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Research report

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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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.

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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.

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

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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-crowed 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 proopiomelanocortin 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 × 47 cm × 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 18 h light (L):6 h 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 >11 h light per 24 h 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