}{\sqrt{(1+E)^{Ct_{Beta\ Actin}} \times (1+E)^{Ct_{HMBS}}}}$$

2.7. Testosterone assay

Plasma T in the pre-experiment and terminal blood samples was measured with a commercial grade competitive immunoassay (Catalog No. 582701; Cayman Chemical, Ann Arbor, MI) used in prior songbird studies, including starlings [22, 66]. Samples were run in duplicate on two plates run simultaneously (using solutions mixed in the same batches), using the manufacturer's protocol at a dilution of 1:8 (selected after pilot tests showed that this dilution would run within the curve) in buffer solution and visualized at 405 nm with a BioTek 800 plate reader (Catalog No. 7331000; BioTek Instruments, Inc., Winooski, VT). A separate assay verified that the standard curve and sample in a dilution series yielded parallel curves implying specificity of this kit to measure T in starling plasma. Sensitivities of the commercial EIA according to the manufacturer's specifications indicate a limit of

detection: 80% B/B_0 : 6 pg/ml and sensitivity: 50% B/B_0 : 32 pg/ml. The assay is specific (cross reactivity to 5 α -DHT is 27.4%, and to 17 β -estradiol <0.01%). All samples were run within a single assay on two plates. The intra-assay Coefficient of Variations (CV) were 5.4% and 14.5%. The inter-assay CV was 8.3%.

2.8. Statistical methods

Data were analyzed using the statistical software program Statistica (StatSoft 2001, Tulsa, OK). Stepwise multiple regression analyses were used to examine statistical relationships between sexually-motivated song, T concentrations, androgen receptor expression and opioid expression (PENK and muOR) in each nucleus. AR expression in mVTA was undetectable, so it was not included in regression analyses of VTA. (This is in agreement with protein data [22] and in situ studies where AR mRNA was found in the caudal extreme of VTA, but not in more rostral portions [55]). Variables for inclusion in multiple regression models were selected based on F values. For backward regression, the F to remove was set to 3. Points that both had a standardized residual >2 and were highly influential (i.e., the removal or retention of the point dramatically altered results) were considered outliers and removed from analysis, resulting in the removal of only a single point from one analysis (detailed in results). Time spent singing in the presence of a female was not normally distributed (Lilliefors $p < 0.01$) and was square root transformed correcting for zeros ($\sqrt{x+0.05}$). This transformation successfully normalized the data (Lilliefors $p < 0.2$). For analysis of each brain region, total time singing in the presence of a female ($\sqrt{x+0.05}$ transformed), was entered as a dependent variable and measures of T (post-experimental), AR expression (not applicable for VTA), PENK and muOR expression were entered as independent variables. Multiple regression analyses were also used to look for relationships between non-courtship behavior (sum of feeding, drinking and preening bouts) and T, ARs, muORs, and PENK. For each analysis, non-courtship behavior (the sum of feeding, drinking and preening) was entered as a dependent variable. Measures of T, AR, PENK and muOR expression for a single brain region (POM, mVTA, HVC, Area X) were entered as independent variables. Preliminary analysis revealed that the only significant independent variable in each nucleus was T. As serum T is not nucleus specific, a simple regression was used to determine the statistical relationship between T and non-courtship behavior (detailed in Results 3.2). Finally multiple regression analyses were used to examine statistical relationships between a ratio of opioid expression (PENK/muOR) and androgens (ARs and T). For this analysis, the dependent variable was a ratio of PENK/muOR based on the results of the first analysis (detailed below 3.2). For each analysis, the opioid expression ratio for a single brain region was entered as a dependent variable. Measures of T (post-experimental) and AR expression for the same region were entered as independent variables. For POM, HVC, and mVTA a single individual (not the same individual) was lost due to missing data for one of the qPCR values resulting in $n=19$ for these nuclei.

3. Results

3.1. Testosterone concentrations

All birds had low (<400pg/ml) or undetectable (<6pg/ml) concentrations of T at the beginning of the experiment; however, after 16 days of exposure to natural light conditions

(approximately 15.5L: 8.5D) in outdoor aviaries mean[sem] T concentration was 1191.2[232.9] pg/ml.

3.2. Gene expression, testosterone, and behavior

To provide insight into the hypothesis that androgen and opioid activity interact to control singing behavior, backward stepwise multiple regression analyses were used to determine the extent to which individual differences in AR, muOR, PENK expression and T contribute statistically to singing behaviors. Backwards stepwise multiple regression analysis indicated that AR and PENK expression in POM contributed positively, whereas muOR expression contributed negatively to variance in time spent singing sexually-motivated song (Table 2A) after removal of a single outlier.

For mVTA, PENK contributed negatively to variance in time spent singing (Figure 2, Table 2B). For the vocal nuclei, HVC and Area X, no models were returned to explain sexually-motivated song. T concentrations did not reach the threshold for inclusion in any of the models.

To see if the relationships identified above were specific to singing behavior, backwards stepwise multiple regression analyses were used to determine whether individual differences in other non-courtship behaviors could be explained by androgens (T concentration and ARs) or opioids (PENK and muORs). Analysis for each brain region returned T as the only significant explanatory variable for nonspecific behavior. Therefore, as serum T concentration is a global measure and not brain region specific, we report here the result of a simple linear regression revealing a positive relationship between T and non-courtship behavior ($n=20$, $r=0.57$, $p=0.01$).

3.3. Androgens and opioids

To provide insight into the hypothesis that androgen activity alters opioid markers (priming the system to facilitate sexually-motivated song), backwards stepwise multiple regression analyses were used to determine whether opioid expression differences could be explained statistically by androgens (T concentration or ARs). Backwards stepwise multiple regression analyses indicated a negative relationship between AR expression and the opioid expression ratio in POM (Figure 3). Testosterone was not found to contribute significantly to the model. No models were returned for the other nuclei. When PENK and muOR expression were entered as dependent variables (rather than the ratio) in separate analyses, no significant models were returned.

4. Discussion

The present study is the first to demonstrate associations between sexually-motivated communication and markers for both androgen- and opioid-related gene expression in the POM. The results support the hypothesis that AR may alter opioid receptor and ligand expression in POM to fine-tune singing behavior to reflect a male's motivational state. The data also suggest that additional bidirectional relationships may exist between opioids and AR in the POM and singing behavior, and support a role for opioids in mVTA in the

production of sexually-motivated song that may be independent of AR activity in this region.

4.1. Androgen receptor and opioid marker expression in POM relate to song

Elevated AR expression in POM was associated with high levels of sexually-motivated song. This is consistent with previous findings demonstrating a positive correlation between AR protein labeling in POM and sexually-motivated singing behavior [22]. In addition to AR expression, opioid marker expression in POM correlated with sexually-motivated singing behavior (PENK positively, muOR negatively). This indicates that birds with high AR expression, along with high PENK and low muOR expression in the POM produced the highest rates of sexually-motivated song. This finding is in agreement with past data showing that labeling for muOR protein is lowest in high singing male starlings [38]. However, the finding that birds singing at high rates had relatively high PENK expression in POM compared to low singing birds somewhat differs from past results. For example, in one study in male starlings immunolabeling for met-enkephalin (a protein product of PENK) in the POM failed to correlate with sexually-motivated singing behavior [32], and in another study, met-enkephalin labeling was found to be highest in males singing at low rates [39]. These apparent inconsistencies may reflect a reduction in met-enkephalin synthesis (reflected in reduced PENK mRNA expression) when the ligand met-enkephalin is already elevated in low singing animals; however additional studies that include measures will be necessary to explain these past results. Additionally, comparison of studies measuring met-enkephalin (the ligand) and PENK (the precursor) is difficult as immunolabeling for met-enkephalin cannot discriminate between stored and released protein, and PENK mRNA indicates an ad hoc increase in ligand, perhaps as a mechanism to auto-regulate the opioid system (discussed below).

The couplings between sexually-motivated song, ARs, PENK and muORs in POM are consistent with the hypothesis that androgens fine-tune opioid activity to promote or inhibit sexually-motivated singing behavior. Although other directional relationships may exist (discussed further below), the possibility that activation of steroid hormone receptors influences opioids is consistent with multiple past studies [67–69]. Although possible, so far it does not appear that ARs influence opioids via the traditional role of ARs as transcription factors, as there are no known AR binding sites on either opioid-related gene measured here at least based on the human genome (2009) using ENCODE (<http://genome.ucsc.edu/>) [70]. The transcription factor binding sites have not been annotated in the zebra finch genome so it is possible that species differences may exist; however, ENCODE allows alignment of the zebra finch genome to the human genome and there do not appear to be AR binding sites on the opioid receptor or preproenkephalin genes for the zebra finch either. Instead, it has been proposed that steroid hormones cause release of opioid ligand and that this in turn causes opioid receptor down regulation [71]. The mechanism behind the potential hormone and opioid ligand release has not been identified, but is an area worthy of further study. Increases in PENK mRNA (in response to methamphetamine) are associated with decreased muOR mRNA, consistent with high PENK resulting in downregulation of muOR [72]. Pharmacological manipulations have also demonstrated that binding of muORs results in internalization and down regulation of the receptor [73]. The data in the present study fit this

model. Specifically, high AR expression is associated with high PENK expression (the ligand precursor) but low muOR expression. This supports the possibility that high AR activity causes the synthesis and release of enkephalin opioids, which in turn cause muOR to downregulate. The finding that AR expression statistically explained the ratio of PENK/muOR offers further evidence in support of this hypothesis.

Although the present and past results support a model in which AR activation then modifies opioid-related gene expression to fine-tune singing behavior, other potentially bi-directional relationships may exist between these variables. Opioids are known to suppress androgen production and release from the gonads through suppression of gonadotropin release [74–77]. As circulating T can alter AR expression [78–80], it is possible that opioids influence ARs indirectly through effects on T. Additionally, it is possible that changes in transcription of ARs, muORs and PENK are caused by the act of singing. This idea is consistent with studies in which the act of singing induces changes in neural plasticity through the actions of T [29]. There is also evidence that vocal production in ring doves can influence activity in opioidergic brain regions [81].

4.2. Opioid markers in mVTA relate to song

PENK expression in mVTA was negatively related to sexually-motivated song. Although a previous study in male starlings found no relationship between met-enkephalin immunolabeling in the VTA and sexually-motivated song [32], another study demonstrated that males singing the highest rates of sexually-motivated song had the lowest levels of muOR immunolabeling [38]. Together the results of the present and this past study suggest that both opioid ligand and receptors may be downregulated in VTA in the highest singing birds. Opioids in the VTA are well known to influence dopaminergic projection neurons and motivated behaviors [82, 83]. However, the current findings were somewhat inconsistent with what would be predicted based on past studies showing that opioids act at muORs to inhibit GABA receptors, which causes a disinhibition of activity in VTA dopamine neurons [84] to facilitate sexually-motivated behavior [85]. However, the mammalian VTA contains three types of neurons, two of which are inhibited by opioid agonists. The avian VTA only contains one of these inhibited neuronal types, suggesting that the proportion of avian VTA neurons inhibited by opioids may not be consistent with rodents [82]. Because ARs are not present (except in low quantities in portions of VTA caudal to the area studied here [55]) our data do not support a direct role for AR in modifying opioid markers in VTA to facilitate singing behavior. However, there is a possibility that alterations to the opioid system in the VTA are modulated by androgenic activity in efferent nuclei as in rats [56].

4.3. AR and opioid expression in song control nuclei do not relate to song

In the present study, neither AR nor opioid mRNA measures in song control regions HVC and Area X related to sexually-motivated song production. The data for AR are somewhat in contrast with past results showing a positive correlation between immunolabeling for AR protein and a measure of courtship song [22]. The reason for this difference is unclear but may relate to differences in the measures of song (in the past study song bout length was measured) or the fact that here we measure AR mRNA expression and in the past study immunolabeled AR was measured. The data for muOR and PENK expression are in

agreement with past studies in which no significant differences were found in these regions in mu receptor binding between singing and non-singing male juncos (*Junco hyemalis*), or between males and females (female juncos do not sing) [86]. Together these findings suggest that individual differences in muOR and PENK expression in the song control system may not necessary for modifying song rate, however their presence in these nuclei suggest that they are important for some song-related activity, perhaps song structure or learning.

4.4. Non-courtship behavior relates to serum testosterone concentrations

An unpredicted (but not unprecedented) positive correlation was found between non-courtship behaviors (sum of feeding, drinking and preening) and T. Previous studies in sparrows and juncos have found that elevated T is associated with an increase in general activity [87, 88]. Thus the present findings are consistent with past literature. While increases in T are associated with seasonal increases in courtship song, we did not find any linear correlations between T and specific song measures, but as all males in this study had seasonally appropriate levels of serum T (low variance among samples), this is not surprising.

5. Conclusions

Our results support the hypothesis that in POM opioids and androgens are both important in the facilitation of sexually-motivated song, and furthermore that androgens may alter opioid systems to promote an ideal receptor/ligand level at which birds are motivated to court a female when the season and social context are appropriate. Additionally, the relationship between opioids in the VTA and song support a role for this nucleus in sexually-motivated song, perhaps via disinhibition of dopaminergic neurons. Future research is needed to examine possible mechanisms for seasonal and context dependent androgen changes to alter the opioid system in songbirds. It is also necessary to determine whether this relationship is bi-directional or whether behavior and/or the environment alter both androgen and opioid transcription.

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Highlights

- Androgen receptor and opioid expression in the preoptic nucleus related to song.
- Gene expression in vocal nuclei did not relate to song production.
- Opioid expression in the preoptic area was explained by androgen receptor expression.
- Results suggest androgens may alter opioids to promote appropriate communication.
- Data suggest possible bidirectional relationships between gene expression and song.

Figure 1. Punch Diagrams

Punch locations for the nuclei used for gene expression analysis. Area X (1.25mm diameter punch); POM (1.25mm diameter punch); HVC (1.25mm diameter punch); mVTA (0.75mm diameter punch). Cb- cerebellum, ICo- nucleus intercollicularis, LS- lateral septum, mMAN- medial portion of the magnocellular nucleus, NIII- third cranial nerve, PAG- periaqueductal grey, Rt- nucleus rotundus, TnA- nucleus taenia of the amygdala.

Figure 2. Penk in VTA

Scatterplot showing relationship between PENK expression (presented as a ratio, calculation specified in text 2.6) in the medial ventral tegmental area (mVTA) and sexually motivated song. Each point represents data from an individual.

Figure 3. opioids and AR

Scatterplot showing relationship between androgen receptor expression and opioid expression ratio (PENK/ μ OR) (expression presented as a ratio, calculation specified in text 2.6) in the medial preoptic area (POM). Each point represents data from an individual.

Table 1

Primers

Gene	Direction	Sequences	Ta	Product
Preproenkephalin NM_001245451.2	Forward	TGTCAGCAAAAGATACGGAGG	58.5°	185bp
	Reverse	GGAGAAATGGAGTCGGCAAAAG		
Androgen Receptor NM_001076688.1	Forward	ACTTGACCGTATCATTTGCCCTG	57.4°	171bp
	Reverse	ATTCTGGGTAGTCCACGCTC		
Mu Opioid Receptor XM_002187352	Forward	GCAGATGCCCTAGCAACAAG	57.0°	165bp
	Reverse	CAGGTAGCGATCCACACTCA		
Beta Actin XM_002192780	Forward	GAGAGGGAAATCGTGCGTG	55.7°	187bp
	Reverse	GGAAAGATGGCTGGAACAGGG		
HMBS XM_002187761.1	Forward	ACCTGCCAAACTTCTCTTCCT	58.0°	140bp
	Reverse	GGTTCCAAATCACACACTCTTTTCA	(20s annealing)	

Table 2

Revised

Regression Analyses						
Dependent Variable	Independent Variables	β	S.E.	t	df	p value
A. POM						
Singing Behavior						
adj. $R^2=0.456$ $p=0.009$ n=18	AR Expression	0.786	0.231	3.40	14	0.004
	PENK Expression	0.819	0.222	3.68	14	0.002
	muOR Expression	-0.710	0.225	-3.17	14	0.007
	Testosterone	excluded from model				
B. mVTA						
Singing Behavior						
adj. $R^2=0.185$ $p=0.038$ n=19	PENK Expression	-0.480	0.222	-2.25	17	0.038
	muOR Expression	excluded from model				
	Testosterone	excluded from model				
C. POM						
Opioid Expression Ratio						
adj. $R^2=0.249$ $p=0.017$ n=19	AR Expression	-0.534	0.204	-2.64	17	0.017
	Testosterone	excluded from model				