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Contributions of testosterone and territory ownership to sexually-motivated behaviors and mRNA expression in the medial preoptic area of male European starlings

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Abstract

Animals integrate social information with their internal endocrine state to control the timing of behavior, but how these signals are integrated in the brain is not understood. The medial preoptic area (mPOA) may play an integrative role in the control of courtship behavior, as it receives projections from multiple sensory systems, and is central to the hormonal control of courtship behavior across vertebrates. Additionally, data from many species implicate opioid and dopaminergic systems in the mPOA in the control of male courtship behavior. We used European starlings to test the hypothesis that testosterone (T) and social status (in the form of territory possession) interact to control the timing of courtship behavior by modulating steroid hormone-, opioid- and dopaminergic-related gene expression in the mPOA. We found that only males given both T and a nesting territory produced high rates of courtship behavior in response to a female. T treatment altered patterns of gene expression in the mPOA by increasing androgen receptor, aromatase, mu-opioid receptor and preproenkephalin mRNA and decreasing tyrosine hydroxylase mRNA expression. Territory possession did not alter mRNA expression in the mPOA, despite the finding that only birds with both T and a nesting territory produced courtship behavior. We propose that T prepares the mPOA to respond to the presence of a female with high rates of courtship song by altering gene expression, but that activity in the mPOA is under a continuous (i.e. tonic) inhibition until a male starling obtains a nesting territory.

Keywords

testosterone; territory; sexual motivation; opioid; dopamine; behavior; vocal communication; medial preoptic area

Introduction

Animals use both hormonal signals and social information to control the timing of breeding behavior. For example, the sex steroid testosterone (T) is necessary to initiate male courtship behavior across many vertebrates (Gwinner and Gwinner, 1994; Leonard and Ferkin, 1999;

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Lindzey and Crews, 1986; Watson and Parr, 1981; Woolley et al., 2004), but social factors such as territory possession or high social status are also critical regulators of courtship behavior (Fuxjager et al., 2009; Maruska, 2015; Ritters et al., 2000). These signals are processed by the brain, but relatively little is known about how the brain integrates hormonal signals and social information to ensure that courtship behavior is produced in an appropriate context.

In European starlings (*Sturnus vulgaris*), increasing day length causes increases in T production and release, which stimulates males to sing high rates of courtship song to females (Dawson et al., 2001; Gwinner and Gwinner, 1994). However, even when T is fixed at breeding-typical levels, males will only sing courtship song to females if they acquire a nesting territory (Heimovics and Ritters, 2005; Kelm et al., 2011). This suggests that in starlings, T and territory possession interact in the brain to activate courtship behavior.

The medial preoptic area (mPOA) is one brain region in which T and territory possession may act to influence courtship behavior in starlings. Across vertebrates, the mPOA is centrally involved in male sexually-motivated behavior, including courtship song in starlings (Alger et al., 2009; Alward et al., 2013; Balthazart and Ball, 2007; Hull et al., 1995; Woolley et al., 2004). Lesions to the mPOA disrupt sexually-motivated behaviors in many species, as well as territorial behavior (Alekseyenko et al., 2007; Alger et al., 2009; Alger and Ritters, 2006; Edwards and Einhorn, 1986; Slimp et al., 1978; Woolley et al., 2004). Additionally, the mPOA contains high densities of steroid receptors (Davis and Moore, 1996; Gahr, 2001; Morrell and Pfaff, 1978; Simerly et al., 1990; Wood and Newman, 1995), receives afferent projections from most sensory systems (Simerly and Swanson, 1986) and projects to brain nuclei implicated in several forms of social behavior (Goodson, 2005; Newman, 1999; Ritters and Alger, 2004). The mPOA is thus a likely site in which T and information about territory possession are integrated to control sexually-motivated behaviors such as courtship song.

It has been proposed that T affects sexually-motivated behaviors by altering activity in opioid and dopaminergic systems in the mPOA (Cordes et al., 2015; Hull et al., 1995; Ritters, 2012). Opioids and dopamine in the mPOA are strongly implicated in male sexual behavior in mammals and birds (Hull et al., 1995; Kleitz-Nelson et al., 2010b; Kotegawa et al., 1997; Van Furth et al., 1995). In starlings, converging data demonstrate that opioids act in the mPOA to modulate sexually-motivated song in a complex manner (Kelm et al., 2011; Kelm-Nelson et al., 2013). For example, blocking opioid receptors with naloxone in the mPOA of male starlings increases courtship song in low-singing birds, but decreases song in high singing birds (Kelm-Nelson et al., 2013), demonstrating that an optimal level of opioid activity in the mPOA is necessary to facilitate male courtship behavior.

With respect to dopamine, extracellular dopamine in the mPOA is released in male rats and quail that anticipate contact with a female (Hull et al., 1995; Kleitz-Nelson et al., 2010a, 2010b), and in starlings, stimulation of dopamine D1 receptors in the mPOA modulates courtship song in an inverted-U shaped manner (Ritters et al., 2014). Correlations between courtship singing behavior, and D1 and D2 dopamine receptor expression in the mPOA also indicate that an optimal level of dopamine receptor expression in the mPOA is needed to stimulate courtship song (DeVries et al., 2015). Given that T and its metabolites can modify

both opioid- and dopamine-related protein concentrations in the mPOA (Chu and Wilczynski, 2002; Eckersell et al., 1998; Watson Jr. et al., 1986), T may alter gene expression in the mPOA to prepare the brain to produce courtship behaviors.

In concert with T, territory possession may control the timing of courtship behaviors by altering steroid hormone and opioid activity in the mPOA. Starlings that successfully acquire and defend a nesting territory have higher concentrations of circulating T (Gwinner et al., 2002, 1987), and winning territorial disputes increases androgen receptor (AR) labeling in the mPOA of California mice (Fuxjager et al., 2010). Additionally, both mu-opioid receptor (MOR) and met-enkephalin protein labeling in the mPOA are diminished in starlings that own a territory compared to non-owners (Kelm et al., 2011; Kelm-Nelson et al., 2013). This suggests that owning a territory may activate courtship behavior by two non-mutually exclusive routes: increasing steroid sensitivity in the mPOA, and decreasing opioid activity in the mPOA.

Overall, we hypothesized that T and territory possession interact to modulate gene expression in the mPOA to control the timing of courtship behavior. To test this hypothesis, male starlings were castrated and received one of four treatments: neither T nor a nesting territory, a nesting territory only, T only, or both T and a nesting territory. We observed male behavior in the presence of a female, and quantitative PCR (qPCR) was run to measure mRNA expression in the mPOA. Specifically, we measured mRNA of steroid hormone-related genes (AR, aromatase (ARO; the enzyme that converts T to estradiol), and estrogen receptor beta (ER β)), opioid-related genes (MOR and preproenkephalin (PENK; the precursor of met-enkephalin)) and dopaminergic-related genes (TH, dopamine D1 and dopamine D2 receptors) to determine the effects of T and territory possession on mRNA expression in the mPOA.

Methods

Animal conditions

Starlings (37 males and 15 females) used in this study were captured on a farm in Madison, WI using baited fly-in traps during the winter of 2013-2014. The birds were housed in the University of Wisconsin's Department of Zoology animal facilities indoors in single sex cages (91 cm $\times$ 47 cm $\times$ 47cm; a maximum of five birds/cage) and provided with food and water ad libitum, as well as daily enrichment (e.g. vegetables, mealworms, toys). All birds were assigned numbered and colored leg bands for individual identification. Birds were housed on artificial photoperiods of 18-h light (L):6-h dark (D) until they became refractory (6 weeks; Dawson et al. 2001). Refractory birds were then moved to short day light (8L:16D) for 6 weeks. This induces a state of photosensitivity, under which the brain will respond to T to initiate breeding activities (Dawson et al., 2001).

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.

Castration

Male starlings were castrated while housed on 18L:6D, when testes were fully regressed. Briefly, birds were anaesthetized using isoflurane gas delivered through a beak cone, and both gonads were removed after making a unilateral incision on the left flank, just posterior to the last rib. A spacer was used to maintain a window into the body cavity, while one pair of curved forceps was used to clamp a testis and another pair was used to remove it from the body cavity. The incision site was sutured and birds recovered on a heating pad. Birds typically exhibited pre-surgery motor behaviors within 5-10 minutes after surgery.

Hormone implants

After birds were photosensitive, they received 2 subcutaneous implants that were empty or were packed with crystalline T (Sigma-Aldrich, St. Louis, MO). Crystalline T was dissolved in 100% ethanol and pulled into silastic tubing (i.d., 1.47mm; o.d. 1.96-mm; Dow Corning, Midland, MI, USA) using a 2mL syringe and an 18 gauge needle. A piece of cotton between the syringe and the testosterone solution allowed most of the ethanol to pass into the syringe while packing T into the tubing. Twenty-four hours later, when any remaining ethanol had completely vaporized, the tubing was cut and sealed on each end using 100% silicone sealant such that each implant was 12mm long and contained 10mm of packed T. To make control implants, empty tubing was cut to 12mm and sealed with 100% silicone sealant (DAP Products Inc., Baltimore, MD).

Study procedures and behavioral observations

Immediately after recovering from the hormone implant surgery, study animals were housed in indoor aviaries (3.5m × 2.25m × 2m) on 13L:11D. Moving photosensitive birds to long light (greater than 11L) causes them to become photostimulated, a condition typical of the spring breeding season in which T stimulates courtship singing and nest building (Dawson et al., 2001). Aviaries had either a nesting territory (a nest box), or a simple perch. Each aviary contained plastic branches and a bath. Food and water were provided ad libitum.

Study animals were all housed separately for behavioral testing, but because starlings are a social species, a castrated companion male was assigned to each aviary to avoid the stress of social isolation. The companion males (n = 6) were treated identically to the study males (n = 26), except they did not undergo a hormone implant surgery. Study males were given between 1-2 hours to habituate to the aviary before the companion male was introduced.

Males spent 7 days habituating to their aviaries, and then underwent 3 consecutive days of behavioral testing (one test per day). Each behavioral test was 20 minutes, and all observations were between 0900 and 1200 hours. Directly prior to each test, a handful of green nesting material and a novel stimulus female starling were introduced to the aviary.

An observer blind to the hormone implant condition of each bird recorded male behavior from behind a one-way mirror. For the study male, the observer recorded measures of courtship behavior, including complete songs, song bout length (in seconds), incomplete song measures (fragments and whistles), bouts of gathering nesting material (bouts were repeated behaviors that were counted as one if they were separated by less than 2 seconds),

and entering the nest box, as well as measures of conspecific displacement (defined as a male approaching within 5 cm of another male and the approached male departing immediately) and general behaviors, including bouts of feeding and drinking, and the number of times a male landed on any perch in the aviary. Behaviors for companion males were recorded if they involved song, the nest box, or displacement of the study male. At the end of the test, the female conspecific was returned to her home cage.

Testosterone assay and tissue preparation for qPCR

Directly following the final test, study males were sacrificed by rapid decapitation. Trunk blood was collected (approximately 1.5mL) to measure plasma T. Blood samples were centrifuged at 4000 rpm for 20 min at 4C°, and the plasma (supernatant) was aspirated and stored at -20C° until the assay. Brains were dissected from the skull and submerged in isopentane (Catalog No. 277258; Sigma-Aldrich, St. Louis, MO) on dry ice for approximately 30 seconds to freeze. The brains were then individually wrapped in foil, labeled, and stored at -80C° until sectioning.

We measured plasma T using a commercial grade competitive immunoassay (Catalog No. 582701; Cayman Chemical, Ann Arbor, MI). The specificity of this kit for measuring T in starling plasma has been verified (Cordes et al., 2015). Cross-reactivity is 27.4% to 5 α -DHT, and <0.01% to 17 β -estradiol. The manufacturer specifies the limits of detection (80% B/B_0 : 6 pg/ml) and sensitivity (50% B/B_0 : 32 pg/ml). All samples were run in duplicate on a single plate, and were diluted at 1:8 in EIA buffer solution as in previous studies (Cordes et al., 2014; EIA buffer concentrate provided in kit; Item No. 400060). Standards and control wells were prepared according to manufacturer's protocol, and the assay was visualized at 405 nm using a BioTek 800 plate reader (Catalog No. 7331000; BioTek Instruments, Inc., Winooski, VT). A standard curve and T measurements were calculated by inputting plate set-up and raw data to Myassays.com.

We sectioned brains coronally on a cryostat at -15C° onto slides. The slides were moved to dry ice, and we used a Fine Science Tools Sample Corer (Item No. 18035-02; Foster City, CA) to collect the mPOA on 3 consecutive 200 μ m thick coronal sections (Fig. 1). Punches were 2mm in diameter, and were stored in capped 2mL centrifuge tubes at -80C°. One sample was lost during this process.

We extracted RNA from tissue punches with the Bio-Rad Aurum Total RNA Fatty and Fibrous Tissue Kit (Catalog No. 73206830; Bio-Rad, Hercules, CA). RNA was isolated as instructed by the manufacturer, and the resulting RNA concentration for each sample was measured using a Nanodrop system (Catalog No. ND-2000; Thermo Scientific, Wilmington, DE). Following RNA extraction, we converted samples to single stranded cDNA using the Invitrogen SuperScript III First-Strand Synthesis System (Cat. #18080-051; Life Technologies, Carlsbad, CA).

Quantitative Polymerase Chain Reaction (qPCR) and Pfaffl data transformation

We used qPCR to measure the relative gene expression for steroid hormone-related genes (AR, ARO, estrogen receptor beta, ER β), opioid-related genes (MOR, PENK), and dopaminergic-related genes (dopamine receptors D1 and D2, TH) in the mPOA. Trial runs

with primers for estrogen receptor alpha did not meet MIQE guidelines (Bustin et al., 2009), and as such, this gene was not reported for this study. Two reference genes, Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) and Hypoxanthine-guanine phosphoribosyltransferase (HPRT) were run to normalize the variation in endogenous gene expression between animals. There was no difference in relative mRNA expression of either reference gene between experimental treatments in this study.

Primers for each gene were obtained using the NCBI Gene Database Primer-Blast for zebra finch (*Taeniopygia guttata*; Table 1). Netprimer (Premier Biosoft) was used to examine any secondary structures of primers. Prior to running primers with sample tissue, primers were run with only standard tissue, and products of successful runs were sent for Sanger sequencing with both forward and reverse primers at the University of Wisconsin Biotechnology Center (Table 2). NCBI BLAST shows that all sequences match our intended targets.

Samples were prepared for cDNA amplification as previously described (Cordes et al., 2015), 5 standards were prepared at a 1:10 dilution series and 1 negative template control was prepared by substituting nuclease free water for cDNA. Samples, standards and controls were plated in triplicate. The final volume of each well was 20 μ L.

Each run was amplified using the BioRad CFX96 Touch Real-Time PCR Detection System (Catalog No. 185-5195; Bio-Rad, Hercules, CA, USA) and consisted of: a 30 second initiation step at 95C°, 40 cycles of 95C° for 5 seconds, 30 seconds annealing step (temperature varied with primer set, see Table 1), and 20 second elongation step at 72C°, followed by a 60C° to 88C° melt curve (5 seconds for each 0.5 degrees). Runs that did not meet the MIQE guidelines were not used (Bustin et al., 2009).

qPCR raw data were transformed according to the Pfaffl Method to obtain relative expression level values (Cordes et al., 2014; Pfaffl, 2001). Briefly, the geometric mean of both reference gene mean CT values (the cycle number at which each sample amplified at 200RFU) was used to normalize the mean CT values of the gene of interest. The resulting values indicate mRNA expression relative to two reference genes. These relative expression values were used for analysis.

Statistical methods

Data were analyzed using Statistica software (version 6.0, StatSoft, Tulsa, OK). Factorial ANOVAs and MANOVAs were used to assess the effect of treatment on behavior and gene expression in the mPOA. We report effect sizes using eta-squared for significant effects in ANOVA and MANOVA analyses and using Cohen's d for post-hoc pairwise comparisons. We tested the assumptions of parametric statistics using Lilliefors's test for normality and Levene's test for homogeneity of variance. When data violated assumptions, they were square root transformed. If transformations did not correct the violations, nonparametric statistics were used. Outliers were identified and removed from analyses if values were greater than 2 standard deviations from the group mean. All data are presented as the mean $\pm$ the standard error of the mean.

Results

Hormone condition and testosterone concentrations

A post-mortem examination confirmed that study males had no gonadal remnants, and that implants were intact. Adequate plasma was not available for 9 animals. All remaining samples (7 control and 10 T treated birds) matched the assigned hormone conditions of the animals. Because the behavioral and mRNA data from the missing T samples were in line with the hormone condition groups they were assigned to, we performed our analysis assuming all males were in their assigned T condition. T concentrations for males given T had a mean of 3614.51 pg/mL, a standard deviation of 2424.11 pg/mL, and a range of 1687.8-9962.8 pg/mL. This last value (9962.8pg/mL) was extreme and when removed, the mean plasma T was 2909.14pg/mL, the standard deviation was 1006.75pg/mL, and the range was 1687.8-4654.40pg/mL. Thus, on average, these levels of plasma T are comparable to results obtained in free-living starlings using radio-immunoassays and chromatography (average of between 2750-3000pg/mL; Ball and Wingfield, 1987; Dawson, 1983). T concentrations for males given blank implants were all below the detection limit of the assay.

Effects of testosterone and nest box treatment on behavior

Only birds treated with T and a nest box displayed high rates of courtship behavior. Both total song and number of times males gathered nest material violated the assumptions of parametric tests, and transformation of the data did not correct this problem. Thus, we used Kruskal-Wallis tests for these behaviors. A Kruskal-Wallis test revealed a significant effect of treatment on total song ($H=19.23$, $p<0.0002$). Post-hoc pair-wise comparisons showed that birds with T and a nest box sang significantly more than any other group ($p<0.00019$ compared to birds with no T and no nest box, Cohen's $d = 1.56$; $p<0.000057$ compared to birds with no T and a nest box, Cohen's $d = 1.50$; $p<0.024$ compared to birds with T and no nest box, Cohen's $d = 1.53$; Fig. 2). Similarly, a Kruskal-Wallis test revealed a significant effect of treatment on bouts of gathering nesting material ($H=12.81$, $p<0.0051$), and post-hoc pair-wise comparisons show birds with T and a nest box differ from birds with neither T nor a nest box ($p<0.0054$, Cohen's $d = 1.03$) and without T but with a nest box ($p<0.0017$, Cohen's $d = 0.99$), but not from birds with T but no nest box ($p>0.05$, Cohen's $d = 0.83$; Fig. 2).

We assessed the effect of the treatments on displacement behavior using a factorial ANOVA with T condition and nest box condition entered as independent variables. Two outliers were identified as greater than two standard deviations from the means of their respective groups, and were removed prior to analysis. The outliers are reported graphically in figure 2C. The analysis revealed a significant main effect of T treatment on displacements ($df=1,20$; $F=10.87$, $p<0.0036$, $\eta^2=0.31$), and a Fisher's LSD post-hoc analysis showed that T treated birds displaced more than birds with blank implants ($p<0.0043$, Cohen's $d=1.35$; Fig. 2).

We also examined whether treatments affected bouts of feeding and drinking, or the number of times males landed on a perch (a general motor behavior). A factorial ANOVA revealed a significant main effect of T treatment on sum of feeding and drinking bouts ($df=1,22$;

$F=7.21$, $p<0.014$, $\eta^2=0.23$), and a Fisher's LSD post-hoc comparison showed that birds with T displayed more feeding and drinking bouts than birds without T ($p<0.018$, Cohen's $d=1.00$). A factorial ANOVA showed no main effect of either treatment on the number of times males landed on perches.

Effects of testosterone and nest box treatment on steroid hormone-related mRNA

To assess how group treatment affected steroid hormone-related mRNA expression in mPOA, we entered the relative mRNA expression of AR, ARO and ER β in mPOA as dependent variables (3 within effects) and T treatment and nest box treatment as categorical factors in a MANOVA. There was a significant main effect of T treatment ($df=1,21$; $F=26.41$, $p<0.000043$, $\eta^2=0.24$) and a GENE X T treatment interaction ($df=2,42$; $F=24.86$, $p<0.00001$, $\eta^2=0.23$). There was no main effect of nest box treatment. A Fisher's LSD post-hoc analysis revealed that T-treated birds had higher levels of AR and ARO mRNA expression in mPOA ($p<0.0028$ and $p<0.000003$ respectively, Cohen's $d=1.24$ and 3.99 respectively; Fig. 3), and no effect of T treatment on ER β mRNA expression in mPOA ($p>0.05$, Cohen's $d=-0.64$; Fig 3).

ARO mRNA expression in the mPOA violated equal variances, but the results of a Kruskal-Wallis agree with results of the MANOVA, and removing ARO from the analysis did not affect the results for AR and ER β mRNA expression in mPOA. Therefore, we report all three gene measures in a single analysis to reduce Type I error.

Effects of testosterone and nest box treatment on opioid and dopamine-related mRNA

To test whether T treatment and nest box treatment affected the mRNA expression of markers known to relate to sexually-motivated song, we entered MOR, PENK, TH, D1 and D2 mRNA expression in mPOA (5 within effects) into a MANOVA, using T treatment and nest box treatment as categorical factors. There was a significant GENE X T treatment interaction ($df=4,80$; $F=6.81$, $p<0.000091$, $\eta^2=0.18$). A Fisher's LSD post-hoc analysis revealed that T treatment increased MOR and PENK mRNA expression in mPOA ($p<0.015$ and $p<0.025$ respectively, Cohen's $d=1.62$ and 0.95 respectively; Fig. 4) and decreased TH mRNA expression in mPOA ($p<0.014$, Cohen's $d=-1.20$; Fig. 5). T treatment did not affect the mRNA expression of D1 or D2 in mPOA ($p>0.05$, Cohen's $d=0.85$ and 0.13 respectively; Fig. 5).

Discussion

In this study, we tested the hypothesis that both T and social factors (i.e., territory possession) interact to alter gene expression in the mPOA, resulting in the stimulation of male courtship behavior. We found strong support for the prediction that T alters steroid-hormone, opioid- and dopamine-related gene expression; however, we did not find any evidence that the presence of a nesting territory further influences gene expression in the mPOA. This was despite the finding that only birds with both T and a nesting territory produced courtship behavior. We interpret these findings to indicate that increases in T prepare the mPOA to stimulate courtship behavior through slow steroid-hormone

modification of steroid-, opioid-, and dopamine-related gene expression, but that in the absence of a nesting territory the mPOA is prevented from stimulating courtship behavior.

Effects of T and territory possession on behavior

We found that T was not sufficient to trigger courtship behavior (i.e. singing and the gathering of nesting material). Instead, both T and territory possession were necessary to induce courtship in response to females. This finding agrees with multiple past studies in starlings (Gwinner et al., 2002; Pinxten et al., 2002; Ritters et al., 2000; Sartor and Ball, 2005), and is consistent with studies of other species, including other birds, fish and primates, in which social ascension, often reflected in territory acquisition, is necessary to trigger sexual and courtship behavior (Abbott, 1984; Collias et al., 2002; Maruska, 2014; Maruska and Fernald, 2013). The control over courtship behavior can be rapid, which allows the brain to adjust behavior quickly in relation to the current environment (in starlings, this appears to occur within 1 hour of being introduced to a nesting territory; Gwinner et al., 2002). This is adaptive as it prevents birds that lack the resources to breed from engaging in energetically costly or risky courtship displays.

In contrast to courtship behavior, birds treated with T displaced conspecifics more than non-T treated birds whether a nesting territory was present or not. This finding is consistent with the well-established role of T in agonistic behavior (Arnold, 1975; Barkley and Goldman, 1977; Moore, 1988; Watson and Parr, 1981).

T alone alters patterns of mRNA expression in the mPOA—Although both T treatment and territory possession were necessary to stimulate courtship behavior, only T treatment altered mRNA expression in the mPOA. Specifically, T increased AR, ARO, PENK, MOR and decreased TH mRNA in the mPOA. It is possible that these T-induced changes function to increase agonistic behavior, and there is some evidence that ARO and opioids modulate the frequency of agonistic behaviors in the Japanese quail (Kotegawa et al., 1997; Schlinger and Callard, 1990). However, a greater body of evidence demonstrates that the products of these genes in the mPOA modify male sexual and male sexually-motivated behaviors in both birds and mammals. For example, blocking AR in the mPOA decreases preference for a sexually receptive female in rats (McGinnis et al., 2002), ARO inhibitors in the mPOA decrease sexual behavior in quail (Balthazart et al., 1990), and, as reviewed in the introduction, both dopamine and opioids act in the mPOA to modulate sexual and sexually-motivated behaviors, including courtship song in male starlings. We thus interpret these findings as evidence that T acts at intracellular receptors to modify gene expression in the mPOA to prepare the brain to stimulate courtship behavior. Because the addition of a nesting territory does not contribute to changes in gene expression in the mPOA, we suggest that the mPOA is prepared by T to stimulate male courtship behavior but is somehow prevented from doing so in the absence of appropriate social factors (i.e. territory possession).

One possibility is that the mPOA is continuously (tonically), inhibited in males that do not possess territories. The idea that a tonic inhibition suppresses male courtship behavior in the absence of appropriate stimuli is supported by studies in both rats and quail (Cornil et al.,

2004, 2002, Hull et al., 1997, 1995). Past data from our lab strongly suggest that opioid activity may, in part, mediate this inhibition. For example, in male starlings, blocking opioid receptors in the mPOA increased courtship song in low singing males (Kelm-Nelson et al., 2013) and many studies demonstrate that opioid activity in the mPOA inhibits male sexual behavior (Furukawa et al., 1995; Kotegawa et al., 1997; Pfaus, 2009). In the present study, T treatment increased opioid-related gene expression, suggesting that in addition to preparing the mPOA for courtship behavior, T also increases inhibition of the mPOA to prevent birds from producing energetically costly courtship behaviors when they lack the resources to breed. We thus propose that once a male acquires a territory, a rapid reduction in opioid release disinhibits the mPOA, allowing a male to produce courtship behavior in response to a female. Other neuromodulators or neurotransmitters, such as GABA (Bhattarai et al., 2011), may also play a role and must be examined in future work.

Relationship of the present findings to past studies of T-induced gene expression in the mPOA

Our findings showing that T increased mRNA expression of AR, ARO and PENK in the mPOA are consistent with past studies in birds and rodents measuring both mRNA and protein (Balthazart and Foidart, 1993; Bittman, 2003; Holland et al., 1998; Meek et al., 1997; Watson Jr. et al., 1986). The T-induced increase in MOR expression in the mPOA is, to our knowledge, the first time that this has been reported. Furthermore, although T has been found to decrease dopamine turnover in the POA of rats (Simpkins et al., 1980a, 1980b), to our knowledge, our data are the first to show a T-induced reduction in TH mRNA in the mPOA. However, other studies show that T can increase TH immunoreactivity in the preoptic area (Chu and Wilczynski, 2002) or have no effect on TH protein immunolabeling in the mPOA (Du and Hull, 1999). Because TH is also a synthetic enzyme of norepinephrine, it is also possible that T is affecting norepinephrine synthesis. Furthermore, dopamine activity may be influenced by nitric oxide release in the mPOA, and nitric oxide synthesis is also regulated by T (Du and Hull, 1999; Lorrain and Hull, 1993). Future studies are needed to understand these relationships.

Finally, T did not alter the expression ER β , D1 and D2 dopamine receptor mRNA in the mPOA. With respect to ER β , our finding agrees with a study in song sparrows showing that ER β mRNA in the mPOA does not change across seasons despite seasonal differences in circulating T (Wacker et al., 2010). Similarly, the findings for D1 and D2 receptors also agree with a study in starlings showing that D1 and D2 receptor densities did not differ in breeding-season condition birds treated with T compared to nonbreeding-condition birds with low T (Heimovics et al., 2009).

Limitations of qPCR

As with any technique, it is important to highlight the limitations of our methodology. qPCR data, though highly quantifiable, represents mRNA expression and not the final protein product. Thus, for example, mRNA measured in this study may not be translated in the mPOA; the final location of translated protein may be in axon terminals outside the mPOA. Future studies manipulating the genes measured in this study in the mPOA are needed to understand their role in courtship behavior.

Concluding remarks

Our findings are consistent with studies showing that T is necessary for the production of courtship song, but that courtship singing will not occur outside the appropriate context. Data from this study suggest that T prepares the mPOA to respond to the presence of a female with high rates of sexually-motivated song once a male acquires a nesting territory. Changes in gene expression induced by T treatment may be necessary for sexually-motivated behavior; however, territory ownership controls the timing of sexually-motivated behavior, perhaps by rapidly inhibiting a tonic inhibition of mPOA neurons. Future work is needed to assess the effect of territory possession on motivation systems in the brain.

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Highlights

- T and territory possession interact to activate courtship song and behavior
- T but not territory possession altered patterns of mRNA expression in the mPOA
- T increased steroid hormone-related mRNA AR and ARO in the mPOA
- T increased mRNA of opioid-related genes MOR and PENK in the mPOA
- T decreased mRNA of dopaminergic-related gene TH mRNA in the mPOA

Figure 1.

Location of intermediate mPOA tissue punch used for mRNA analysis. The approximate punch size for mPOA was 2mm diameter. One 2mm punch directly rostral and one 2mm punch directly caudal of this coronal section were also obtained and pooled for qPCR analysis. Abbreviations: M = mesopallium; N = nidopallium; LSt = striatum laterale; GP = globus pallidus; Rt = nucleus rotundus; GLv = nucleus geniculatus lateralis, pars ventralis; CO = chiasma opticum; SL = nucleus septalis lateralis; SM = nucleus septalis medialis; mPOA = area preoptica medialis.

Figure 2.

Mean total song (A), nest material collection (B), and displacements (C) + SEM across all treatment conditions (testosterone abbreviated as T; nest box abbreviated as NB). Sample sizes for each group are indicated at the bottom of each bar. Empty square box points in (C) indicate outliers that were removed from analysis. * indicates $p < 0.05$, ** indicates $p < 0.01$, and *** indicates $p < 0.001$ by Fisher's LSD post-hoc tests.

Figure 3.

Relative mRNA expression measures of androgen receptor (A), aromatase (B) and estrogen receptor β (C) in the medial preoptic area (mPOA) for all treatment conditions (testosterone abbreviated as T; nest box abbreviated as NB). Sample sizes for each group are indicated at the bottom of each bar. ** indicates $p < 0.01$, and *** indicates $p < 0.001$ by Fisher's LSD post-hoc tests.

Figure 4.

Relative mRNA expression measures of mu-opioid receptor (A) and preproenkephalin (B) in the medial preoptic area (mPOA) for all treatment conditions (testosterone abbreviated as T; nest box abbreviated as NB). Sample sizes for each group are indicated at the bottom of each bar. * indicates $p < 0.05$ by Fisher's LSD post-hoc tests.

Figure 5. Relative mRNA expression measures of tyrosine hydroxylase (A), dopamine D1 receptor (B) and dopamine D2 receptor (C) in the medial preoptic area (mPOA) for all treatment conditions (testosterone abbreviated as T; nest box abbreviated as NB). Sample sizes for each group are indicated at the bottom of each bar. * indicates $p < 0.05$ by Fisher's LSD post-hoc tests.

Table 1

Details of primers used for reference genes and genes of interest.

Gene	Direction	Sequences	Annealing temperature (°C)	Product (bp)
GAPDH	Forward	AGCAATGCTTCCTGCACTAC	57.5	121
NM_204305.1	Reverse	CTGTCTTCTGTGTGGCTGTG		
HPRT	Forward	GACCTGGACTTGTCTGCAT	57	107
NM_204848.1	Reverse	ATTTCACGTGCCAGTCTCTC		
AR	Forward	ACTTGACCGTATCATTGCCTG	57.4	171
NM_001076688.1	Reverse	ATTCTGGGTAGTCCACGCTC		
ER β	Forward	GGAACATGCTGGAATTGAGGA	60	171
From sequence	Reverse	TTTTTGCCCCAGACCTTGTA		
ARO	Forward	TGGTGATGATGAAGGCAATCC	62	173
NM_001076691.2	Reverse	ATTCTCGTTTGCGTTTCCCCT		
DAR1	Forward	ACGAGAGGAAAATGACCCCC	58	112
NM_001243833.1	Reverse	GTTGTAGCCTTGTGCCAGTT		
DAR2	Forward	TACCAGTCCCCCTGAGAAAG	58	96
XM_002191611.2	Reverse	GTAGAGTTGTTGCCCCGATT		
TH	Forward	GCCATGCTGAACCTCTTCTT	60	298
XM_002198931.2	Reverse	GATGGTGGCACTTGTCCAAT		
MOR	Forward	GCAGATGCCCTAGCAACAAG	57	165
XM_002187352	Reverse	CACGTAGCGATCCACACTCA		
PENK	Forward	TGTCAGCAAAAGATACGGAGG	58.5	185
NM_001245451.2	Reverse	GGAGAATGGAGTCGGCAAAG		

Table 2
Sanger sequencing results of the qPCR product for each reference gene and gene of interest.

Gene	Sequence
GAPDH	GGGCTGGCAGGTATCCATGACACTTTGGCATCTCTGGAGGGTCTCATGACCACTGGTCCA TGCCATCACAGCCACACAGAAAGACAGAT
HPRT	ATTTGGAAAGTCTATAATTCTCTCATGGGCTCATCATGGGACAGGCACCGGNAGAGACTGGCA CGNTGAAATTA
AR	CACCACCTCCGGGCTCAGGGAGGTTTCCAGGCCCTCACCAAGGGTCTCTGGGACTCCGGCTGC ATCCGATTGGCCAAAGNACCTGCATCAGTTTACATTGTATCTCTGATCAAGGCACACATGG NTGAGCGTGGACTACCCAGAAANA
ERβ	AATCTCCCTTNNGCTGCAGCCTTCAGACTCTCGGGGAACCTCGGAGGATCATGCGGCCAGG GAGCATATCAAAAGATCCTCCAAATTCCTCTACACATTTCCCTCATCCCGTGATCTAATA CAAGGTCTGGGGCAAAAA
ARO	CGAAGTCCACACGAANGAAAGGGGAAGAGGCCCTCAACAACATCCAGTAAACAAATGGAC TTATCCATGCACCCCATAGAAAGGCAGCCTCTGCTGGAGATGGTTTTTACACCCAGGGGA AACGCAAAACGAGAATA
DAR1	GAGGAANACCGGGACAAAGGTCCACGGCCACGCCCTGGATCATGGATGAAGGGCTGCC TTGGGGGGGTCAITTTTCCCTCTI
DAR2	GGGNTNNTTGGTGTCTGTGGNNTTTGAAGGGCGCTTTTCTCAGGGGGACCTGGATAA
TH	CTTCCCCTGGTCCGGGTCTTAAAGGTGTTTGAGACATTTGAAGCTAAATTCACCAACC TGAGACGAGGCTTAGCCGAAGCCCGGTGAAGGGACTGCTGAACCTGGAATCTTTGTG CGCTGTGAAGTCCACAGCTCAGACCTCAATACTTTCATTAGCTCCATCAAGAGAGTGGA GAAGATGTGAGGACTACTAAGGAGGACAAATTTCACTGGTTTCCCAAGAAAGATCTGTGAA TTTGACAAAGTGCCACCATCA
MOR	GGATACCTTGAGGGACGTGGCCATTCTGGTACCATCTTTGTAGATTGTTATATCCATAGAC TACTACAAATATGTTTCAACAGTATCTTTCACACTCTGCACCATGATGTGGATCGTACGTGA
PENK	AGCGCAGCCCTGAATTGGAGATGAGGCCAAAGGAGCTGCAAAAGAGATACGGTGGCTTC ATGAGAAAGATGGGCAAGCCGGAGTGGTGGGTATACCAGAAACGATATGGTGGGT TTCCTTAAGCGCTTTGCCGACTCCCAITTCCTCAA