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Co-localization patterns of neurotensin receptor 1 and tyrosine hydroxylase in brain regions involved in motivation and social behavior in male European starlings

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Abstract

Animals communicate in distinct social contexts to convey information specific to those contexts, such as sexual or agonistic motivation. In seasonally-breeding male songbirds, seasonal changes in day length and increases in testosterone stimulate sexually-motivated song directed at females for courtship and reproduction. Dopamine and testosterone may act in the same brain regions to stimulate sexually-motivated singing. The neuropeptide neurotensin, acting at the neurotensin receptor 1 (NTR1), can strongly influence dopamine transmission. The goal of this study was to gain insight into the degree to which seasonal changes in physiology modify interactions between neurotensin and dopamine to adjust context-appropriate communication. Male European starlings were examined in physiological conditions that stimulate season-typical forms of communication: late summer/early fall non-breeding condition (low testosterone; birds sing infrequently), late fall non-breeding condition (low testosterone; birds produce non-sexually motivated song), and spring breeding condition (high testosterone; males produce sexually-motivated song). Double fluorescent immunolabeling was performed to detect co-localization patterns between tyrosine hydroxylase (TH; the rate-limiting enzyme in dopamine synthesis) and NTR1 in brain regions implicated in motivation and song production (the ventral tegmental area, medial preoptic nucleus, periaqueductal gray, and lateral septum). Co-localization between TH and NTR1 was present in the ventral tegmental area for all physiological conditions, and the number of co-localized cells did not differ across conditions. Immunolabeling for TH and NTR1 was also present in the other examined regions, although no co-localization was seen. These results support the hypothesis that interactions between NTR1 and dopamine in the ventral tegmental area may modulate vocalizations, but suggest that testosterone- or photoperiod-induced changes in NTR1/TH co-localization do not underlie seasonally-appropriate adjustment of communication.

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Declaration of Interest

The authors declare no conflicts of interest.

Ethical Statement

All procedures and protocols followed 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.

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Keywords

Neurotensin; Dopamine; Testosterone; Songbird; Vocal Communication

1. Introduction

Animals communicate in distinct social contexts to convey information specific to those contexts, such as sexual or agonistic motivation (Bradbury and Vehrencamp, 2011). In seasonally breeding male songbirds, as the breeding season approaches, changes in photoperiod and circulating testosterone (T) stimulate the production of sexually-motivated song that is directed at females for courtship (Alward et al., 2017; Ball et al., 2004; Ball and Balthazart, 2010; Harding, 2004). Outside of a breeding context, some songbirds also produce song that is not used to immediately attract mates (Dunn and Zann, 1996; Immelmann, 1962; Merullo et al., 2016). In seasonally breeding species, this often occurs at high rates during the non-breeding season when T concentrations are low (Ball et al., 2004; Dawson et al., 2001; Feare, 1984). Seasonal changes in photoperiod and circulating T may alter behavior by modifying neuromodulatory systems involved in motivation (Ball and Balthazart, 2010). Dopaminergic systems are central to motivation (Berridge and Kringelbach, 2015), and dopamine (DA) is strongly implicated in motivated behaviors across vertebrates, including sexually- and non-sexually motivated song in male songbirds (Kubikova and Kostal, 2010; Ritters, 2012; Simonyan et al., 2012). Neurotensin (NT) is a neuropeptide involved in song and motivation that strongly interacts with DA (Binder et al., 2001; Ferraro et al., 2016; Merullo et al., 2016). DA and NT systems are both present in steroid-sensitive brain regions involved in motivation (Binder et al., 2001; O'Connell and Hofmann, 2011), which suggests that T may modify interactions between these two systems to alter motivation.

One site in which DA and NT may interact to influence singing behavior is the medial preoptic nucleus (often abbreviated POM in birds). This region across vertebrates is implicated in sexually-motivated male behaviors (Goodson, 2005; Newman, 1999; O'Connell and Hofmann, 2011), including male courtship song (Ritters, 2012). Both T and DA act in POM to stimulate sexually-motivated male song. For example, T implants targeting the POM increase courtship song rates in male canaries (*Serinus canaria*) (Alward et al., 2013; Alward et al., 2016). In male starlings, optimal levels of D1 dopamine receptor stimulation in POM increase sexually-motivated song (Ritters et al., 2014). Although POM facilitates courtship song, it also inhibits non-sexually motivated song (Alger and Ritters, 2006), and therefore it has been proposed to function as a critical node in adjusting song to match specific seasonal contexts (Ritters, 2012). D1-like receptor density in POM correlates negatively with sexually-motivated song, when T is high, but correlates positively with non-sexually motivated song, when T is low (Heimovics et al., 2009). T treatment decreases mRNA expression of tyrosine hydroxylase (TH; the rate-limiting enzyme in DA synthesis) in POM (Spool et al., 2016), and TH immunolabeling in POM negatively correlates with courtship song, when T is high (Heimovics and Ritters, 2008). All of this evidence suggests that seasonal changes in circulating T and/or photoperiod may modify DA systems in POM to adjust vocal communication so that it is appropriate for a given season.

Singing behavior has also been linked to DA-related markers in additional steroid-sensitive brain regions that are implicated in motivation, such as the ventral tegmental area (VTA), lateral septum (LS), and periaqueductal gray (PAG) (Ball et al., 2004; Ball and Balthazart, 2004; Ball et al., 2003; Merullo et al., 2016; Riters, 2012). Relationships between singing behavior and DA markers in these areas differ between birds with high T concentrations singing sexually-motivated song and birds with low T singing non-sexually motivated song. In male European starlings, TH immunolabeling in VTA, along with DA concentrations in VTA and PAG, relate only to song produced by males with high T in a breeding context (Heimovics and Riters, 2008; Heimovics et al., 2011). In male Bengalese finches, neurons positive for TH in VTA show greater expression of the immediate early gene EGR-1 after the production of sexually-motivated song than after the production of non-sexually motivated song (Matheson and Sakata, 2015). D1-like receptor densities in PAG and LS of male European starlings also show correlations that differ depending upon whether song is sexually- or agonistically-motivated (Heimovics et al., 2009). These context-specific associations between DA-related markers and song suggest that seasonal changes in T and photoperiod may underlie these different relationships to facilitate distinct forms of vocal communication.

T may also modify DA activity by influencing NT. In male mice, a high proportion of cells that express NT also co-express estrogen and androgen receptors (Tsuneoka et al., 2017). Also in mice, a steroid-responsive set of NT cells in the preoptic area has been found to project to and modulate activity in VTA, thereby leading to increased striatal DA release and sexually-motivated behavior (McHenry et al., 2017). In male starlings, NT immunolabeling in VTA correlates with sexually-motivated song (Merullo et al., 2015), suggesting that T-dependent modulation of NT may alter VTA activity and DA release to stimulate song in this context. NT in other brain regions has also been implicated in sexually-motivated and non-sexually motivated song in starlings, with NT immunolabeling in the rostral portion of LS found to correlate positively with sexually-motivated song (Merullo et al., 2015), and mRNA expression for NTR1 in septum found to correlate positively with non-sexually motivated song (Merullo et al., 2016). These studies suggest that T, along with photoperiod, may alter NT activity in several steroid-sensitive brain regions to facilitate dopamine release and sexually-motivated song.

The goal of this study was to gain insight into the hypothesis that seasonal changes in T and photoperiod modify interactions between NT and DA to adjust the control of context-appropriate communication. To test this, we examined separate groups of male European starlings in different physiological conditions that stimulate seasonally distinct forms of communication (Dawson et al., 2001; Feare, 1984): 1) photorefractory and molting (late summer/early fall non-breeding condition; low T; birds sing infrequently), 2) photosensitive (late fall non-breeding condition; low T; birds produce non-sexually motivated song), and 3) photosensitive with T (spring breeding condition; high T; males produce sexually-motivated song). Double fluorescent immunolabeling was performed to observe the degree to which co-localization patterns of TH and NTR1 differed across conditions in brain regions that are steroid-sensitive, contain DA and NTR1 and have been implicated in song (i.e., VTA, POM, PAG, and LS).

2. Materials and Methods

2.1. Animals

Seventeen male European starlings were caught during 2013–14 using baited fly-in traps at a single location in Madison, WI and brought to the University of Wisconsin-Madison. Birds were initially housed indoors in stainless steel cages ($91 \times 47 \times 47$ cm) in groups no larger than five. Food and water were provided *ad libitum*. All procedures and protocols followed 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. Experimental Housing Conditions

Birds were randomly assigned to one of three experimental conditions: photorefractory ($n = 7$), photosensitive ($n = 5$), and photosensitive with T ($n = 5$). Photorefractoriness is a photoperiodic condition in starlings that is characterized by regressed gonads and low circulating T concentrations, representing the post-breeding season and the time of year starlings begin to molt (~late July-August) and do not sing frequently (Böhner et al., 1990; Dawson et al., 2001; Eens, 1997; Feare, 1984; Nicholls et al., 1988). Photosensitivity is a different photoperiodic condition in which the gonads are also regressed and circulating T concentrations are low, but the gonads will increase in size and release circulating T following exposure to day lengths greater than 11 hours (Dawson et al., 2001). This mimics late fall and winter, when wild starlings flock together and produce high rates of non-sexually-motivated song (Feare, 1984). Treatment of photosensitive birds with T stimulates sexually-motivated song and mimics conditions observed during the spring breeding season (Alger and Ritters, 2006; Nowicki and Ball, 1989). To begin the study, all birds were transferred from their initial housing conditions into groups of three in the same room and kept on a photoperiod of 18-hour light:6-hour dark for six weeks to induce photorefractoriness. Then birds in the photosensitive and photosensitive with T groups were moved to a different room and maintained on a photoperiod of 8-hour light:16-hour dark for six weeks to induce photosensitivity. Birds in the photorefractory group remained on the photorefractory photoperiod for the remainder of the study. After six weeks, all birds received subcutaneous implants (detailed below). All birds were euthanized and tissue collected (described below) 8–11 days after receiving implants. This time course has been shown in starlings to be sufficient to observe elevated levels of circulating T and the effects of T on behavior (Spool et al., 2016) (see section 4.2 in the Discussion for more details).

2.3. Testosterone Implants

Birds were implanted with two subcutaneous implants twelve weeks after the start of the study (after photoperiod manipulations). Implants were either empty (for the “photosensitive” and “photorefractory” groups) or contained T (for the “photosensitive with T” group; catalog No. T1500, Sigma-Aldrich, St. Louis, MO) and remained in the animal for at least eight days before tissue collection. Testosterone was mixed in 100% ethanol and the resulting slurry was packed into silastic tubing. The tubing was left to dry overnight so that all ethanol evaporated, and then implants were cut to 12 mm in size, yielding 10 mm of packed T, and sealed with 100% silicone sealant (DAP Products Inc., Baltimore, MD).

Empty implants were also cut to 12 mm in size and sealed. The presence of implants, and whether they contained T, was confirmed at the time of tissue collection. Plasma T concentrations were measured for a subset of samples using an enzyme immunoassay kit for T (catalog No. 1-2402, Salimetrics, Carlsbad, CA) following the manufacturer's instructions. This kit has been validated previously for measuring T in several songbird species (Guigueno et al., 2015; Washburn et al., 2007). All samples were run on a single plate and visualized at 405 nm with a BioTek Cytation 3 cell imaging multi-mode reader (catalog No. BTCYT3MV, BioTek Instruments, Winooski, VT). The intra-assay coefficient of variation was 10.5%. We checked and verified parallelism in starlings using a standard curve for samples pooled from five birds in a 2.5x standard dilution series. T levels were elevated only in individuals implanted with T. Although these values fell outside of the standard curve, they are within the physiological range of circulating T in starlings (Bernard and Ball, 1997; Duffy et al., 2000). The mean pg/mL of T $\pm$ standard deviation for each group was: Photorefractory (432.05 ± 205.08 , $n = 4$); Photosensitive (351.58 ± 185 , $n = 4$); Photosensitive with T (5065.5 ± 176.07 , $n = 2$). All T implants had T remaining at the time of collection.

2.4. Tissue Collection

Brain tissue was collected over four consecutive days. The collection order was alternated across conditions ("photosensitive," "photosensitive with T," and "photorefractory") so that the amount of time that implants remained in the animals was counterbalanced across conditions throughout each collection day. Each individual was injected with 0.2 mL of dexmedetomidine and then deeply anaesthetized with isoflurane (supplied in a 1.5x concentration relative to oxygen). Birds were transcardially perfused through the left ventricle with approximately 100 mL saline followed by approximately 150 mL of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were removed from the skull, placed in the same fixative overnight at 4 °C, and then placed in 30% sucrose in 0.1 M PB at 4 °C for three consecutive days in a fresh sucrose solution each day. Tissue was then stored at -80 °C until sectioning. Brains were sectioned coronally at 40 μ m on a cryostat at -20 °C and stored at -20 °C in antifreeze cryoprotectant (0.2 M phosphate buffer, polyvinylpyrrolidone, sucrose, and ethylene glycol solution).

2.5. Validation of Antibody Specificity

Antibody specificity for TH and NTR1 was confirmed with Western immunoblotting. Hypothalamic tissue from a female starling (not part of this study) was homogenized in a mixture of RIPA buffer (catalog No. BP-115D, Boston BioProducts, Ashland, MA, USA), protease inhibitor cocktail (catalog No. P8340, Sigma-Aldrich, St. Louis, MO, USA), phosphatase inhibitor (catalog No. P0044, Sigma-Aldrich), and PMSF (catalog No. P7626, Sigma-Aldrich). Supernatant was collected after centrifugation at 12,000 RPM at 4 °C for 10 min. Protein was run in 1:5 dilution. Samples were diluted in a 1:1 ratio with β -mercaptoethanol (161-0710, Bio-Rad Laboratories, Hercules, CA, USA) and sample buffer (catalog No. 161-0737, Bio-Rad), heated for 5 min at 95 °C, cooled to room temperature, and centrifuged at 12,000 RPM at 4 °C for 3 min. Samples were loaded into 4–20% precast gels (catalog No. 4561093S, Bio-Rad) along with pre-stained standard ladder (catalog No. 26634, Thermo Scientific, Madison, WI, USA) and unstained marker (catalog No. LC5602,

Invitrogen, Carlsbad, CA, USA). Gel electrophoresis was performed in 0.1 M tris/glycine/SDS buffer (catalog No. 1610732, Bio-Rad) at 100 V for 10 min and then at 150 V for 50 min.

Protein was transferred to an Immobilon-FL PVDF membrane (catalog No. IPFL00010, Millipore, Billerica, MA, USA) in 0.1 M tris/glycine buffer (catalog No. 1610734, Bio-Rad) and 20% methanol (catalog No. A456-1, Fisher Scientific, Fair Lawn, NJ, USA) at 350 mA for 1 h, and then washed in 0.1 M tris-buffered saline with 0.05% Tween 20 (catalog No. BP337-100, Fischer Scientific) (TBST) at room temperature. The membrane was incubated in blocking buffer (0.1 M TBST and 5% non-fat dry milk (catalog No. 170-6404, Bio-Rad)) with agitation for 1 h at room temperature, washed in 0.1 M TBST, and incubated overnight at 4 °C in primary antibody for either 1:500 dilution of anti-TH in mouse (catalog No. MAB318, Millipore) or 1:10,000 dilution of anti-NTR1 in rabbit (LS-B5255, LifeSpan BioSciences, Seattle, WA, USA). The following day, the membrane was washed in 0.1 M TBST, incubated in HRP-conjugated secondary antibody for either horse anti-mouse antiserum (catalog No. 7076s, Cell Signaling Technologies, Danvers, MA, USA) or goat anti-rabbit antiserum (catalog No. 7074s, Cell Signaling Technologies), washed again in 0.1 M TBST, and then washed in 0.1 M tris-buffered saline (TBS). Detection reagents (catalog No. RPN2232, GE Healthcare, Buckinghamshire, UK) were mixed in a 1:1 ratio and added to the membrane to incubate for 5 min at room temperature. Bands were visualized with a C-DiGit blot scanner (catalog No. 3600, LI-COR, Lincoln, NE, USA) and ImageStudio (version 3.1.4, LI-COR, Windows 7).

For NTR1, a single band appeared at approximately 70 kDa (Figure 1A). The molecular weight of NTR1 has been reported to be 72 kDa (in bovine) when a reducing agent was used (Mills et al., 1988), as was used in the present study, and this weight represents the glycosylated form of the receptor (Boudin et al., 1995). (The molecular weight of the non-glycosylated form of NTR1 (in rats) is 47 kDa (Boudin et al., 1995; Tanaka et al., 1990). The distribution of immunolabeling with this antibody is consistent with NTR1 observed in rodents (see Results) (Binder et al., 2001). For TH, a dense band was visible at approximately 60 kDa (Figure 1B), which is within the expected range of 55–62 kDa for TH (Grima et al., 1985).

2.6. Double Fluorescence Immunohistochemistry

Tissue slices containing brain regions of interest were identified using neuroanatomical landmarks (Figure 2). Slices were washed 5× 5 min 0.02 M PBS, incubated for 30 min in 1.5% H₂O₂/50% methanol, rinsed 3× 10 min in 0.02 M PBS/0.3% Triton X-100/0.05% normal goat serum (NGS), incubated for 60 min in blocking solution (10% NGS/0.3% Triton X-100/0.02 M PBS), and incubated overnight at 4 °C with antibodies (mouse anti-TH, catalog No. MAB318, Millipore, Billerica, MA, USA, diluted 1:500; rabbit anti-NTR1, catalog No. LS-B5255, LifeSpan BioSciences, Seattle, WA, diluted 1:10,000) in primary antibody incubation solution (0.3% Triton X-100/1% NGS/1% blocking reagent/0.02 M PBS). These antibody concentrations were determined after pilot testing with serial dilutions.

Tissue was washed 3× 10 min in 0.1 M TBST, incubated for 60 min in HRP-conjugated goat anti-rabbit antiserum (catalog No. 7074s, Cell Signaling Technologies) diluted 1:100 in 0.1 M TBST, washed 3× 10 min in 0.1 M TBST, and incubated for 10 min in Cy3-conjugated tyramide (TSA Plus Cyanine 3 kit, catalog No. NEL744001KT, PerkinElmer, Waltham, MA, USA) for NTR1 labeling diluted 1:50 in amplification diluent from the TSA kit. Then, tissue was washed 3× 10 min in 0.1 M TBST, incubated for 30 min in 3% H₂O₂ in 0.1 M TBS, washed 3× 5 min in 0.1 M TBST, incubated for 60 min in blocking solution, incubated for 60 min in HRP-conjugated horse anti-mouse antiserum (catalog No. 7076s, Cell Signaling Technologies) diluted 1:100 in 0.1 M TBST, washed 3× 10 min in 0.1 M TBST, and incubated for 30 min in Alexa Fluor 488-conjugated tyramide (catalog No. T20932, Molecular Probes, Eugene, OR, USA) for TH labeling diluted 1:100 in 0.0015% H₂O₂ and amplification diluent from the Alexa Fluor kit. Lastly, tissue was washed 3× 10 min in 0.1 M TBST. As controls, quenching of HRP activity prior to incubation with Cy3- or Alexa Fluor 488-conjugated tyramide, or omission of the primary antibodies or HRP-linked secondary antibodies, completely abolished corresponding specific labeling. Tissue slices were mounted on gelatin-coated slides in 0.1 M TBST, cover slipped with DePeX mounting medium (catalog No. 18243.01, Crescent Chemical Co., Islandia, NY, USA), and stored in dark at 4 °C.

2.7. Quantification of Immunolabeling

All images were taken with the Zeiss Elyra PS.1 system with LSM780 and ZEN software. Cell counts were taken as the average of two hemispheres for three consecutive slices of each region (i.e. six images total were used and the cell count was averaged across hemispheres and sections). For PAG, cell counts were divided by half because a single imaged area contained both hemispheres. Fewer images total were taken in cases where tissue damage prevented quantification. Cell counts were normalized to the area of each region by dividing the cell count by the total area in mm². Anatomical guidelines were used to position the microscope for image capture (Figure 2). VTA was imaged laterally to the third cranial nerve. POM was imaged along the medial hemispheric division at the level of anterior commissure and rostral of commissure. PAG was imaged ventral to the cerebellum and dorsal to the third cranial nerve. LS was imaged along the lateral ventricle on the same slices that contained POM. In LS, a single image frame (area: 212.55 μm × 212.55 μm) was taken in three subregions as in (Goodson et al., 2004): LSr (lateral septum, rostral), LScv (lateral septum, caudal ventral) and LScvl (lateral septum, caudal ventrolateral). In VTA and POM, a 1×3 vertically tiled image frame (214.62 μm × 618.13 μm) was captured; in PAG, a 3×1 vertically tiled image frame (618.13 μm × 214.62 μm) was captured. However, the image frame for PAG extended outside of the boundaries of this region. The quantified portion containing cells in PAG was about two-thirds of the entire frame area (i.e. only 88,442 μm² compared to 132,663 μm²). Cells counts in PAG were normalized to this reduced area size.

Cell counts were performed by an experimenter blind to the experimental conditions of the animals. Cells labeled for TH appeared green and cells labeled for NTR1 appeared red. Double-labeled cells contained both green and red and the resulting label appeared yellow. TH-labeled cells were readily identifiable based on the visibility of cell bodies and

connected fibers (Fig. 3). The NTR1 label had high background and there was difficulty with reliably identifying single-labeled cells (Figs. 3 and 4). However, TH-NTR1 co-labeled cells were identifiable based on the presence of yellow, with reference to single-labeled images to ensure that yellow coloring contained cells of both labels (Fig. 3). Thus, we only quantified singly-labeled TH cells and co-labeled TH-NTR1 cells, and we did not quantify single-labeled NTR1 cells. TH labeling in LS was not as clear as in the other regions because of higher background, less clear cell bodies, and a lack of visibly connected fibers (Fig. 4). Therefore, labeling in LS was not quantified.

Color contrast of all images was adjusted in ImageJ (version 1.51a). All adjustments were performed by the same experimenter blind to the condition of the individuals during analysis. Contrast was adjusted so that background color was the same value (black) for all images; i.e. the adjusted contrast values differed between images depending on the particular level of background staining for a given image, but all images were adjusted so that backgrounds matched. Since staining in some regions was unquantifiable due to tissue damage, the amount of quantifiable tissue differed for each region. The number of individuals with quantifiable cell count data for each region in each experimental condition was: VTA (photorefractory = 4, photosensitive = 4, photosensitive with T = 4); POM and PAG: (photorefractory = 5, photosensitive = 4, photosensitive with T = 5).

2.8. Statistics

Data were analyzed in R v3.3.1 (R Core Team, 2016) and RStudio v.1.0.136 (RStudio Team, 2016). A linear mixed model with restricted maximum likelihood was used to account for the nested nature of the data because repeated measures were taken from the same individuals (Aarts et al., 2014; Wilson et al., 2017). TH cell count was entered as the dependent variable. Brain region (VTA, POM, PAG), physiological condition (photorefractory, photosensitive, photosensitive with T), and the interaction between region and condition were entered as fixed effects. Each individual was entered as a random effect. Normality assumptions were checked with visual inspections of normal QQ plots and histograms, and the Shapiro-Wilk test. Variance assumptions were assessed with visual inspections of residual plots and Levene's test. Post-hoc comparisons were made with Tukey's test using Satterthwaite's degrees of freedom. Co-labeling was not included in the model because regions other than VTA contained only zero values, which reduced the variance of the model to zero, so a separate analysis was performed for co-labeling. In VTA, a comparison of raw numbers of co-localized cells across physiological conditions was performed with a one-way ANOVA. Another one-way ANOVA was used to compare the proportion of co-localized cells to total TH cells across physiological conditions.

R packages that were used include: agricolae (de Mendiburu, 2016), car (Fox and Weisberg, 2011), cowplot (Wilke, 2016), dplyr (Wickham et al., 2017), ggplot2 (Wickham, 2009), lme4 (Bates et al., 2015), lmerTest (Kuznetsova et al., 2016), lsmeans (Lenth, 2016), and reshape2 (Wickham, 2007).

3. Results

NTR1 and TH-positive neurons were present in every region analyzed (Figures 3 and 4). Neurons that showed co-localization of NTR1 and TH labeling occurred exclusively in VTA (Figure 3). Not all labeled neurons exhibited co-localization, but there was a consistent proportion of co-localized cells to TH cells in VTA across all physiological conditions (mean $\pm$ SE: photorefractory = 0.18 ± 0.058 ; photosensitive = 0.19 ± 0.058 ; photosensitive with T = 0.16 ± 0.049). One-way ANOVAs showed that there was no change across conditions in the proportion of co-localized cells ($F_{2,9} = 0.05$, $p = 0.95$) or raw numbers of co-localized cells ($F_{2,9} = 0.24$, $p = 0.79$). In the model for TH cell counts per mm², there was a significant effect of region ($F_{2,22,49} = 67.67$, $p < 0.001$). Post-hoc tests revealed that there were more TH cells per mm² in VTA compared to both POM ($t_{25,87} = 11.10$, $p < 0.001$) and PAG ($t_{24,47} = 5.32$, $p < 0.001$). There also were more TH cells per mm² in PAG compared to POM ($t_{25,88} = 6.05$, $p < 0.001$). There was no significant interaction between region and condition ($F_{4,22,1} = 1.61$, $p = 0.21$), indicating that TH cell counts in all regions did not differ between groups (Figure 5). However, an independent samples t-test in PAG suggested a difference in TH cell counts between the photosensitive group and the photosensitive with T group ($t_7 = 2.71$, $p = 0.03$).

4. Discussion

This study examined the extent to which co-localization patterns of NTR1 and TH varied in brain regions involved in song and motivation across physiological conditions that stimulate distinct forms of communication in male European starlings. We found that co-localization of these markers occurred exclusively in VTA, and this co-localization did not differ significantly across physiological conditions. NTR1 and TH were also present in PAG, POM, and LS; however, they did not co-localize and no differences in cell counts for TH were observed across physiological conditions in POM or PAG (LS was not quantified). These results suggest that interactions between NTR1 and DA in VTA could influence behavior, but that T- or photoperiod-dependent changes in these interactions, at least at the level of protein labeling, are not likely to underlie seasonal changes in singing or other behaviors.

4.1. Co-localization of NTR1 and TH in VTA

The co-localization of NTR1 on TH-positive neurons in VTA suggests a potential mechanism by which NT can influence DA transmission and behavior. Of the three NT receptor subtypes, NTR1 has the highest binding affinity and the greatest effect on DA transmission (Binder et al., 2001). NTR1 is also the only identified avian NT receptor subtype (Numao et al., 2011). In VTA, the activation of NTR1 located on DA neurons can depolarize those neurons and lead to the release of DA (Binder et al., 2001; Ferraro et al., 2016; Lu et al., 2009; Mercuri et al., 1993). VTA sends dopaminergic projections to other regions, including POM, LS, and PAG, where DA activity is implicated in singing behavior (Kubikova and Kostal, 2010; Riters, 2012; Simonyan et al., 2012). Thus, VTA is well-positioned to be a target region for NT to bind to NTR1 to influence dopaminergic transmission and motivated behaviors. However, the lack of T or photoperiod effects on

NTR1/TH labeling suggest that seasonal changes in these co-localization patterns do not underlie seasonal changes in singing behavior. This does not exclude the possibility that seasonal changes in song are influenced by other seasonal changes in NT/DA interactions. For example, activated NTR1 can allosterically reduce the affinity of inhibitory D2 receptors, thereby leading to overall increases in DA activity (Binder et al., 2001; Ferraro et al., 2016; Fuxe et al., 1992). Thus, T or photoperiod dependent changes in NTR1/D2 receptor interactions may be another mechanism underlying seasonal changes in singing behavior that could be investigated in future studies by co-labeling for NT and D2. Furthermore, it is possible that the time course for T exposure in the present study was not sufficient to observe differences in protein labeling, or that effects would be observed in a larger sample size. We discuss these possibilities in Section 4.2 below.

4.2. Labeling in POM, PAG, and LS

It is well-known in rodents that NTR1 co-localizes with DA neurons in VTA (reviewed in Binder et al., 2001). To our knowledge, previous studies in mammals have not examined co-localization of NTR1 and TH in POM, PAG, and LS. Here we did not see co-localization between these markers in POM, PAG, or LS. The lack of co-localization in these regions between NTR1 and TH-positive neurons could indicate that NTR1 does not modify DA activity here by depolarizing DA neurons. However, interactions could occur via other mechanisms, such as allosterically through D2 receptors as described above.

Although TH cell counts did not differ significantly in the overall model, a separate pairwise comparison suggested that, in PAG, TH cell counts were lowest in photosensitive birds implanted with T, compared to photosensitive birds without T. Little is known about the effects of T on the DA system in PAG. A previous study in starlings found that D1-like receptor density in PAG correlated negatively with song produced in a breeding context (i.e. individuals had high circulating levels of T), but no correlation was found for song outside of the breeding context (when circulating T levels are low) (Heimovics et al., 2009). The robust nucleus of the arcopallium (RA), a nucleus involved in vocal production, receives a TH-positive projection from PAG (Appeltants et al., 2002), suggesting that changes in densities of TH in PAG might impact vocal production. Few TH or NTR1 labeled cells were observed in LS compared to the other regions, which is consistent with past studies that have also found lower numbers of TH and NTR1 labeled cells in LS. For example, a previous study in rats also found fewer cell bodies labeled for NTR1 in LS (Boudin et al., 1996), and TH labeling in zebra finches is generally more dense in the rostral portion of the septum, compared to the lateral portion (Bottjer, 1993).

4.3. Consistency of labeling patterns across physiological conditions

No group differences were found in labeling patterns for any region between birds that were photorefractory, photosensitive, or photosensitive with T (except for the pairwise comparison in PAG noted above). This suggests that interactions between NTR1 and TH do not change in response to photoperiod or T. However, it is possible that the time course for T exposure in the present study was not sufficient to observe differences in protein labeling. Although this is possible, T implants similar to those used here have been found to stimulate singing behavior in 3–7 days (Sartor et al., 2005; Spool et al., 2016; Tramontin et al., 2000).

and to induce changes in protein within 7 days (Matragrano et al., 2013). Thus, we expect that the 8–11 days of T exposure used here should be sufficient to alter behavior and TH labeling. It is also possible that significant effects could be observed with a larger sample size. However, our findings for POM and VTA are consistent with past studies in male European starlings and rats with larger sample sizes which show that even two weeks of T exposure did not alter TH immunolabeling in POM or VTA (Du and Hull, 1999; Heimovics and Ritters, 2008). Nonetheless, our study indicates that if T or photoperiod do alter patterns of TH or TH/NTR1 labeling in POM, VTA, PAG or LS in starlings, this response is not robust or observed uniformly across animals.

4.4. Comparisons to past studies

The effect of T on dopamine systems in the brain regions examined in this study is not universally clear. For example, in one study of male European starlings, T decreased mRNA expression of TH in POM (Spool et al., 2016), whereas in a study in leopard frogs, T increased measures of TH immunoreactivity in POM (Chu and Wilczynski, 2002), and in a study in Japanese quail, increased cloacal gland size due to T action correlated with increased DA levels in POM prior to copulation (Kleitz-Nelson et al., 2010). For VTA, results of studies on male rats, sheep, and leopard geckos generally show that T increases TH immunolabeling measures, although some of these studies only focused on prenatal T exposure (Brown et al., 2015; Dias et al., 2007; Espinosa et al., 2016). These inconsistent findings may indicate that T does not modify TH immunolabeling, or they could reflect a more complex curvilinear relationship between T and DA related to motivational state (Kleitz-Nelson et al., 2010) that should be examined in future studies. It is also possible that TH immunolabeling could reflect a role for norepinephrine instead of dopamine, since TH is the rate-limiting enzyme for both products. This could be clarified by labeling for dopamine-beta-hydroxylase as a marker for noradrenergic neurons (Mello et al., 1998).

Little is known about the influence of T on NT systems. Androgen and estrogen receptors have been shown in mice to be present on NT neurons (Tsuneoka et al., 2017). Estradiol priming can increase interactions between NT and DA, such that DA release in the nucleus accumbens is enhanced following stimulation of NT-expressing neurons in POM that project to VTA (McHenry et al., 2017). A study in rats did not find an effect of T on NT immunoreactivity in POM (Diez-Guerra et al., 1987). Two studies in Japanese quail found that gonadally-intact females have greater NT immunoreactivity in POM compared to males (Absil and Balthazart, 1994a), but that T treatment did not influence NT immunoreactivity in POM in either sex (Absil and Balthazart, 1994b). While the results of the present study do not suggest a role for T in modifying DA or NT systems, work that fully explores the impact of T on DA, NT, and their receptors is needed to fully understand this.

4.5. Potential mechanisms of seasonal and individual differences in singing behavior

Although increases in circulating T in photosensitive birds cause increases in sexually-motivated singing behavior, other factors beyond photoperiod and hormones also contribute to changes in behavior. In starlings, dominance status (as reflected in the acquisition and defense of a nesting site) stimulates sexually-motivated behavior in males with controlled levels of circulating T (Cordes et al., 2014; Feare, 1984; Kessel, 1957; Ritters et al., 2000;

Sartor and Ball, 2005; Spool et al., 2016). Therefore, it is possible that factors beyond T and photoperiod that were not considered in the present study, such as dominance status, are important for physiological differences that characterize seasonal conditions. The presence of NTR1 and TH in all of the examined regions for all of the physiological conditions could indicate that interactions between these systems contribute more to individual differences in behavior, rather than drive seasonal shifts in behavior. These hypotheses could be tested in future behavioral studies. Additionally, the present study only examined cell counts. It is possible that T could affect other DA- and NT-related measures such as density of labeling, mRNA expression, receptor ratios, and protein concentrations.

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Highlights

- Double fluorescent immunolabeling was performed for NTR1 and TH in male starlings
- NTR1 cells and TH cells were present in VTA, POM, PAG, and LS
- Co-localization of NTR1 and TH was seen in VTA only
- No labeling differences were found for birds in distinct seasonal conditions
- Interactions between NTR1 and dopamine in VTA may modulate starling behavior

Figure 1.

A Western blot of analysis of antibodies for NTR1 (Panel A) and TH (Panel B). Protein samples were run in 1:5 dilution. A single band for NTR1 is present at approximately 70 kDa, which represent the glycosylated form of the receptor (see text for details of interpretation). For TH, the clearest band is present at approximately 60 kDa, which is within the expected weight range of 55–62 kDa.

Figure 2.

Coronal illustrations of the approximate locations for the regions imaged as indicated by boxes in the diagram. Only cells within a given region were analyzed. Abbreviations: A, arcopallium; Cb, cerebellum; CO, optic chiasm; DM, dorsomedial intercollicular nucleus; ICo, intercollicular nerve; GLV, lateral geniculate nucleus, ventral part; GP, globus pallidus; HVC, letter-based proper name; LScv, lateral septum, caudal division, ventral zone; LScvl, lateral septum, caudal division, ventrolateral zone; LScv, lateral septum, rostral division; LSt, lateral striatum; M, mesopallium; N, nidopallium; NC, caudal nidopallium; NIII, third cranial nerve; PAG, periaqueductal gray; POM, medial preoptic nucleus; Rt, nucleus rotundus; TnA, nucleus taeniae of the amygdala; VTA, ventral tegmental area.

Figure 3.

Representative microscope images of portions of VTA, POM, and PAG for TH, NTR1, and the resulting merged image. The entire area analyzed for each region is larger than displayed in the figure. Co-localization is apparent in VTA in the neurons that are positively labeled for both TH and NTR1, and appear yellow when those respective images are merged. Arrows indicate a sample of representative TH cells that were examined for co-labeling and co-labeling was found to be present. Arrowheads indicate cells that were found to only be single-labeled for TH. The experimental group to which a row of images belongs is indicated in the upper-left corner of the image in the TH column. Scale bars indicate 50 μm .

Figure 4.

Representative microscope images of portions of LSr, LScv, and LScvl for TH, NTR1, and the resulting merged image. The entire area analyzed for each region is larger than displayed in the figure. Arrowheads indicate a sample of representative TH cells that were examined for co-labeling, but only single-labeling for TH was found to be present. The experimental group to which a row of images belongs is indicated in the upper-left corner of the image in the TH column. Scale bars indicate 50 μ m.

Figure 5.

Cell counts per mm² (± SE) across physiological conditions for TH and co-localized cells in VTA (Panel A), POM (Panel B), and PAG (Panel C). No statistical differences were seen between groups in an overall model. Data points represent individuals. Cell counts were performed for the entire imaged region (see Section 2.7).