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Anna Grozhik April 18, 2012

Steroidal regulation of vasotocin receptor mRNA in a seasonally breeding songbird

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Abstract

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Behaviors associated with breeding are often seasonally modulated in a variety of species. These seasonal changes in behavior are mediated by gonadal steroids, levels of which likewise vary with season. The effects of androgens on behaviors associated with breeding may in turn be partly mediated by the neurohypophyseal nonapeptides vasopressin (VP) and oxytocin (OT) in mammals, and vasotocin (VT) in birds. The effects of testosterone (T) on production of these neuropeptides has been well-investigated; however, the regulation of VT receptors by T is not well understood. In this study, I investigated steroidal regulation of VT receptor (VTR) mRNA in a seasonally breeding songbird, the white-throated sparrow (*Zonotrichia albicollis*). VTRs and their mammalian homologues occur as several subtypes, and I focused on subtypes that have been most strongly implicated in social behavior: V1a, which bears the same name as its mammalian homologue, and OT-like receptor, which is homologous to mammalian OT receptor (OTR). Using *in situ* hybridization, I showed that in response to T treatment, V1a and OT-like receptor expression was altered in several regions associated with seasonal reproductive behaviors such as song. In some regions, the effects of T treatment depended on the presence or absence of a chromosomal rearrangement that affects singing behavior, plasma testosterone, and VT immunolabeling in this species. Overall, the results of this study strengthen evidence that VT may mediate T's behavioral effects in songbirds, and suggest that the chromosomal rearrangement in this species may affect the sensitivity of the vasotocin system to seasonal changes in T.

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INTRODUCTION

Many behaviors associated with breeding are seasonally modulated in a variety of species. The effects of season on behavior are often mediated by gonadal steroids, levels of which likewise vary with season. For example, exogenous testosterone (T) treatment has been shown to increase territorial aggression in male Syrian hamsters (*Mesocricetus auratus*) (Delville et al. 1996) and white-throated sparrows (*Zonotrichia albicollis*) (Archawaranon & Wiley 1988). Furthermore, T treatment increased territorial song production in male dark-eyed juncos (*Junco hyemalis*) (McGlothlin et al. 2007) and white-throated sparrows (Wiley et al. 1993). Gonadal steroids are known to be necessary for pair bond formation in monogamous voles (*Microtus ochrogaster*) (Sue Carter et al. 1995) and expression of sexual behavior in quail (*Coturnix japonica*) (Adkins & Pniewski 1978). These examples reinforce the idea that behaviors associated with breeding are under the influence of androgens in males.

What neural mechanisms may be mediating T's behavioral effects? A candidate family of neuropeptides is vasopressin (VP) and oxytocin (OT), and their nonmammalian homologues vasotocin (VT) and mesotocin (MT), respectively (Goodson & Bass 2001) (**Figure 1**). Like T, these neuropeptides have been strongly implicated in behaviors associated with breeding. For example, central infusions of VP have been shown to increase territorial aggression in prairie voles (Young et al. 1997) and Syrian hamsters (Ferris et al. 1984; Ferris et al. 1997), and central OT infusions increased sexual behavior in male rats (*Rattus norvegicus*) (Argiolas et al. 1986). VP and OT are also strongly implicated in pair bond formation in prairie voles (Williams et al. 1994). In nonmammals, the behavioral role of VT has been better investigated than that of MT; the role of VT in behavior overlaps the roles of both VP and OT in mammals (Goodson & Bass 2001). In amphibians, VT is known to regulate sexual behavior (Moore & Miller 1983; Moore et al. 2005a). In birds, VT infusions have been shown to increase territorial behavior, such as aggression (Goodson et al. 2004b), and song (Maney et al. 1997; Goodson 1998). VT may therefore be the neural mediator of T's behavioral effects in songbirds.

To further support the idea that behavioral effects of androgens are mediated by these neuropeptides, evidence demonstrates that T regulates the production of VP, OT, and VT. For example, in gerbils (*Meriones unguiculatus*), castration reduced the number of VP-IR cells the hypothalamus and lateral septum (LS), and T treatment reinstated it (Wang & De Vries 1993). T does not appear to regulate OT-IR fiber density (De Vries et al. 1986), although T may regulate expression of OT mRNA (Nomura et al. 2002). VT immunoreactivity in birds, like VP immunoreactivity in mammals, has been shown to be modulated by T. For example, treating zebra finches or canaries (*Serinus canaria*) with T increased the density of VT-IR fibers in the bed nucleus of the stria terminalis (BSTm) and LS (Voorhuis et al. 1988b; Kimura et al. 1999). Similarly, in male Japanese quail, T treatment increased expression of VT mRNA in the medial preoptic nucleus (POM) and BSTm, and enhanced VT immunoreactivity in the BSTm, LS, and POM (Panzica et al. 1999, 2001, 2002). T therefore modulates VP and VT production in mammals and birds.

The effect of T on expression of VP and VT is well-documented. Several previous studies have assessed the effect of T on expression of VP, OT, and VT receptors (VPR, OTR, and VTR, respectively) using radioligands. For example, T is known to regulate OT binding in the ventromedial hypothalamus (VMH) of rats (Johnson et al. 1991). Delville et al. (1996) showed that castration reduced, and T treatment increased VP binding in the ventrolateral hypothalamus (VLH) of Syrian hamsters. In canaries, T treatment increased VT-like binding in the robust nucleus of the arcopallium (RA), a song control region (Voorhuis et al. 1988a). Results of these studies suggest that T regulates expression of VPR and VTR, again strengthening evidence that VP/VT mediate T's behavioral effects. Notably, VPRs and VTRs occur as several receptor subtypes—which have unique gene sequences but are structurally similar—and the effect of T on expression of individual subtypes, particularly those implicated in social behaviors, has not been previously investigated in songbirds. In this study, I used *in situ* hybridization (ISH) to assess whether T regulates expression of two VTR receptor subtypes in a songbird model of

social behavior, the white-throated sparrow. These songbirds breed seasonally with accompanying changes in androgen levels and behavior (see Falls & Kopachena 2010 for review) and serve as an appropriate model for steroidal regulation of neural plasticity and social behavior.

To test the effect of T on VTR expression in this species, I focused on the two receptor subtypes that have been well investigated in the context of social behavior: V1a and oxytocin-like (OT-like) receptor. The V1a receptor binds VP-like ligands, which include both VP and VT, whereas the OT receptor ("OT-like" receptor in birds)binds OT-like ligands, which include VT and MT (Chini et al. 1995, Acharjee et al. 2004; Leung et al. 2009). The distributions of the mRNA of these receptors have been mapped by Leung et al. (2011) in the white-throated sparrow using ISH, and both V1a and OT-like receptor are now known to be highly expressed in several regions implicated in social behaviors such as song. OT-like receptor is expressed in the LS in the white-throated sparrow and zebra finch, and V1a is expressed in the VMH—in the white-throated sparrow (Leung et al. 2011). Several other regions with intense labeling of receptor mRNA belong to the song system (**Figure 2**), which is involved in song learning (Bottjer & Johnson 1997), maintenance (Bottjer 2004), and production (Wild 1990; Wild 1993). In the white-throated sparrow, VTR mRNA is found in several nuclei of the song system: OT-like receptor is expressed in HVC, RA, and the tracheosyringeal and lingual portions of the hypoglossal nucleus (nXIIts and nXIII, respectively), whereas V1a is expressed in the lateral and medial magnocellular nuclei of the anterior nidopallium (LMAN and MMAN, respectively) (Leung et al. 2011). Because results of previous studies show that the VT system is sensitive to T (Voorhuis et al. 1988a, 1988b; Johnson et al. 1991; Wang & DeVries 1993; Delville et al. 1996; DeVries et al. 1996; Kimura et al. 1999; Panzica et al. 1999, 2001; Nomura et al. 2002; Panzica et al. 2002), I hypothesized that in songbirds, T leads to seasonal changes in behavior in part by regulating expression of VTR subtypes.

It should be noted that the white throated sparrow occurs in two morphs that exhibit alternative life-history strategies (Kopachena 1992). Males of the white morph have higher plasma T during the breeding season (Spinney et al. 2006), and are more aggressive, provide less parental care, engage in more copulations outside of the pair-bond, and sing more than males of the tan morph (see Falls & Kopachena 2010 for review). Interestingly, morph differences in behavior correlate with neuroendocrine differences; males of the white morph and have denser VT staining in the BSTm and ventrolateral portion of LS (LSc.vl) (Maney et al. 2005), regions implicated in agonistic behavior and territoriality (Goodson 1998). Because the morphs exhibit differences in plasma T, social behavior and the neural VT system, I balanced the number of white and tan males in each treatment group and tested for effects of morph as well as treatment.

METHODS

Capture and housing

Animal capture and housing was done in accordance with guidelines for animal care established by Emory University, the Institutional Animal Care and Use Committee (IACUC), the National Institutes of Health, and federal and state laws.

Twenty male and ten female white-throated sparrows were captured during Fall migration in 2010 with mist nets, and blood samples were collected to confirm sex and morph via polymerase chain reaction (PCR) as established by Griffiths et al. (1998). After capture, the birds were housed in flight cages on short photoperiod (8.5 hours of light) to ensure their gonads were regressed (Wolfson 1952; Wingfield et al. 1997) and endogenous sex hormone levels therefore remained low.

Birds were removed from flight cages when needed for the study and thereafter housed in individual cages in sound-attenuated booths. The birds continued to be housed on short photoperiod at this time and for the remainder of the investigation.

Hormone manipulation

Once in individual cages, half of the males (N=10) received subcutaneous silastic capsules (length 12 mm, I. D. 1.46 mm, O. D. 1.96 mm) (Dow Corning, Midland, MI) filled with T (Steraloids, Newport, RI) and sealed at both ends with silicone adhesive. The remaining males (N=10) received empty capsules. Each treatment group contained six birds of the tan morph and four birds of the white morph. Males were housed in mixed treatment groups, with four to six individual cages in each sound booth.

We also manipulated hormone levels in ten conspecific females to be used as social stimuli during behavioral observation of the males (see below). Each female received a 12 mm silastic capsule filled with estradiol (E2) (Steraloids) to stimulate receptivity (Moore, 1983). Each female was then housed in an individual cage adjacent to one other treated female for two weeks before the behavioral observations.

Behavioral observations

The evening before tissue collection, each male was placed into a sound-attenuated booth equipped with a camera and microphone forty five minutes before lights-out. An E2-treated female of the opposite morph was placed inside the booth, but outside visual contact with the male. Each male within a treatment group (T or control) was exposed to a unique female. The camera began recording at lights-on the following morning, and recording proceeded for one hour. At that time, the female was placed in view of the male, and recording continued for another hour.

Tissue collection

After behavioral recording, each male was deeply anesthetized with isoflurane. A blood sample was collected from the jugular vein for assaying plasma T levels, and then the male was rapidly decapitated and the brain was removed and frozen on dry ice. All brains were subsequently stored at -80ºC, and then sectioned into 20 µm sections in the coronal plane. Sections were mounted on Superfrost plus microscope slides (Fisher, Pittsburgh, PA) and kept at -80ºC until ready to use for ISH.

Testosterone assay

Plasma T was assayed at Virginia Tech using a commercially available RIA kit, and methods of the assay have been previously described by Moore et al. (2005b). All samples were run in a single assay, and the intra-assay coefficient of variation was 4%. The lower limit of detection was 0.05 ng/ml. Effects of treatment on plasma T were analyzed using a *t*-test.

Cloning

Total RNA was extracted from white-throated sparrow brain with a RNEasy minikit (QIA-GEN, Valencia, CA), and was reverse transcribed with SuperscriptIII reverse transcriptase (Invitrogen, Carlsbad, CA) into cDNA. The cDNA product was used as template in PCR amplification of the genes of interest. Two separate PCRs with two different primer pairs were performed for two genes of interest: V1a and OT-like receptor. The primer sequences for V1a were GCTCGTACGGGATGATCG (forward) and CGGGTTACAGCAACTGTTCA (reverse). Primer sequences for OT-like receptor were ACATCACCTTCAGGTTCTACGG (forward) and ATGTAGATCCACGGGTTACAGC (reverse). The resulting fragments (477 bp for V1a and 677 bp for OT-like receptor) were cloned into pCRII plasmid vectors using a TOPO TA Cloning Kit (Invitrogen). The plasmids were transformed into One Shot Mach 1-T1 *E. coli* (Invitrogen), and the bacteria were grown overnight. The following morning, plasmids were isolated from bacterial

cells using a QIAprep Spin Midiprep Kit (QIAGEN) and sequenced by Agencourt (Danvers, MA). Sequences were confirmed using the basic local alignment search tool (BLAST; NIH) by aligning with known homologous genes in the zebra finch. After sequencing, plasmids were linearized with either *BamH1* or *EcoR1* depending on the orientation of the insert as determined by plasmid sequence.

Tissue preparation

The day before ISH, slides with brain sections were defrosted to room temperature and dried with a conventional blow-dryer. The sections were then fixed for 5 min in 4% paraformaldehyde (pH=7.4) and washed of fix with PB twice for 5 min each. Slides were then dipped into water, washed in 0.1M triethanolamine (TEA) with 0.25% acetic anhydride for 10 min, and then in 2x sodium saline citrate (SSC) for 3 min. The tissue was then dehydrated with 70%, then 95%, then 100% ethanol for 3 min each, and delipidated with chloroform for 5 min. Finally, the sections were rehydrated with 100%, then 95% ethanol for 3 min each. Slides were then left to air-dry until the following day.

***In situ* hybridization**

ISH procedures have been previously described (Leung et al. 2011) and were adapted from Burmeister et al. (2008) and Wiemann et al. (1990). Linearized plasmids from the cloning process were transcribed to produce complementary riboprobes. Transcription was done using a T7/SP6 Riboprobe *In Vitro* Transcription Kit (Promega, Madison, WI), and incorporated ^{35}S-labeled UTP (Perkin Elmer, Waltham, MA) which was concentrated via vacuum drying. The transcription reaction proceeded for one hour, at which time unused template was destroyed with DNase and the reaction was terminated with EDTA. Riboprobes were purified using Illustra ProbeQuant G-50 Microcolumns (GE Healthcare, Piscataway, NJ). After purification, riboprobes were denatured at 80ºC for 5 min, and diluted to a final concentration of $1x10^7$cpm/ml in

hybridization buffer, which was composed of 50% deionized formamide, 10% dextran sulfate, 300 mM NaCl, 10 mM Tris, 1mM EDTA, and 10 mM dithiothreitol (DTT). The hybridization buffer and riboprobe mixture was then applied to the slides at a volume of 100 µl per slide. Slides were immediately covered with glass coverslips, placed into metal racks, and submerged in a prewarmed mineral oil bath. The slides remained in the mineral oil bath overnight at temperature appropriate for specific hybridization—67ºC for V1a or 64ºC for OT-like receptor—which was determined by the length and GC content of the riboprobes (Sambrook & Russell 2001).

The following morning, slides were removed from the mineral oil bath, washed of oil in chloroform twice for 10 min each, separated from the coverslips, and washed with 4x SSC containing 2 mM DTT twice for 15 min each. Riboprobes that did not hybridize were destroyed with RNase by incubating the slides in RNase buffer (0.5 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA) with RNase (0.03mg/ml; Roche Applied Science, Indianapolis, IN) for 30 min at 37ºC. The slides were then washed of RNase in RNase buffer with 1 mM DTT for 30 min at 37ºC. Next, slides were processed in washes of 2x SSC with 1 mM DTT for 30 min at room temperature, 0.1x SSC with 1 mM DTT for 30 min in a water bath at hybridization temperature, and 0.1x SSC for 3 min at room temperature. Finally, the slides were dehydrated with 50% and 85% ethanol with 300 mM ammonium acetate for 3 min each, and 100% ethanol for 3 min.

Imaging

Dry slides were placed into film cassettes and covered with Kodak BioMax Maximum Resolution autoradiographic film (Kodak, Rochester, NY). Cassettes were protected from light and the film was exposed for five days. Films were developed with a Konica SRX101-A developer and scanned at 1600 dpi for viewing with an Epson V700 scanner. For four of the birds (two from each treatment group), alternate sets of sections were fixed, and stained with

toluidine blue (Nissl stain) for clarification of cell groups. These slides were scanned at 20x resolution with an Olympus Nanozoomer system (Olympus, Center Valley, PA).

Determination of regions of interest

Regions of interest included intensely labeled areas known to be implicated in reproductive behaviors. Several of these areas were strongly implicated in social behavior, and others belonged to the song system. VTR expression in all regions of interest has been previously described by Leung et al. (2011). In regions implicated in social behavior, V1a mRNA was expressed in the VMH. Expression of OT-like receptor mRNA was found in three distinct sub-regions of the septal area: the dorsal portion of LS (LS.d), the medial septum (MS), and the ventral LS (LS.v) (Goodson et al. 2004a). The song system likewise contained V1a and OT-like receptor mRNA expression in several nuclei. V1a mRNA in the song system was expressed in LMAN and MMAN. Regions expressing OT-like receptor mRNA included RA, HVC, and nXIIts.

Two additional areas, which fell outside the song control system and social behavior network, were intensely labeled. The dorsal arcopallium (Ad) contained a high density of OT-like receptor mRNA. A particularly dense area of V1a labeling was observed in the lateral forebrain bundle (FPL) cell group, which was described by Leung et al. (2011).

Quantification of signal and background correction

Before quantifying the VTR signal in each region of interest, I first confirmed the anatomical location of the signal by superimposing digital images of scanned films and the corresponding images of Nissl-stained slides in Powerpoint (version 12.3.2). To quantify the area encompassed by the label and its optical density, the film images were opened with Image J (National Institutes of Health) and converted to 8-bit grayscale. The labeled area was then traced with the lasso tool (see **Figure 3** for examples). Labeled areas were traced in the left and right hemispheres in every section in which they were unambiguously present except in Ad,

where mRNA expression was quantified only in sections caudal to RA to prevent sampling from RA. For every selection, area in pixels and mean optical density (OD) were recorded. The OD of every selection was then corrected by subtracting the OD of an area (0.18 mm^2) adjacent to the sampled area but which contained no obvious label. The corrected OD measurements were then averaged across sections for each region of interest and converted to positive values for the statistical analysis. Area measurements were summed across sections for each region of interest in each animal.

Statistics

Area and corrected OD measurements were analyzed using a repeated-measures MANOVA with measure (area or OD) and region of interest (12 regions) as the repeated measures. MANOVAs were then run on area and OD separately. Finally, univariate F-tests were performed to determine the effects of treatment and morph in each region. In regions where significant effects were observed, post hoc pairwise comparisons were conducted between treatment groups within morphs and vice-versa.

RESULTS

Plasma T

Birds that received implants filled with T had significantly higher plasma T than controls ($P<0.001$). In the T-treated group, plasma T averaged 9.998 $\pm$ 0.697 ng/ml and ranged from 7.264 ng/ml to 12.466 ng/ml. In the control group, plasma T averaged 0.079 $\pm$ 0.012 ng/ml and ranged from 0.063 ng/ml to 0.186 ng/ml.

Overall effects

The results of this experiment support the hypothesis that T changes VTR expression in regions implicated in social behaviors, such as song. Overall, a MANOVA testing for effects of treatment and morph on expression of OT-like receptor and V1a showed no main effects of treatment ($F_{1,16}$=0.073, *P*=0.790) or morph ($F_{1,16}$=0.254, *P*=0.621), but there was a significant region × treatment × morph interaction ($F_{11,6}$=6.343, *P*=0.017). A MANOVA testing for effects of treatment and morph on the area covered by VTR expression showed no main effects of treatment ($F_{12,5}$=2.614, *P*=0.149) or morph ($F_{12,5}$=3.196, *P*=0.104), but there was a significant treatment × morph interaction ($F_{12,5}$=5.090, *P*=0.042). Finally, a MANOVA testing for effects of treatment and morph on the optical density of VTR expression revealed no main effects of treatment ($F_{12,5}$=1.235, *P*=0.435) or morph ($F_{12,5}$=1.682, *P*=0.295), but there was a significant treatment × morph interaction ($F_{12,5}$=5.433, *P*=0.037). Results specific to each region of interest are presented in **Table 1**, and significant effects are discussed below.

VMH

T treatment significantly changed receptor expression in the VMH, and these effects are presented in **Figure 4**. In this region, T-treated birds had significantly greater density of V1a mRNA (*P*=0.008) compared to controls. Moreover, there was a significant treatment × morph interaction (*P*=0.002), suggesting that that effect of treatment depended on morph. Indeed, the effect of treatment on V1a density in the VMH was driven by males of the tan morph (*P*<0.001). A main effect of morph was observed as well; control males of the white morph had significantly higher density of V1a mRNA (*P*=0.001) than those of the tan morph.

Song system

T treatment significantly changed VTR expression in two regions of the song system: HVC and nXIIts. In HVC, there were no main effects, but there was a significant treatment × morph

interaction (P=0.020), indicating that the effect of treatment depended on morph. A post-hoc pairwise comparison revealed that treatment affected birds of the white morph only; T-treated males of the white morph had greater area covered by OT-like receptor mRNA compared to controls (P=0.049). Likewise, in nXIIts, there was a significant treatment × morph interaction (P=0.041), indicating that the effect of treatment depended on morph in this region. A post-hoc pairwise comparison revealed that here, T treatment decreased the density of OT-like receptor mRNA in only in males of the white morph (P=0.014). Notably, I observed a borderline significant treatment × morph interaction in LMAN (P=0.060), and a post-hoc pairwise comparison showed that in this region, T treatment increased the area covered by V1a only in birds of the white morph (P=0.026). These results are summarized in **Figure 5**.

Other regions

I tested for effects of treatment and morph in two additional areas, which were strikingly labeled: Ad and the FPL cell group. I found that T treatment significantly decreased the density of OT-like receptor mRNA in Ad (P=0.043) compared with controls. In the FPL cell group, T treatment significantly increased the area covered by V1a mRNA (P=0.009) compared with controls. Although the treatment × morph interaction in this region was not significant, a post-hoc pairwise comparison revealed that the effect of treatment was driven by males of the white morph (P=0.012). These results are summarized in **Figure 6**.

DISCUSSION

In this study I have shown that T regulates transcription of V1a and OT-like receptor in regions implicated in social behaviors, including song, in a seasonally breeding songbird. In the VMH, T upregulated expression of V1a. In the song system, T upregulated expression of OT-like receptor in HVC and downregulated expression of this receptor in nXIIts. Outside of the

VMH and song system, T upregulated V1a expression in the FPL cell group, and downregulated OT-like receptor expression in Ad. These results strengthen evidence that nonapeptides may mediate T's behavioral effects.

Several known mechanisms may explain the regulatory effects of T. Transcriptional effects of T usually occur through androgen receptor (AR), which migrates from the cell cytosol to the nucleus and binds to androgen-responsive elements in promoters of genes, acting as a transcription factor. T can also modulate gene transcription through estrogen receptors (ERs) after being aromatized to 17β-estradiol (E2); in fact, the restoring effects of T in castrated animals can occur via aromatization to E2 (Pak et al. 2007). For example, in male rats, castration eliminated VP immunoreactivity in the LS, and E2 reversed this effect (De Vries et al. 1986). In other words, T can change gene transcription via AR and ERs.

AR, ERs, and aromatase are expressed in some of the regions in which I observed effects of T on VTR expression, providing some insight into how androgens may regulate VTRs. Balthazart et al. (1992) found AR in several regions of the song system, including HVC, in the zebra finch and canary. AR was also found in the VMH and the PVN in the zebra finch (Balthazart et al. 1992). These results suggest that T may regulate VTR mRNA via AR in HVC, the VMH, and the PVN. Like the expression of AR, the expression of aromatase and ERs is found in several regions in which I observed effects of T. In zebra finches, aromatase has been found in the VMH (Saldanha et al. 2000). The α subtype of ER has been found in HVC, whereas the β subtype of ER has been found in the VMH and the PVN (see Gahr 2001 for review). The overlap of the distributions of AR, aromatase, and ER in the VMH suggests that T's effects could have been mediated by AR as well as ERs in this region.

There was little overlap between regions in which T is known to regulate production of VT and in which T regulated VTR expression. As has been suggested previously in mammals and

birds, peptides and their receptors may be independently regulated. For example, in prairie voles and rats, T affected VP immunoreactivity in the LS (De Vries et al. 1984; Wang & De Vries 1993). Likewise, T regulated VT immunoreactivity in the LS in zebra finches (Kimura et al. 1999), canaries (Voorhuis et al. 1988b), and quail (Panzica et al. 1996). In studies of the effect of T on VPR and VTR expression, however, T treatment did not alter expression of these receptors in the LS of either mammals (Delville et al. 1996; Young et al. 2000) or birds (Voorhuis et al. 1988a). Likewise, in this study, I did not find an effect of treatment on OT-like receptor expression in the LS. Furthermore, T has not been found to regulate VT in any of the regions in which I observed significant effects of treatment on VTR expression (De Vries et al. 1984; Voorhuis et al. 1988b; Panzica et al. 1996; Kimura et al. 1999;). The regulation of peptides and their receptors in disparate regions is further exemplified in studies of sex differences in VP/VT immunoreactivity and VPR/VTR expression. For example, sex differences have been found in VP immunoreactivity in the BSTm, medial amygdala (MA), LS, and mPOA of prairie voles (Bamshad et al. 1993); however, no sex differences were found in the expression of V1a in this species (Lim et al. 2003). Likewise, in songbirds, sex differences have been found in VT immunoreactivity in the LS (Voorhuis et al. 1988b), but no sex differences have been found in the expression of VTR subtypes (Leung et al. 2011). In other words, sex differences in the VP and VT systems are driven by regulation of peptide but not receptor. The finding that VT immunoreactivity in the LS is sensitive to androgens, but VTR expression is not is therefore consistent with literature showing that within a given region, peptides and their receptors are independently regulated.

White-throated sparrows occur in two plumage morphs that differ with respect to circulating T and T-dependent behaviors. Because I balanced the number of white and tan males in each treatment group, I was able to test for effects of morph on VTR expression as well as for interactions between morph and treatment. In all of the regions in which I observed

significant effects of treatment, except Ad and the FPL cell group, the effect of treatment depended on morph. For example, T altered the expression of OT-like receptor in HVC and nXIIts only in birds of the white morph (**Figure 5**), suggesting a morph-dependent sensitivity to T treatment in these areas. Notably, T increased only the area covered by—not the density of—OT-like receptor label in HVC. Song production is known to increase HVC volume (Alvarez-Borda & Nottebohm 2002), and T treatment induces a higher song rate in birds of the white morph than in birds of the tan morph. In this study, T-treatment may have increased song rate, and thus HVC volume, without an increase in the number of receptors. In nXIIts, I observed a decrease in the density of labeling in response to treatment, and this finding could likewise indicate growth of a nucleus. T treatment is known to increase the number and size of cells within song nuclei (Tramontin et al. 2000), meaning receptors on these cells could be distributed less densely. nXIIts is known to be larger in males of the white morph—which typically have higher levels of plasma T—than those of the tan morph (DeVoogd et al. 1995). Consequently, the number of OT-like receptors could be comparable between morphs, and birds of the white morph could exhibit lower receptor density, as I found in this study. To check for treatment and morph effects on volume of these nuclei, HVC and nXIIts size could be measured in the Nissl-stained sections from this study.

Morph differences in behavior in this species have sometimes been attributed to morph differences in plasma T, as males of the white morph exhibit higher levels of plasma T during the breeding season (Spinney et al. 2006). In a study by Maney et al. (2009), however, behavioral differences between morphs persisted after males received T treatment that equalized their plasma T levels. These results suggest that the alternative reproductive strategies may be better explained by differential sensitivity to T in brain regions regulating those behaviors. For example, in the VMH, birds of the white morph expressed more V1a than those of the tan morph; furthermore, T upregulated V1a receptor expression in birds of the tan

morph to the level observed in control birds of the white morph. These findings show a clear evidence supporting the hypothesis that morph differences in behavior are driven in part by differential responses to plasma androgens.

Morph-dependent sensitivity to T treatment likely originates in a chromosomal inversion that segregates absolutely between morphs, as several genes in this inversion are involved in reproductive hormone action in the brain (Maney 2008). Because the white morph carries the inversion and the tan morph does not, expression levels of certain genes may be morph-dependent. Two genes located in the inversion are of particular interest: 5α-reductase (JW Thomas, unpublished), an enzyme that converts T to dihydrotestosterone (DHT), which cannot be aromatized, and the α subtype of ER (ERα) (Thomas et al. 2008). Expression of ERα has been shown to vary between morphs (BM Horton, unpublished), and although there were no morph differences in plasma DHT in the birds from this study, expression of 5α-reductase may be morph-dependent in the brain, where it is strongly expressed in HVC (BM Horton, unpublished). These observations suggest that effects of T treatment can consequently be morph-dependent. The regulation of neural receptor expression by androgens and season has been previously studied in mammals and birds and is a common theme in the field of neuroendocrinology. In rats, for example, levels of androgen receptor (AR) mRNA in the hypothalamus and septum increased significantly after castration, and this effect was abolished with T treatment (Krey & McGinnis 1989; Burgess & Handa 1993). In another study, castrated rats had increased levels of serotonin receptor 1A (HTR1A) and decreased serotonin receptor 2A (HTR2A) mRNA in the VMH, and all changes were reversed with T treatment (Zhang et al. 1999). Androgens likewise regulate neural receptor expression in birds. For example, in starlings (*Sturnus vulgaris*), levels of α2-noradrenergic receptor protein in HVC and RA were found to be lowest when T level was highest (Riters et al. 2002). AR has likewise been found to be regulated by T in songbirds; in white-crowned sparrows, T treatment rapidly increased AR

protein labeling in HVC (Soma et al. 1999). Similarly, the level of AR mRNA in the para-HVC (pHVC) of song sparrows (*Melospiza melodia morphna*) was highest when circulating T level was high (Wacker et al. 2010). Results of these studies demonstrate that T regulates transcription as well as translation of several of neural receptors in brain areas implicated in reproductive behavior. It should be noted that in this study I assessed the levels of VTR mRNA. It remains possible that T regulates translation and transcription of VTRs independently. Nevertheless, this study of steroidal regulation of VTR mRNA adds to the existing body of literature and enhances our understanding of steroidal and seasonal regulation of receptor expression.

Finally, although I observed an effect of T on VTR expression, in this study, I did not determine whether the T-induced changes in VTR were accompanied by changes in behavior. Even if a link were established, however, it would be premature to conclude that changes in VTR expression drive seasonal changes in behavior. Like plasma reproductive hormones, neuropeptide and receptor expression are known to be affected by behavior. For example, in prairie voles, mating induced an increase in production of dopamine and dopamine receptors (Young & Wang 2004). Therefore, as an alternative explanation for the observations described here, it is possible that T leads to behavioral changes through another neural pathway. These behavioral changes could then produce the changes in VTR expression I observed here. To investigate the functional significance of changes in VTR expression, future studies should focus on developing and using subtype-specific VTR antagonists to better understand the role of VTR in social behaviors in birds.

Summary

Here I have shown that plasma androgens regulate the expression of VTR mRNA in brain regions implicated in seasonal behaviors in a seasonally breeding songbird. Moreover, I have

demonstrated that in this species, neural responses to T are morph-dependent, suggesting that morph differences in behavior may be driven by morph-dependent sensitivity to androgens. This study suggests that seasonal behaviors are modulated in part via changes in VTR expression, and adds to a growing body of literature demonstrating that neuropeptides and their receptors are sensitive to gonadal steroids.

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TABLES

TABLE 1. F and *P* Levels for the Effects of Treatment and Morph

	Area covered						Corrected optical density						Post-hoc pairwise comparisons (*P*)			
	Treatment		Morph		Treatment × Morph		Treatment		Morph		Treatment × Morph		T vs. control		Tan vs. white	
Region	F1,16	*P*	F1,16	*P*	F1,16	*P*	F1,16	*P*	F1,16	*P*	F1,16	*P*	Tan	White	T	Control
Social behavior network																
Hypothalamus	1.216	0.286	3.424	0.083	1.391	0.255	9.031	**0.008**	6.185	**0.024**	13.460	**0.002**	**<0.001**	0.677	0.486	**0.001**
LSc.d	1.670	0.215	0.054	0.820	0.019	0.891	0.948	0.345	0.041	0.842	0.012	0.913	-	-	-	-
LSc.v	1.991	0.177	1.393	0.255	1.712	0.209	0.000	0.996	0.005	0.944	0.183	0.675	-	-	-	-
MS	0.092	0.766	0.874	0.364	0.348	0.564	0.114	0.740	0.644	0.434	0.074	0.789	-	-	-	-
Song system																
HVC	0.048	0.829	0.117	0.737	6.738	**0.020**	0.177	0.680	2.891	0.108	1.645	0.218	0.123	**0.049**	0.107	0.102
LMAN	2.135	0.163	0.052	0.823	4.038	0.062	0.780	0.390	0.272	0.609	1.233	0.283	0.706	**0.026**	0.306	0.089
MMAN	0.008	0.932	0.510	0.486	2.634	0.124	0.015	0.904	1.019	0.328	0.788	0.388	-	-	-	-
nXIIl	1.780	0.201	0.812	0.381	0.012	0.915	0.201	0.660	1.894	0.188	3.071	0.099	-	-	-	-
nXIIts	0.553	0.468	0.170	0.686	0.074	0.790	4.185	0.058	0.064	0.803	4.915	**0.041**	0.905	**0.014**	**0.043**	0.285
RA	0.137	0.716	0.239	0.632	0.918	0.352	1.517	0.236	0.300	0.592	0.924	0.351	-	-	-	-
Other regions																
Ad	1.242	0.282	0.767	0.394	0.855	0.369	4.809	**0.043**	0.286	0.600	0.889	0.360	0.052	0.324	0.742	0.383
FPL cell group	**8.876**	**0.009**	1.620	0.221	0.846	0.371	0.077	0.784	0.526	0.479	0.217	0.648	0.183	**0.012**	0.787	0.195

Table 1. F and *P* levels for the effects of treatment and morph. Univariate F-tests were conducted for each region, and post-hoc pairwise comparisons were performed for regions in which the F-tests showed significant or borderline significant effects.

FIGURES

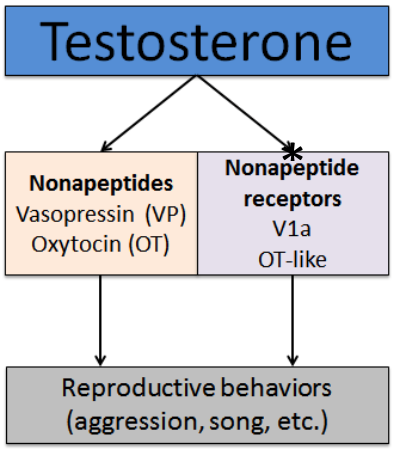

Figure 1. A model for the mediation of T's behavioral effects by the nonapeptides VP and OT, and their receptors. The regulation of VP and OT and their nonmammalian homologues by androgens is well-investigated, and the effects of T on V1a and OT-like receptor in songbirds (marked with asterisk) were assessed in this study.

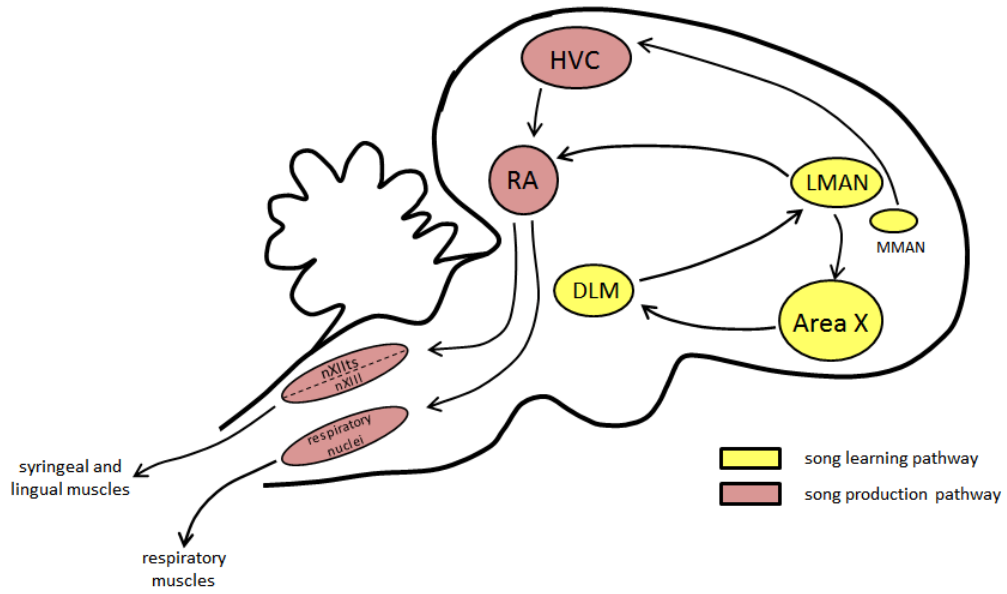

Figure 2. Sagittal view of the song system, a network of nuclei involved in song learning (yellow) and production (red). VTRs are expressed in the lateral and medial magnocellular nuclei of the anterior nidopallium (LMAN and MMAN, respectively), HVC, the robust nucleus of the arcopallium (RA), and the tracheosyringeal and lingual portions of the hypoglossal nucleus (nXIIts and nXII, respectively) (Leung et al. 2011).

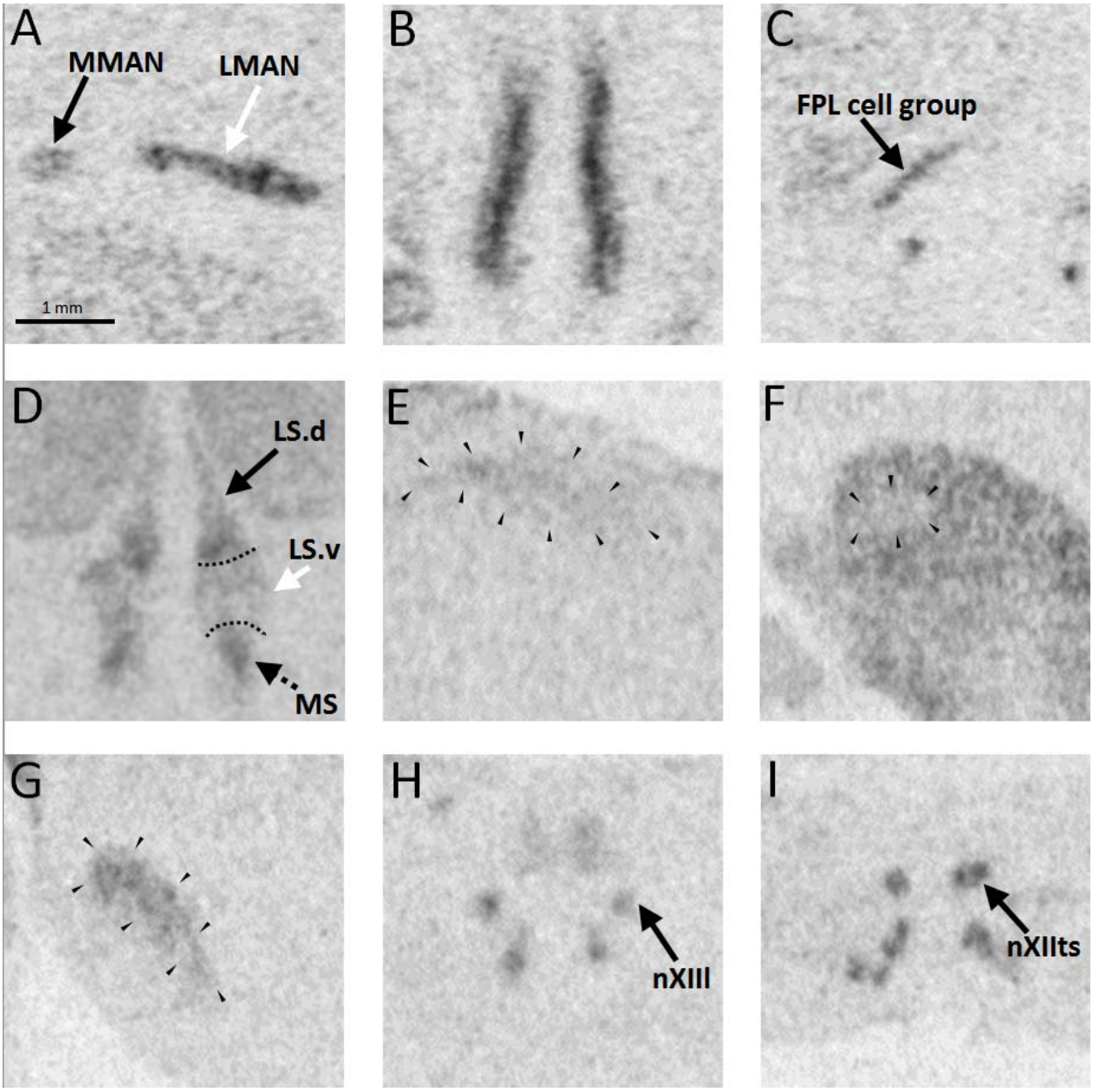

Figure 3. Example images from one individual (blank implant) showing regions of interest. Sections are in the coronal plane and progress from caudal to rostral. Panels B, D, H, I: midline is in center. Panels A, C, E, F, G: midline at left border of panel. Panels E, F, G: arrows point at border of label. A) V1a labeling in MMAN (black arrow) and LMAN (white arrow). B) V1a labeling in the VMH. C) V1a labeling in the FPL cell group. D) OT-like receptor labeling in the LS.d (black arrow), LS.v (white arrow), and MS (dotted arrow). E) OT-like receptor labeling in HVC. F) OT-like receptor labeling in RA (borders marked), which is surrounded by more

intensely labeled Ad. G) OT-like receptor labeling in Ad. H) OT-like receptor labeling in nXIII. I) OT-like receptor labeling in nXIIts.

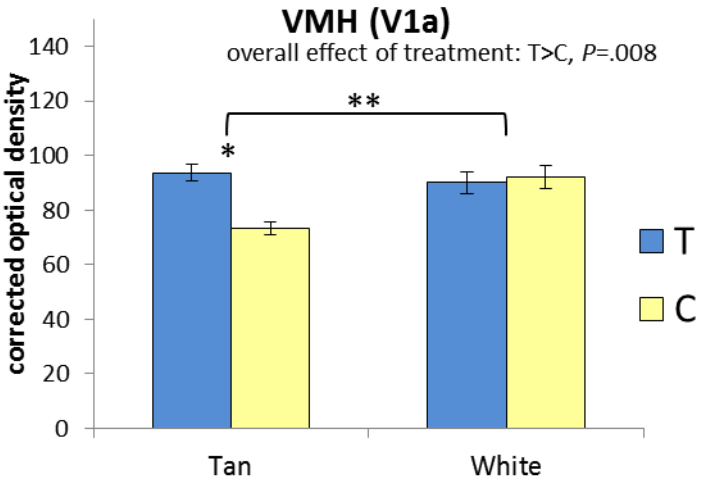

Figure 4. V1a expression in the VMH. In this region, there was an overall effect of treatment: T-treated birds had higher density of V1a mRNA compared to controls. A post-hoc pairwise comparison showed that this effect of treatment was driven by males of the tan morph (*). Furthermore, there was a main effect of morph (**); males of the white morph had higher V1a density than males of the tan morph.

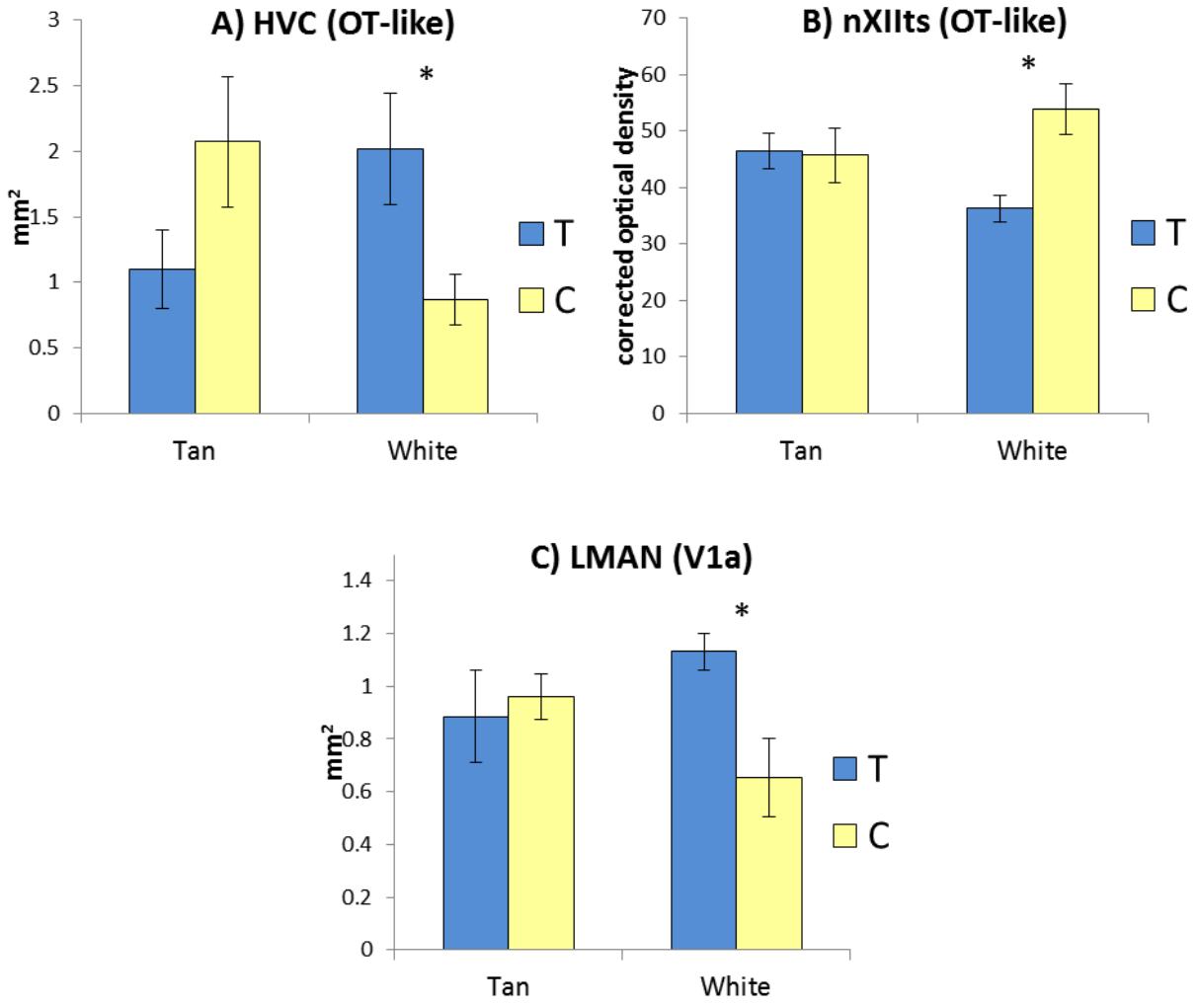

Figure 5. VTR expression in three regions of the song system. A) In HVC, there were no main effects of treatment or morph, but there was a significant treatment × morph interaction. A post-hoc pairwise comparison revealed that T treatment increased the area covered by of OT-like receptor mRNA only in birds of the white morph. B) In nXIIts, there were no main effects of treatment or morph, but there was a significant treatment × morph interaction. A post-hoc pairwise comparison showed that T treatment decreased the area covered by OT-like receptor mRNA only in birds of the white morph. C) In LMAN, there were no main effects of treatment or morph, but there was a borderline significant treatment × morph interaction. A post-hoc pairwise comparison revealed that T treatment increased the area covered by V1a only in birds of the white morph.

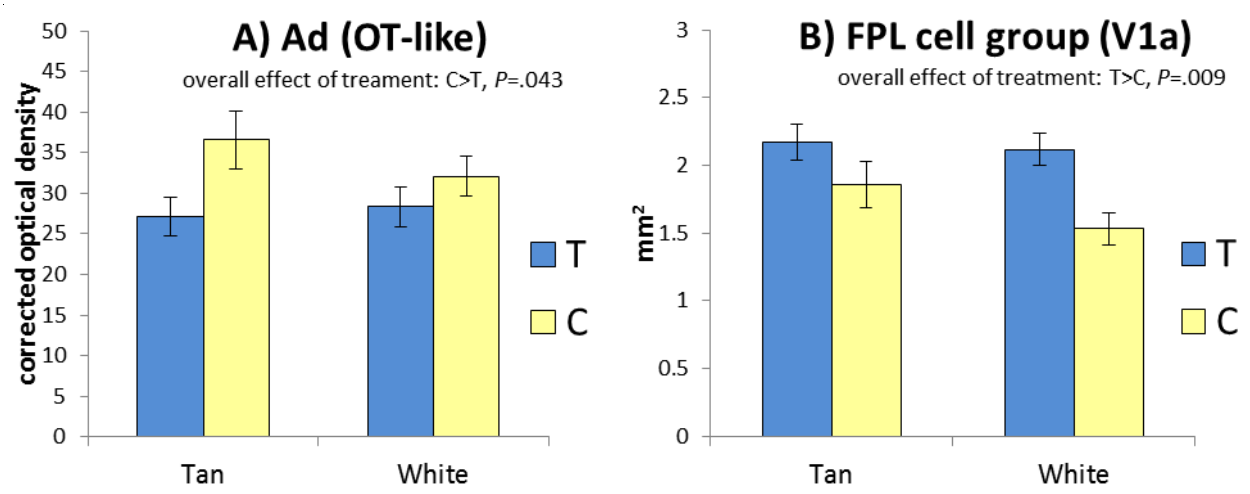

Figure 6. VTR expression in Ad and the FPL cell group. A)T treatment decreased the density of OT-like receptor mRNA in Ad compared to controls. B) In the FPL cell group, T treatment significantly increased the area covered by V1a mRNA compared to controls. Although the treatment × morph interaction in this region was not significant, a post-hoc pairwise comparison revealed that the effect of treatment was driven by males of the white morph.