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Co-localization of mu-opioid and dopamine D1 receptors in the medial preoptic area and bed nucleus of the stria terminalis across seasonal states in male European starlings

Jeremy A. Spool*[,1], Devin P. Merullo[2], Changjiu Zhao, Lauren V. Riters

Department of Integrative Biology, University of Wisconsin, Madison, WI 53706, USA

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ABSTRACT

In seasonally breeding animals, changes in photoperiod and sex-steroid hormones may modify sexual behavior in part by altering the activity of neuromodulators, including opioids and dopamine. In rats and birds, activation of mu-opioid receptors (MOR) and dopamine D1 receptors in the medial preoptic area (mPOA) often have opposing effects on sexual behavior, yet mechanisms by which the mPOA integrates these opposing effects to modulate behavior remain unknown. Here, we used male European starlings (*Sturnus vulgaris*) to provide insight into the hypothesis that MOR and D1 receptors modify sexual behavior seasonally by altering activity in the same neurons in the mPOA. To do this, using fluorescent immunohistochemistry, we examined the extent to which MOR and D1 receptors co-localize in mPOA neurons and the degree to which photoperiod and the sex-steroid hormone testosterone alter co-localization. We found that MOR and D1 receptors co-localize throughout the mPOA and the bed nucleus of the stria terminalis, a region also implicated in the control of sexual behavior. Numbers of single and co-labeled MOR and D1 receptor labeled cells were higher in the rostral mPOA in photosensitive males (a condition observed just prior to the breeding season) compared to photosensitive males treated with testosterone (breeding season condition). In the caudal mPOA co-localization of MOR and D1 receptors was highest in photosensitive males compared to photorefractory males (a post-breeding season condition). Seasonal shifts in the degree to which neurons in the mPOA integrate signaling from opioids and dopamine may underlie seasonal changes in the production of sexual behavior.

1. Introduction

Seasonal changes in photoperiod and sex-steroid hormone concentrations prepare seasonally breeding animals to engage in behavior appropriate for the time of year. For example, increasing photoperiods and concentrations of testosterone (T) or estradiol underlie the activation of sexual behaviors in many species at a time of year when conditions favor breeding (Ball and Balthazart, 2004; Ball and Ketterson, 2008; Bronson, 2009; Dawson et al., 2001; Wingfield and Kenagy, 1991). In contrast, outside the breeding season, when sex-steroid hormone concentrations are low and resources are scarce, animals refrain from producing sexual behaviors (Dawson et al., 2001; Kriegsfeld et al., 2015; Wingfield and Kenagy, 1991). Sex-steroid hormones and photoperiod are proposed to alter behavior in part by modifying the activity of other neuromodulators (Ball and Balthazart, 2010); however, mechanisms by which they do so are still unclear (e.g., Cornil and de Bournonville, 2018; Hellier et al., 2018).

Songbirds such as European starlings (*Sturnus vulgaris*) provide an excellent study system in which to explore neural mechanisms underlying seasonal changes in behavior. Starlings are temperate zone seasonally breeding songbirds. During the spring breeding season, seasonal increases in photoperiod stimulate the gonads to produce and release T (Ball and Wingfield, 1987; Dawson, 1983), which permits males to guard nest cavities and to sing female-directed courtship song (Gwinner and Gwinner, 1994; Kessel, 1957; Pinxten et al., 2002; Spool et al., 2016). At the conclusion of the breeding season in the mid-to-late summer, although the photoperiod is still long, males enter a state of photorefractoriness during which long photoperiods no longer stimulate the gonads to release T. As a result, T concentrations plummet and males cease courting females and defending nesting sites (Ball and

* Corresponding author.
E-mail addresses: spool@umass.edu (J.A. Spool), devin.merullo@utsouthwestern.edu (D.P. Merullo), czhao23@wisc.edu (C. Zhao), LVRiters@wisc.edu (L.V. Riters).
[1] Current address: Department of Psychological and Brain Sciences, University of Massachusetts, Amherst, MA 01003, USA.
[2] Current address: Department of Neuroscience, University of Texas Southwestern Medical Center, Dallas, TX 75390, USA.

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Wingfield, 1987; Dawson, 1983). As the photoperiod decreases in the fall and winter, starlings again become photosensitive, yet gonads remain small and T remains low until the photoperiod increases again in spring (Ball and Wingfield, 1987; Dawson, 1983).

The medial preoptic area (mPOA) is a well-known critical node in adjusting courtship and sexual behaviors so that they are seasonally appropriate. For example, lesions to the mPOA in male starlings decrease courtship singing but either increase or have no effect on singing outside the breeding season (i.e., singing not used for courtship; Alger and Riters, 2006; Hausberger et al., 1995; Riters et al., 2017). Furthermore, in male starlings the mPOA is largest when days are long and T is elevated compared to outside the breeding season (Riters et al., 2000). In quail, T acts to increase the size and number of synaptic connections in the mPOA (Castagna et al., 1999; Panzica et al., 1987; Panzica et al., 1991), and T in the mPOA of castrated male canaries is sufficient to stimulate courtship song (Alward et al., 2013). Additionally, expression of suites of genes in the mPOA changes across seasonal states in starlings (Stevenson et al., 2012). This suggests that changes induced by photoperiod and T-dependent changes in the mPOA play a role in seasonal changes in behavior.

Opioids and dopamine are neuromodulators that are well-known to act in the mPOA to modify courtship and sexual behaviors in both birds and mammals, including courtship singing and copulation in songbirds. In general, opioids acting at mu-opioid receptors (MOR) in the mPOA inhibit sexual behaviors (Hughes et al., 1987; Kotegawa et al., 1997; Matuszewich et al., 1995), and blocking opioid receptors in the mPOA of low-singing male starlings increases courtship singing (Kelm-Nelson et al., 2013). Conversely, dopamine acting at D1 receptors in the mPOA generally stimulates sexually-motivated behaviors in rodents and courtship singing in starlings (Hull et al., 1995; Kleitz-Nelson et al., 2010a, 2010b, 2010c; Riters et al., 2014a), as well as copulation in rats and quail (Balthazart et al., 1997; Markowski et al., 1994). Correlations between singing and both opioid- and dopamine-related genes in the mPOA also change seasonally (Cordes et al., 2015; Heimovics et al., 2009; Riters et al., 2014b), suggesting that steroid hormones and photoperiod may alter these systems to facilitate seasonal changes in behavior.

Despite parallel lines of research demonstrating roles for opioids and dopamine in the mPOA in courtship and sexual behavior, mechanisms by which the mPOA integrates signaling of these neuromodulators remain unexplored. MOR and D1 receptors are found to be expressed in the same neurons in cortex and striatum (Juhasz et al., 2008) and to form oligoheteromers (Juhasz et al., 2008; Tao et al., 2017), and so, one possibility is that the mPOA is capable of integrating opioid and dopamine signaling through MOR and D1 receptors located on the same post-synaptic cells.

In this study we used fluorescent double-immunohistochemistry with tyramide signal amplification to visualize MOR and D1 receptors on cell bodies. Co-localization of MOR and D1 receptors would suggest mPOA neurons are capable of directly integrating neuromodulatory inputs from both opioid and dopamine-releasing neurons, while finding little to no co-localization would suggest integration occurs through a less direct mechanism, elsewhere in the brain, through different receptors, or that signals are mostly processed in parallel. Following our finding that MOR and D1 receptors do co-localize, we assessed the degree to which co-localization in the mPOA is altered by photoperiod and T to gain insight into seasonal changes to possible integration of opioid and dopamine signaling.

2. Methods

2.1. Animal conditions and hormone implants

Seventeen male starlings were used in this study. All birds were caught on a single farm in Madison, WI using baited fly-in traps in 2014–2015 winter seasons. We used photoperiod manipulations (detailed below) to put 12 males into physiological conditions observed outside the breeding season (from late fall to early spring), in which circulating T is low, but the brain will respond to T by stimulating courtship behaviors (photosensitive condition; Dawson et al., 2001). We treated half of these males with T (photosensitive + T condition; detailed below). We also used photoperiod manipulations to put 5 males into a condition mimicking late summer through early fall, when circulating T is low and the brain does not respond to T by increasing breeding behavior (photorefractory condition; Dawson et al., 2001). Specifically, all 17 starlings were housed on a photoperiod of 18L:6D for 6 weeks. At this point, 12 males were moved to a photoperiod of 8L:16D for 6 weeks while concurrently, the other 5 males remained on a photoperiod of 18L:6D (Dawson, 2006). These manipulations induce a photorefractory state for birds that remained on 18L:6D for all 12 weeks, and a photosensitive state for birds that were switched to 8L:16D (Dawson, 2006; Dawson et al., 2001).

At the end of these 12 weeks, birds were anesthetized under 4% isoflurane and maintained on 1.5% isoflurane, and each male received 2 subcutaneous silastic implants (internal diameter, 1.47 mm; outer diameter, 1.96 mm; Dow Corning, Midland, MI, USA) over the dorsal left breast. Half of the photosensitive males were given implants packed with crystalline T (Sigma-Aldrich, St. Louis, MO) while the other half were given empty implants. Implants were created as in Spool et al., 2016. All photorefractory males were given empty implants. This resulted in 3 experimental groups: photorefractory (n = 5), photosensitive (n = 6), and photosensitive + T males (n = 6).

All protocols and procedures were approved by the University of Wisconsin Institutional Animal Care and Use Committee and followed the guidelines of the *National Institutes of Health Guide for the Care and Use of Laboratory Animals*.

2.2. Tissue collection

Between 8 and 11 days post-implantation, birds were injected with 0.1 mg/kg dexmedetomidine, anesthetized with 4% isoflurane and then perfused with 0.9% saline followed by 4% paraformaldehyde (the order in which birds were perfused was balanced across treatments). This time frame of T treatment is sufficient for T to activate courtship singing in European starlings (Spool et al., 2016) and induce changes in neuromodulatory-related protein expression in the songbird brain (e.g., serotonin transporter, norepinephrine synthetic enzyme dopamine beta hydroxylase; Matragrano et al., 2013). Brains were removed, post-fixed overnight in 4% paraformaldehyde, then submerged in a 30% sucrose solution each day (i.e., transferred to a fresh 30% solution every 24 h) until they sank. Brains were then frozen on dry ice and stored at −80 °C until sectioning. We sectioned tissue coronally at 40 μm, and stored sections in cryoprotectant solution at −20 °C until performing immunofluorescent labeling. Immediately prior to perfusion with saline, a terminal blood sample was collected from the right alar wing vein in a subset of birds and stored at −20 °C until hormone analysis. All subcutaneous implants (T or empty) were intact and T was still present in the T-filled implants at the time of perfusion.

2.3. Testosterone assay

We used a commercial grade competitive immunoassay (#1-2402, Salimetrics, Carlsbad, CA) to confirm that plasma T levels of birds were in line with hormonal treatment in a subset of birds (photorefractory birds, n = 4; photosensitive birds, n = 4; photosensitive + T birds; n = 2). Serum was not available for all birds included in this study. The results of this assay were already published as part of another study using the same animals (Merullo et al., 2018). The assay confirmed that androgens for birds treated with T were elevated (average: 5066 pg/mL, standard deviation = 176 pg/mL), whereas T concentrations for photosensitive (average = 640.9 pg/mL, standard deviation = 666 pg/mL) and photorefractory (average = 432 pg/mL, standard deviation = 205 pg/mL) were low (Merullo et al., 2018).

2.4. Antibody characterization

Immunolabeling for MOR was run using a rabbit polyclonal anti-MOR antibody. This antibody has been validated in starlings in a previous study (anti-MOR from Abcam, Cambridge, MA; Cat # ab10275; RRID: AB_2156356; corresponds to the rat mu opioid receptor C terminal amino acids 384–398; antibody sequence has 80% similarity to predicted mu-type opioid receptor in starlings; Kelm et al., 2011). Immunolabeling for D1 dopamine receptors was run using a rabbit polyclonal antibody (anti-D1 from LifeSpan BioSciences, Inc., Seattle, WA; Cat # LS-B13217; RRID: AB_2722508; corresponds to the human dopamine D1 receptor amino acids 187–232; antibody sequence has 91% similarity to predicted D1A sequence in starlings). We validated the D1 receptor antibody using a Western immunoblot (methods detailed below), which resulted in a dark band and a lighter band at ~50 kDa, which is the expected molecular weight of D1 receptor protein in various states of phosphorylation and palmitoylation (Ng et al., 1994; Rothmond et al., 2012). When either antibody was omitted from the immunofluorescent protocol (described below) no labeling above background resembling cell bodies was detected.

2.5. Western blot

To validate the D1 receptor antibody, a Western immunoblot was performed using hypothalamic tissue collected from a female starling and flash frozen using isopentane (#277258, Sigma-Aldrich, St. Louis, MO) as in Merullo et al., 2018. Approximately 30 mg of hypothalamic tissue was homogenized in RIPA buffer (#BP-115D, Boston BioProducts, Ashland, MA), a phosphatase inhibitor (#P0044, Sigma-Aldrich, St. Louis, MO), and two protease inhibitors (#P8340 and #P7626, Sigma-Aldrich, St. Louis, MO). The mixture was centrifuged at 12,000 RPM at 4 °C for 10 min and the resulting supernatant was aspirated and diluted 1:5 using deionized water. This extracted protein was diluted 1:1 with a 1:20 mixture of β-mercaptoethanol (#161-0710, Bio-Rad Laboratories, Hercules, CA) and sample buffer (#161-0737, Bio-Rad Laboratories, Hercules, CA; i.e., one part β-mercaptoethanol, 19 parts sample buffer), heated for five min at 95 °C, allowed to cool to room temperature, and centrifuged at 12,000 RPM for 2 min.

A detailed protocol for a similar set of tissue is described in (Merullo et al., 2018). Briefly, protein and ladders were run on a 4–20% precast gel (#4561093S, Bio-Rad, Hercules, CA) at 100 V for 10 min followed by 150 V for 50 min. Protein on the gel was transferred onto an Immobilon-FL PVDF membrane (#IPFL00010, Millipore, Billerca, MA) at 350 mA for 1 h. Following transfer, the membrane was blocked in 5% non-fat dry milk (170-6404, Bio-Rad, Hercules, CA) and incubated overnight on a shaker at 4 °C in anti-D1 diluted 1:100 in the above blocking solution.

The next day, the membrane was washed in 0.1 M tris-buffered saline with 0.5% Tween 20 (#BP337-100, Fischer Scientific, Fair Lawn, NJ; TBST) incubated in horseradish peroxidase (HRP)-conjugated goat anti-rabbit antiserum for 1 h at room temperature (1:100 in TBST; Cell Signaling Technologies, Danvers, MA). Detection reagents (#RPN2232, GE Healthcare, Buckinghamshire, UK) at room temperature were mixed in a 1:1 ratio immediately before use, and added to the membrane for 5 min at room temperature. Membranes were immediately visualized using a C-DiGit blot scanner (#3600, LI-COR, Lincoln, NE), and images were collected using ImageStudio (version 3.1.4, LI-COR, Lincoln, NE).

2.6. Double immunofluorescent labeling with tyramide signal amplification

For each bird, three tissue sections were labeled for MOR and D1 receptors: two sections caudal to the tractus septomesencephalicus and rostral to the anterior commissure, as well as one section where the anterior commissure is observed to cross brain hemispheres. Tissue sections were rinsed 5× for 5 min in 0.02 M phosphate-buffered saline (PBS) and then incubated in 1.5% H_2O_2/50% methanol for 30 min to inhibit endogenous peroxidase activity. Tissue was then washed 3× for 10 min with 0.05% normal goat serum (NGS)/0.3% Triton X-100/0.02 M PBS. Following washes, tissue was blocked for 1 h using 10% NGS/0.3% Triton X-100/0.02 M PBS and incubated at 4 °C overnight in rabbit anti-D1 (1:100 in 0.3% Triton X-100/1% NGS/1% blocking reagent (Roche Diagnostics Corporation, Indianapolis, IN)/0.02 M PBS (PAIS)).

After primary incubation, sections were washed 3× for 10 min in TBST, then incubated in HRP-conjugated Goat anti-rabbit antiserum for 1 h (1:100 in TBST). Sections were again washed 3× for 10 min in TBST, and labeled by incubation in Cy3-conjugated tyramide (TSA Plus Cyanine 3 kit; Perkin Elmer; Waltham, MA), followed by 3 × 10 min washes in TBST. Because the MOR antibody and D1 receptor antibody were both raised in rabbit, we blocked cross-reactivity by heating sections in a citric acid buffer (10 mM citric acid, pH 6.0) at 98 °C for 5 min (Tóth and Mezey, 2007). Heated sections were washed in TBST 3× for 5 min, blocked for 1 h using the blocking solution as described above, and incubated in anti-MOR for 72 h at 4 °C (1:2000 in PAIS).

Following the second primary incubation, sections were washed 3× for 10 min in TBST, incubated in HRP-conjugated Goat anti-rabbit antiserum for 1 h, washed again in TBST 3× for 10 min, and labeled by incubation in Alexa Fluor 488-conjugated tyramide (Alexa Fluor 488 TSA kit; Molecular Probes, Eugene, OR). Sections were then washed 3× for 10 min in TBST, mounted on subbed slides, allowed to dry in a dark room for 24 h, and coverslipped using Serva DePeX (Crescent Chemical Company, Islandia, NY).

To ensure the efficacy of our protocol and that co-localization was not the result of cross-reactivity of antibodies, adjacent tissue sections from three males in the present study were labeled as described above except the primary antibody for MOR was omitted (i.e., sections were incubated in PAIS overnight with no anti-MOR). Cells were labeled with Cy3 but no labeling with Alexa488 was apparent in any part of the sections. This indicates that (1) anti-rabbit secondary did not react with the D1 receptor primary antibody following heat treatment in citric acid, and (2) HRP activity linked to D1 receptor primary antibody labeling was eliminated following heat treatment in citric acid.

2.7. Imaging and quantification

We imaged cells using the Zeiss LSM 780 (Elyra; Carl Zeiss Inc., Gottingen, Germany), and all images were 212.55 × 212.55 μm. For the mPOA, one image was taken on each side of the third ventricle on all three sections, yielding a total of 6 images for each animal. Because the rostral and caudal portions of the mPOA play different roles in the control of song and sexual behavior in birds (Balthazart et al., 1998a; Balthazart and Ball, 2007; Riters and Ball, 1999), we examined co-localization patterns in each sub-region (i.e., cells were counted separately for each sub-region). Of the three tissue sections imaged from the mPOA, two were representative of the rostral mPOA (the two sections rostral to the anterior commissure) and one was representative of the caudal mPOA (the one section collected at the level of the anterior commissure; Fig. 1).

To provide initial insight into the regional specificity of our findings, we quantified labeling in the medial portion of the bed nucleus of the stria terminalis (BNSTm), a region that in birds has been implicated in the modulation of agonistic, sexual, affiliative, and parental behaviors (Goodson and Wang, 2006; Heimovics and Riters, 2006; Ruscio and Adkins-Regan, 2004; Taziaux et al., 2006). One image was taken in each hemisphere directly above anterior commissure, halfway between the tip of the lateral ventricle and the midline (Fig. 1), yielding a total of 2 images for each animal. The location of BNSTm was based on anatomical and neurochemical evidence that this area in birds is homologous to the mammalian medial BNST (Fig. 1; Aste et al., 1998). Confocal imaging was performed at the Newcomb Imaging Center, Department of Botany, UW Madison.

For all images, one person blind to the experimental conditions of all animals counted all cells manually using the count tool in Adobe Photoshop 2015 (Adobe Systems, Inc., San Jose, CA). Contrast on all

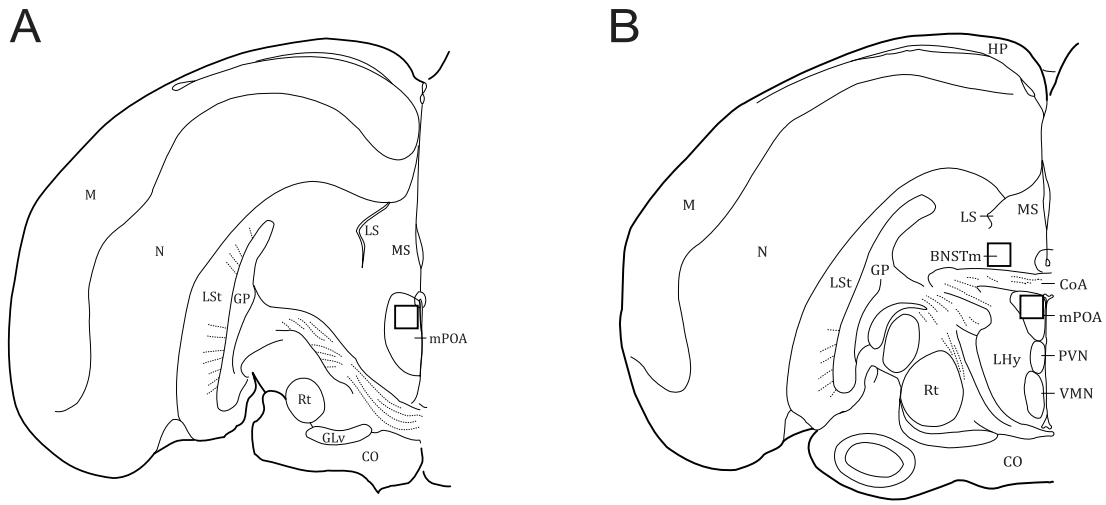

Fig. 1. Approximate location of imaged regions on coronal illustrations of the starling brain (A) at the level of the rostral medial preoptic area and (B) at the level of the caudal medial preoptic area and the bed nucleus of the stria terminalis. Abbreviations: BNSTm, bed nucleus of the stria terminalis, medial; CO, optic chiasm; CoA; anterior commissure; GLv, lateral geniculate nucleus, ventral; GP, globus pallidus; HP, hippocampus; LHy, lateral hypothalamus; LS, lateral septum; LSt, lateral striatum; M, mesopallium; MS, medial septum; N, nidopallium; mPOA, medial preoptic area; Rt, nucleus rotundus; PVN, periventricular nucleus of the hypothalamus; VMN, ventromedial nucleus of the hypothalamus.

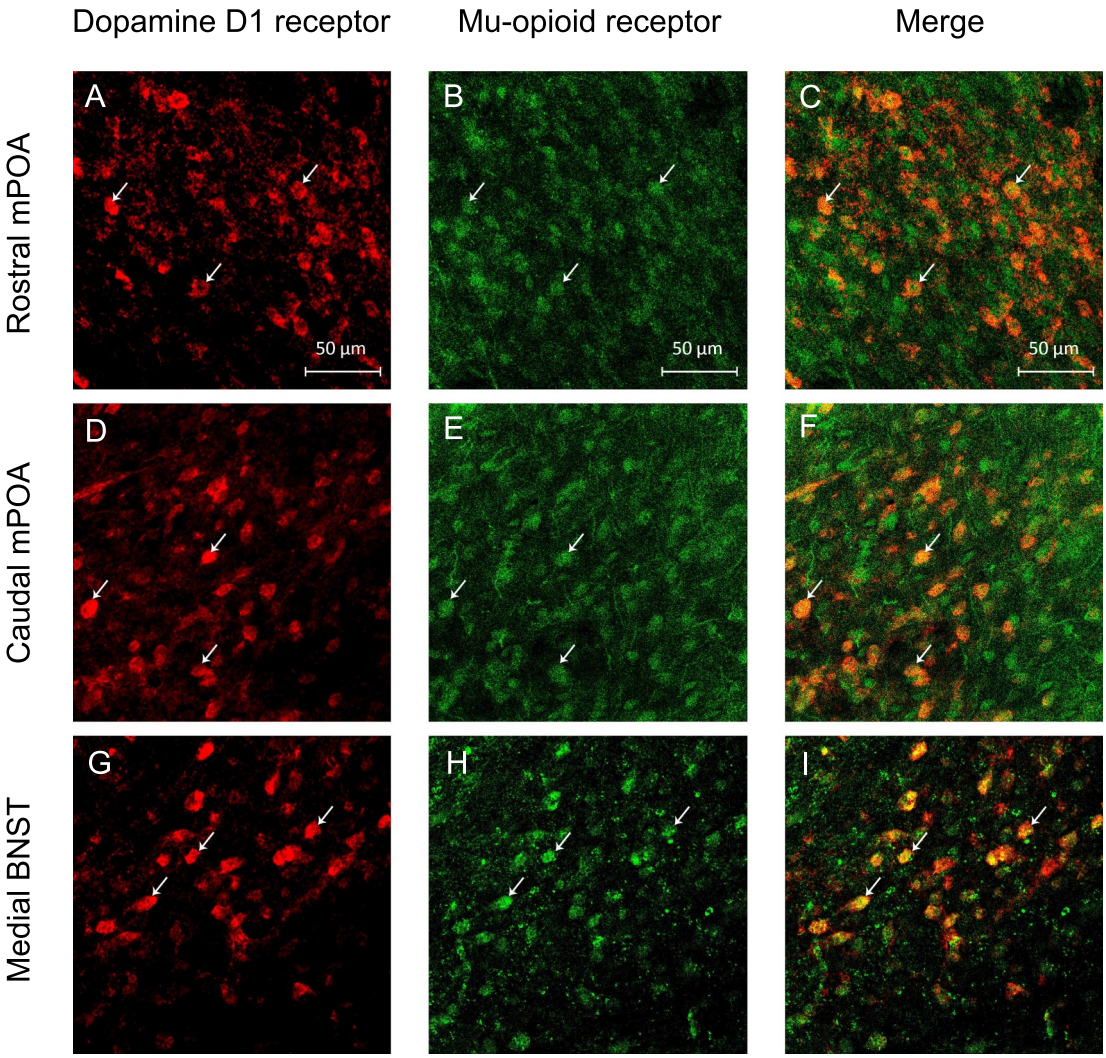

Fig. 2. Representative confocal images of (A,D,G) dopamine D1 receptors, (B,E,H) mu opioid receptors (MOR), and (C,F,I) overlaid images of D1 receptors and MOR. (A–C) rostral medial preoptic area; (D–F) caudal medial preoptic area; (G–I) medial bed nucleus of the stria terminalis. Confocal images were taken at 212.55 × 212.55 µm. Scale bars indicate 50 µm and arrows highlight examples of cells that express both D1 receptors and MOR.

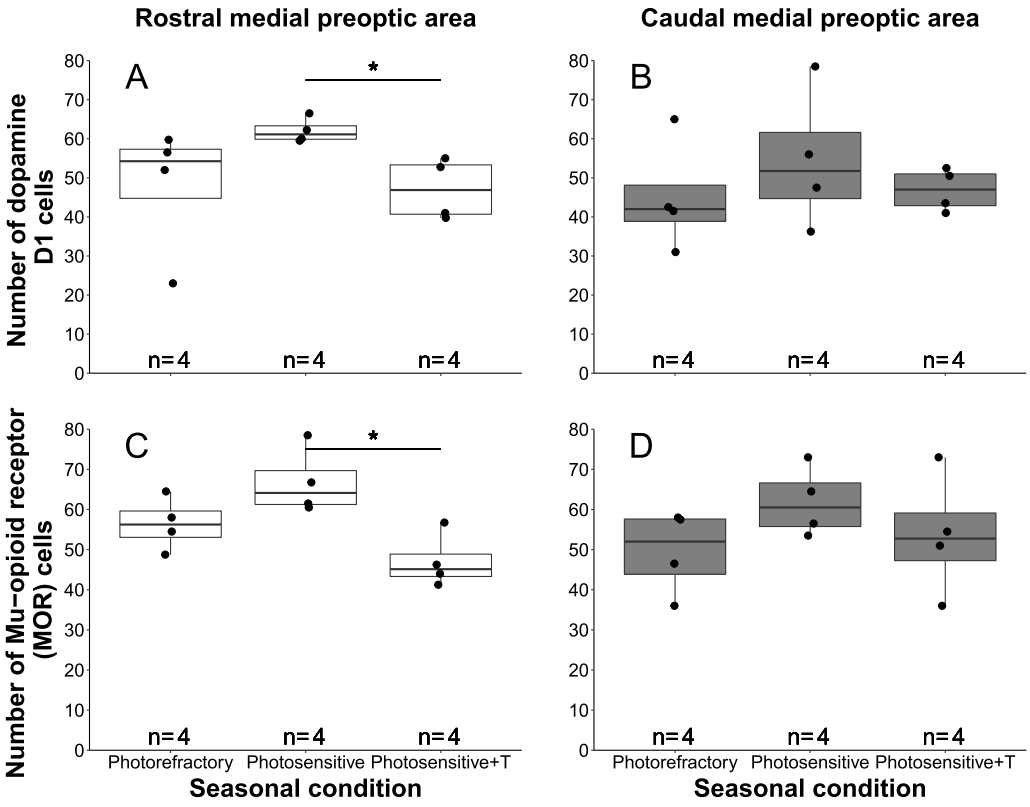

Fig. 3. Quantification of immunofluorescent labeling in the rostral (white boxplots on the left) and caudal (gray boxplots on the right) medial preoptic area (mPOA). Boxplots represent data distribution around the median for each experimental group. Sample sizes for each group are indicated below each boxplot. Individual data points are plotted over boxplots. Cell counts for (A–B) dopamine D1 receptors and (C–D) mu-opioid receptors (MOR) are shown. *Indicates a significant difference between two experimental groups ($p < 0.05$).

photos was adjusted so the image background was black. Cells were counted for D1 (labeled in Cy3 fluorescent red) and MOR (labeled in Alexa 488 fluorescent green) separately by placing a counter in the middle of each labeled cell. Then, images of D1 and MOR were overlaid, and co-labeling was determined by whether counters had been placed on the same cells in both images (co-labeled cells appeared yellow). For details on how labeled cell counts were used in analyses, see the next section (Statistical analysis). While D1 receptor labeling was always clear, MOR labeling was unreliable in some samples, producing faint labeling and high background. Thus we took a conservative approach and included only birds where cell bodies were clearly visible above background for both the D1 receptor and the MOR label. Final sample sizes for each brain region were mPOA: photorefractory = 4, photosensitive = 4, photosensitive + T = 5; BNSTm: photorefractory = 3, photosensitive = 4, photosensitive + T = 5.

2.8. Statistical analysis

Data were analyzed using R v. 3.4.1 with RStudio v. 1.0.143 (R Core Team, 2017; RStudio Team, 2017). There were no significant differences between hemispheres in the number of labeled MOR, D1 receptors, and co-labeled cells in any region examined. Cell counts were averaged across hemispheres for all animals. With respect to the mPOA, counts were also averaged between sections rostral to anterior commissure. Each bird, therefore, had two sets of cell counts: one rostral of anterior commissure (i.e., rostral mPOA), and one at anterior commissure (i.e., caudal mPOA). Side-by-side boxplots identified one bird in the photosensitive + T group that was consistently well outside the interquartile range in almost all analyses in the mPOA and thus this bird was excluded from mPOA analyses (average rostral cell counts: D1 = 81; MOR = 72.25; co-label = 51. Average caudal cell counts: D1 = 80; MOR = 68; co-label = 53.5). Due to low sample sizes and thus poor approximations of normality, we ran Kruskal-Wallis tests to identify differences in medians between experimental conditions. Following significant main effects, we conducted Dunn's tests using the Benjamini-Hochberg False Discovery Rate for multiple comparison corrections to assess median differences between individual groups. Less conservative analyses (e.g., using ANOVAs) revealed similar results; and without correcting for multiple comparisons, results are the same with one exception: single counts of MOR cells in the rostral mPOA (in this case, photosensitive birds had significantly higher cell counts compared to photorefractory birds; $Z = 1.96$; $p = 0.049$). R packages used include dunn.test (Dinno, 2017), dplyr (Wickham et al., 2017), ggplot2 (Wickham, 2009), and cowplot (Wilke, 2017).

3. Results

Single labeled cells and frequent co-localization of MOR and D1 receptors in cell soma were observed in both the rostral and caudal mPOA (Fig. 2A–F) as well as in the BNSTm (Fig. 2G–I) across all experimental conditions.

3.1. Effect of experimental condition on numbers of MOR and D1 single labeled cells in the mPOA and BNSTm

A Kruskal-Wallis test on the number of D1 receptor labeled cells in the rostral mPOA was significant ($H_2 = 6.73$, $p = 0.035$), and post-hoc Dunn's tests revealed that T-treated photosensitive birds had significantly lower counts of D1 receptor labeled cells compared to photosensitive birds ($Z = 2.45$, $p = 0.042$; Fig. 3A). Comparisons between other groups for D1 receptor labeled cells in the rostral mPOA were not significant (photosensitive versus phororefractory birds: $Z = 1.96$, $p = 0.074$; photosensitive + T versus photorefractory birds: $Z = 0.49$, $p = 0.62$). Similarly, a Kruskal-Wallis test on the number of MOR labeled cells in the rostral mPOA was significant ($H_2 = 7.54$, $p = 0.023$) with T-treated photosensitive birds having significantly lower counts of MOR labeled cells compared to photosensitive birds ($Z = 2.75$, $p = 0.018$; Fig. 3C). Comparisons between other groups for numbers of MOR labeled cells in the rostral mPOA were not significant (photosensitive versus phororefractory birds: $Z = 1.37$, $p = 0.17$;

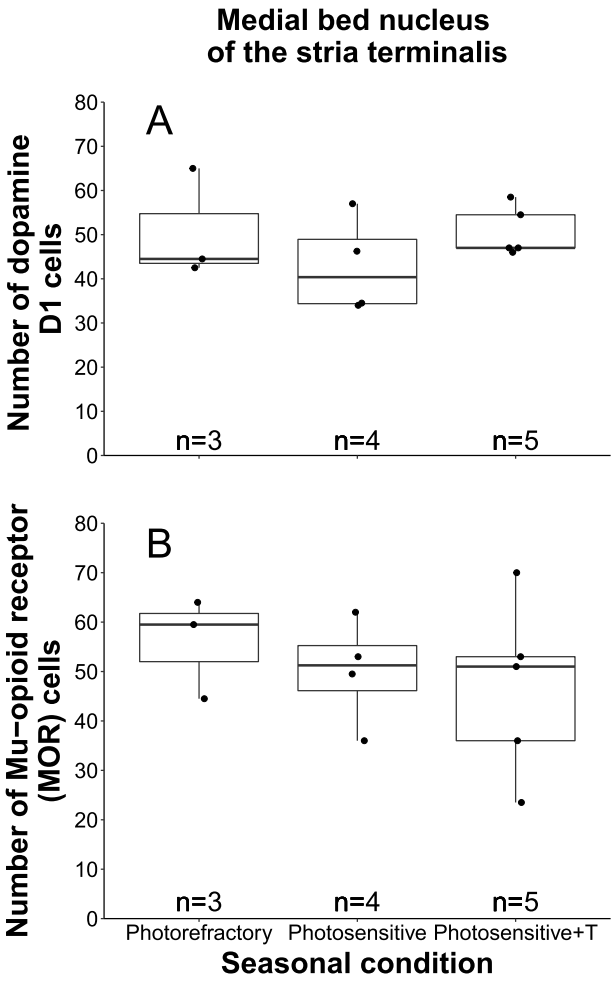

Fig. 4. Quantification of immunofluorescent labeling in the medial bed nucleus of the stria terminalis (BNSTm). Boxplots represent data distribution around the median for each experimental group. Sample sizes for each group are indicated below each boxplot. Individual data points are plotted over boxplots. Cell counts for (A) dopamine D1 receptors, (B) mu-opioid receptors (MOR) are shown.

photosensitive + T versus photorefractory birds: Z = 1.37, p = 0.26). There were no differences in the number of MOR or D1 receptor labeled cells in the caudal mPOA across experimental conditions (p > 0.44; Fig. 3B,D). Finally, the number of D1 and MOR labeled cells did not differ across experimental conditions in the BNSTm (p > 0.4; Fig. 4A,B).

3.2. Co-labeled cells for MOR and D1 receptors differ across experimental conditions in subregions of the mPOA

Kruskal-Wallis tests revealed subregion-specific differences in the number of co-labeled cells across experimental conditions. There were significant main effects of condition on the number of co-labeled cells in both the rostral (H_2 = 8.12, p = 0.017) and the caudal (H_2 = 7.65, p = 0.022) mPOA. Corrected post-hoc Dunn's tests revealed that in the rostral mPOA, photosensitive birds treated with T had fewer co-labeled cells compared to photosensitive birds (Z = 2.84, p = 0.013; Fig. 5A), but other comparisons were not significant (photosensitive versus phororefractory birds: Z = 1.57, p = 0.18; photosensitive + T versus photorefractory birds: Z = 1.27, p = 0.20). Conversely, in the caudal mPOA, photosensitive birds had higher numbers of co-labeled cells than photorefractory birds (Z = 2.75, p = 0.018; Fig. 5B), but other comparisons were not significant (photosensitive versus photosensitive + T birds: Z = 1.08, p = 0.28; photosensitive + T versus photorefractory birds: Z = 1.67, p = 0.14).

Because the volume of the mPOA has been shown to change seasonally in male starlings (Riters et al., 2000), and can increase within a day of T treatment (Shevchouk et al., 2017), we also ran tests using the proportion of co-labeled cells (i.e., number of co-labeled cells divided by the total number of cells labeled) as the dependent variable. A Kruskal-Wallis using proportion of co-labeled cells in the rostral mPOA was not significant (H_2 = 3.73, p = 0.15; Fig. 5C). However, a Kruskal-Wallis using proportion of co-labeled cells in the caudal mPOA was similar to findings using counts of co-labeled cells (H_2 = 9.85, p = 0.007) in that photosensitive birds had higher proportions of co-labeled cells compared to photorefractory birds (Z = 3.14, p = 0.005; Fig. 5D), with no other group differences (photosensitive versus photosensitive + T birds: Z = 1.57, p = 0.18; photosensitive + T versus photorefractory birds: Z = 1.57, p = 0.12).

No group differences were identified for the number of co-labeled cells across experimental conditions in the BNSTm (p = 0.33; Fig. 6). Finally, we observed a tendency for the proportion of D1 receptor labeled cells co-labeled to be greater than the proportion of MOR labeled cells co-labeled, though none of these comparisons were statistically significant (Table 1) and are not discussed further.

4. Discussion

Several studies in rats and birds show that opioids and dopamine in the mPOA tend to have opposing effects on sexual and sexually-motivated behaviors, with opioids inhibiting and dopamine stimulating these behaviors (Hughes et al., 1987; Hull et al., 1995; Kelm-Nelson et al., 2013; Kleitz-Nelson et al., 2010c; Matuszewich et al., 1995; Parra-Gámez et al., 2013; Pehek et al., 1988; Riters et al., 2014a; Scaletta and Hull, 1990; van Furth et al., 1995). However, mechanisms by which the mPOA integrates these opposing signals to influence sexual behavior have not been examined in any species. The results of our present study are the first to suggest that populations of neurons in both the rostral and caudal mPOA are capable of making calculations about relative levels of opioid and dopamine signaling to impact courtship and sexual behavior by activation of co-localized receptors. Our study is also the first to show that MOR and D1 receptors co-localize in the BNSTm, which in birds has been implicated in the modulation of agonistic, sexual, affiliative, and parental behaviors (Goodson and Wang, 2006; Heimovics and Riters, 2006; Ruscio and Adkins-Regan, 2004; Taziaux et al., 2006).

4.1. Effects of T and photoperiod on co-expression of MOR and D1 receptors in the mPOA

In the mPOA we found subregion-specific effects of our experimental conditions on the number of cells co-labeled for MOR and D1 receptors. The rostral and caudal mPOA play different roles in the control of song and sexual behavior in birds (Balthazart and Ball, 2007; Riters and Ball, 1999). Specifically, the rostral mPOA is thought to control sexually-motivated, “appetitive” behavior (e.g., courtship singing in starlings); whereas caudal mPOA is proposed to underlie “consummatory” behavior (e.g., copulation). In the caudal mPOA, birds had fewer co-labeled cells in a post-breeding season condition (i.e., when sexual behavior cannot be stimulated by T; Dawson et al., 2001) compared to birds in the pre-breeding season condition (i.e., late winter, early spring, when T-treatment can cause birds to begin courtship singing; Dawson et al., 2001). Upon normalizing cell counts in the caudal mPOA to control for potential effects of nuclear volume changes, the proportion of co-labeled cells in photorefractory birds remained significantly lower compared to photosensitive birds. Because MOR in the caudal mPOA and D1 receptors in the mPOA are both implicated in sexual behavior (Balthazart et al., 1997; Matuszewich et al., 1995), it is possible that this decrease in MOR and D1 receptor co-

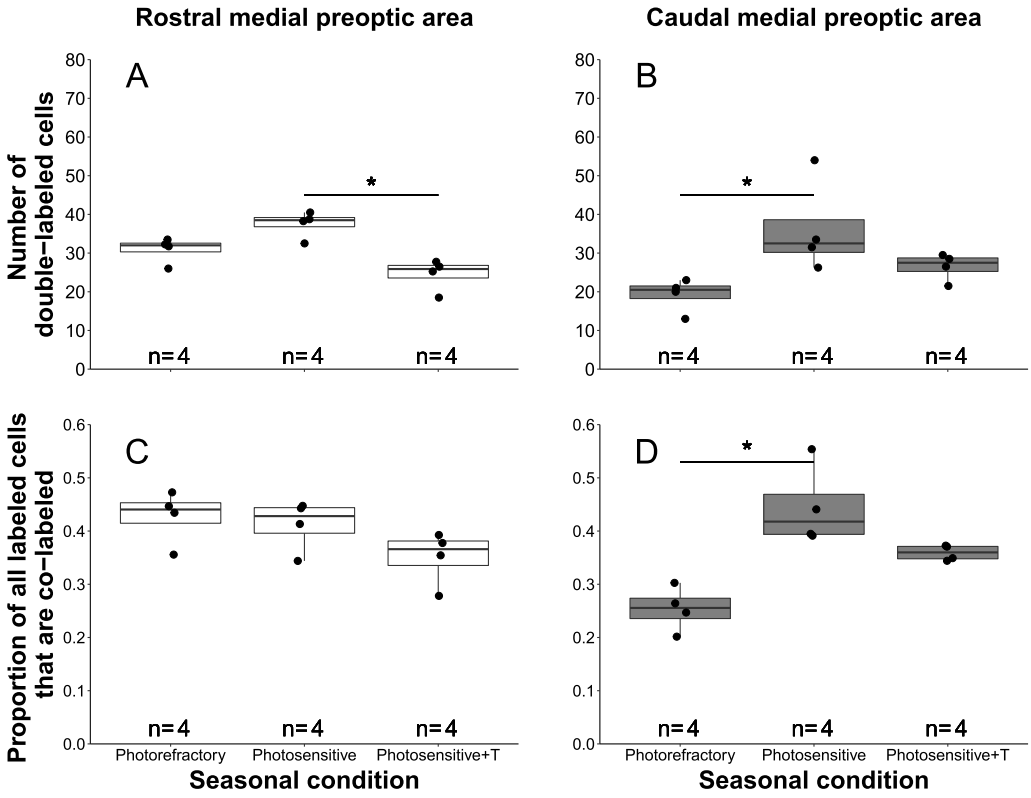

Fig. 5. Quantification of immunofluorescent labeling in the rostral (white boxplots on the left) and caudal (gray boxplots on the right) medial preoptic area (mPOA). Boxplots represent data distribution around the median for each experimental group. Sample sizes for each group are indicated below each boxplot. Individual data points are plotted over boxplots. Cell counts for (A–B) double-labeled cells, (C–D) proportion of co-labeled cells for D1 receptors and MOR are shown. *Indicates a significant difference between two experimental groups ($p < 0.05$).

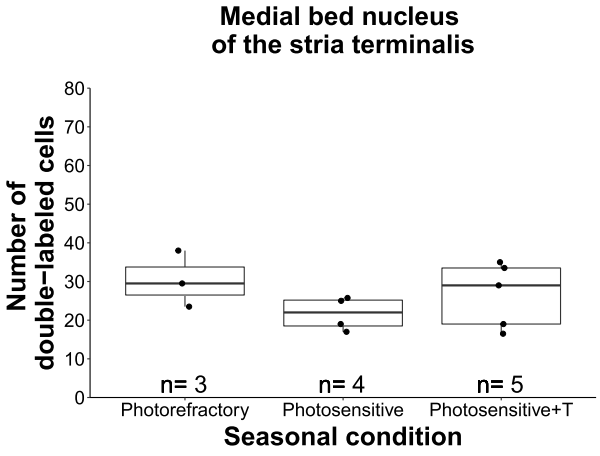

Fig. 6. Counts of cells double-labeled for dopamine D1 receptors and mu-opioid receptors in the medial bed nucleus of the stria terminalis (BNSTm). Boxplot represents data distribution around the median for each experimental group. Sample sizes for each group are indicated below the plot. Individual data points are plotted over boxes.

labeling functions to prevent birds from copulating at the end of the breeding season.

An intriguing possibility is that MOR and D1 receptors are co-expressed on aromatase neurons in the caudal mPOA. In male starlings the mPOA contains dense aromatase immunolabeling during but not outside of the breeding season (Riters et al., 2000). Aromatase activity can rapidly alter sexual behavior in males (reviewed in Balthazart and Foidart, 1993; Cornil et al., 2018). The degree to which MOR are found on aromatase neurons in mPOA is not known; however, catecholaminergic fibers are closely associated with aromatase cells in the mPOA (Balthazart et al., 1998b) and dopamine acting at D1 receptors may alter aromatase activity in the mPOA (Baillien and Balthazart, 1997; Cornil et al., 2005). It is thus possible that aromatase neurons in the caudal mPOA integrate opioid and dopamine signaling to fine-tune sexual behavior. Future work is needed to characterize the neurochemical identity of the cells that co-label for both D1 receptors and MOR.

In the rostral mPOA in the present study, birds in the condition mimicking the breeding season (photosensitive + T birds) had significantly fewer co-labeled cells than birds in a pre-breeding state (photosensitive birds). However, when we normalized co-labeled cells

Table 1
The proportion of total dopamine D1 and mu-opioid receptor (MOR) labeled cells that also showed labeling for the other marker (i.e., that were co-labeled) for each experimental group in the medial preoptic area (mPOA) and the medial bed nucleus of the stria terminalis (BNSTm). For rostral and caudal mPOA, n = 4 for each experimental treatment. For the BNSTm, n = 3 for the photorefractory condition, n = 4 for the photosensitive condition, and n = 5 for the photosensitive + T condition. Means ± one standard error of the mean (SEM) are shown.

Marker	Experimental group	Rostral mPOA mean ± SEM	Caudal mPOA mean ± SEM	BNSTm mean ± SEM
Proportion of D1 cells co-labeled	Photorefractory	0.72 ± 0.14	0.44 ± 0.053	0.60 ± 0.042
	Photosensitive	0.61 ± 0.041	0.67 ± 0.027	0.51 ± 0.029
	Photosensitive + T	0.52 ± 0.039	0.57 ± 0.052	0.52 ± 0.067
Proportion of MOR cells co-labeled	Photorefractory	0.55 ± 0.053	0.40 ± 0.058	0.54 ± 0.025
	Photosensitive	0.56 ± 0.024	0.58 ± 0.064	0.44 ± 0.022
	Photosensitive + T	0.53 ± 0.055	0.51 ± 0.040	0.59 ± 0.044

T = testosterone.

by dividing number of co-labeled cells by the total number of cells labeled in our images to control for volume changes to the mPOA (Panzica et al., 1996, 1987; Riters et al., 2000), this finding was no longer significant. Thus it is possible that a reduction in MOR and D1 co-localization in the rostral mPOA plays a role in facilitating sexual motivation during the breeding season; however, it is also possible that lower cell counts in the rostral mPOA may at least in part represent increases in nuclear volume that increase space between labeled cells.

4.2. Individual MOR or D1 receptor labeled cells across experimental conditions

We found that the number of cells labeled for MOR and D1 receptors in the rostral mPOA was lower in photosensitive animals treated with T compared to photosensitive animals without T. In a previous study, T increased mRNA for MOR in the mPOA in male starlings (Spool et al., 2016). In dark-eyed juncos, MOR specific binding was higher at the height of the breeding season compared to during the fall migration in the caudal mPOA (Woods et al., 2010). Such seasonal differences were not observed in the present study. With respect to dopamine D1 receptors in the rostral mPOA, previous data in starlings suggest that D1 receptor binding does not differ across seasons in the mPOA (Heimovics et al., 2009). Additionally, in female turkeys, D1 receptor mRNA did not significantly change throughout the reproductive cycle (Schnell et al., 1999). One possibility is that translational and post-translational control of protein availability explains differences between our findings and those of previous studies. Another possibility is that MOR and D1 protein expression within individual cells (not quantified in this study; discussed below), rather than total number of cells that express MOR and D1 protein, would match trends identified by quantification of mRNA and protein specific binding in previous studies. Finally, it is possible that increases in nuclear volume in the rostral mPOA cause cells to appear less dense (i.e. spread further apart) as may be the case for our counts of double-labeled cells (discussed above).

4.3. MOR and D1 receptor labeling in the BNSTm

Our experimental conditions did not alter cell counts of either MOR or D1 receptors individually in the BNSTm, or counts of MOR and D1 receptor double-labeled cells in the BNSTm; thus our findings in the mPOA are not observed throughout the brain and may represent changes in function that are specific to the mPOA. Future studies are needed to understand the individual and combined effects of dopamine and opioid signaling in the BNSTm on social behavior.

4.4. Methodological considerations

It is important to note that low sample sizes in the present study may have obscured possible group differences that could have become evident with larger group sizes (i.e., a Type II error). For example, caution should be taken in interpreting lack of group differences in mPOA cell counts between photorefractory birds and photosensitive + T birds as strong evidence that no group differences exist. Despite the limitation of sample size, our findings provide the first evidence that MOR and D1 are found on the same neurons in mPOA and that the number of cells co-expressing MOR and D1 changes across seasonal states.

In all regions examined, we quantified cell numbers and thus do not have insight into the quantity of MOR and D1 receptor protein within individual cells across our experimental conditions. The quantities of protein within cells likely influence the extent to which cells integrate dopamine and opioid signaling. For example, in a previous study T treatment upregulated levels of MOR mRNA in the mPOA (Spool et al., 2016). Future studies are needed to gain insight into how protein levels of MOR and D1 receptors within individual cells impact opioid-dopamine interactions.

5. Conclusions

The present study is the first to show that cells in brain regions that control sexual behavior are capable of integrating opioid and dopamine signaling through co-expressed receptors. Our study also demonstrates that photoperiod may play a key role in regulating the number of cells co-expressing MOR and D1 receptors, and this should be examined in future studies. Future studies are also needed to test how integration of information from opioid and dopamine signaling alters the production of sexual behavior.

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References

Alger, S.J., Riters, L.V., 2006. Lesions to the medial preoptic nucleus differentially affect singing and nest box-directed behaviors within and outside of the breeding season in European starlings (*Sturnus vulgaris*). Behav. Neurosci. 120, 1326–1336. https://doi.org/10.1037/0735-7044.120.6.1326.

Alward, B.A., Balthazart, J., Ball, G.F., 2013. Differential effects of global versus local testosterone on singing behavior and its underlying neural substrate. Proc. Natl. Acad. Sci. U. S. A. 110, 19573–19578. https://doi.org/10.1073/pnas.1311371110.

Aste, N., Balthazart, J., Absil, P., Grossmann, R., Mu, E., Panzica, G.C., 1998. Anatomical and neurochemical definition of the nucleus of the stria terminalis in Japanese quail (Coturnix). J. Comp. Neurol. 396, 141–157.

Baillien, M., Balthazart, J., 1997. A direct dopaminergic control of aromatase activity in the quail preoptic area. J. Steroid Biochem. Mol. Biol. 63, 99–113. https://doi.org/10.1016/S0960-0760(97)00080-0.

Ball, G.F., Balthazart, J., 2004. Hormonal regulation of brain circuits mediating male sexual behavior in birds. Physiol. Behav. 83, 329–346. https://doi.org/10.1016/j.physbeh.2004.08.020.

Ball, G.F., Balthazart, J., 2010. Seasonal and hormonal modulation of neurotransmitter systems in the song control circuit. J. Chem. Neuroanat. 39, 82–95. https://doi.org/10.1016/j.jchemneu.2009.08.005.

Ball, G.F., Ketterson, E.D., 2008. Sex differences in the response to environmental cues regulating seasonal reproduction in birds. Philos. Trans. R. Soc. Lond. Ser. B Biol. Sci. 363, 231–246. https://doi.org/10.1098/rstb.2007.2137.

Ball, G., Wingfield, J., 1987. Changes in plasma levels of luteinizing hormone and sex steroid hormones in relation to multiple-broodedness and nest-site density in male starlings. Physiol. Zool. 60, 191–199.

Balthazart, J., Ball, G.F., 2007. Topography in the preoptic region: differential regulation of appetitive and consummatory male sexual behaviors. Front. Neuroendocrinol. 28, 161–178. https://doi.org/10.1016/j.yfrne.2007.05.003.

Balthazart, J., Foidart, A., 1993. Brain aromatase and the control of male sexual behavior. J. Steroid Biochem. Mol. Biol. 44, 521–540. https://doi.org/10.1016/0960-0760(93)90256-V.

Balthazart, J., Castagna, C., Ball, G.F., 1997. Differential effects of D1 and D2 dopamine-receptor agonists and antagonists on appetitive and consummatory aspects of male sexual behavior in Japanese quail. Physiol. Behav. 62, 571–580. https://doi.org/10.1016/S0031-9384(97)00163-7.

Balthazart, J., Absil, P., Gérard, M., Appeltants, D., Ball, G.F., 1998a. Appetitive and consummatory male sexual behavior in Japanese quail are differentially regulated by subregions of the preoptic medial nucleus. J. Neurosci. 18, 6512–6527.

Balthazart, J., Foidart, A., Baillien, M., Harada, N., Ball, G.F., 1998b. Anatomical relationships between aromatase and tyrosine hydroxylase in the quail brain: double-label immunocytochemical studies. J. Comp. Neurol. 391, 214–226. https://doi.org/10.1002/(SICI)1096-9861(19980209)391:2<214::AID-CNE5>3.0.CO;2-5.

Bronson, F.H., 2009. Climate change and seasonal reproduction in mammals. Philos. Trans. R. Soc. Lond. Ser. B Biol. Sci. 364, 3331–3340. https://doi.org/10.1098/rstb.2009.0140.

Castagna, C., Obole, A., Viglietti-Panzica, C., Balthazart, J., Panzica, G.C., 1999. Effects of testosterone on the synaptology of the medial preoptic nucleus of male Japanese quail. Brain Res. Bull. 50, 241–249. https://doi.org/10.1016/0361-9230(95)02135-3.

Cordes, M.A., Stevenson, S.A., Driessen, T.M., Eisinger, B.E., Riters, L.V., 2015. Sexually-motivated song is predicted by androgen-and opioid-related gene expression in the medial preoptic nucleus of male European starlings (*Sturnus vulgaris*). Behav. Brain Res. 278, 12–20. https://doi.org/10.1016/j.bbr.2014.09.029.

Cornil, C.A., de Bournonville, C., 2018. Dual action of neuro-estrogens in the regulation of male sexual behavior. Gen. Comp. Endocrinol. 256, 57–62. https://doi.org/10.1016/j.ygcen.2017.05.002.

Cornil, C.A., Dalla, C., Papadopoulou-Daifoti, Z., Baillien, M., Dejace, C., Ball, G.F., Balthazart, J., 2005. Rapid decreases in preoptic aromatase activity and brain monoamine concentrations after engaging in male sexual behavior. Endocrinology 146, 3809–3820. https://doi.org/10.1210/en.2005-0441.

Cornil, C.A., Ball, G.F., Balthazart, J., 2018. Differential control of appetitive and consummatory sexual behavior by neuroestrogens in male quail. Horm. Behav. https://doi.org/10.1016/j.yhbeh.2018.02.006.

Dawson, A., 1983. Plasma gonadal steroid levels in wild starlings (*Sturnus vulgaris*) during the annual cycle and in relation to the stages of breeding. Gen. Comp. Endocrinol. 49, 286–294.

Dawson, A., 2006. Control of molt in birds: association with prolactin and gonadal regression in starlings. Gen. Comp. Endocrinol. 147, 314–322. https://doi.org/10.1016/j.ygcen.2006.02.001.

Dawson, A., King, V.M., Bentley, G.E., Ball, G.F., 2001. Photoperiodic control of seasonality in birds. J. Biol. Rhythm. 16, 365–380. https://doi.org/10.1177/074873001129002079.

Dinno, A., 2017. dunn.test: Dunn's Test of Multiple Comparisons Using Rank Sums.

Goodson, J.L., Wang, Y., 2006. Valence-sensitive neurons exhibit divergent functional profiles in gregarious and asocial species. Proc. Natl. Acad. Sci. 103, 17013–17017. https://doi.org/10.1073/pnas.0606278103.

Gwinner, H., Gwinner, E., 1994. Effects of testosterone on nest-box occupation and associated behaviours by male European starlings (*Sturnus vulgaris*). Behaviour 129, 141–148.

Hausberger, M., Richard-Yris, M.A., Henry, L., Lepage, L., Schmidt, I., 1995. Song sharing reflects the social organization in a captive group of European starlings (*Sturnus vulgaris*). J. Comp. Psychol. 109, 222–241. https://doi.org/10.1037/0735-7036.109.3.222.

Heimovics, S.A., Riters, L.V., 2006. Breeding context-dependent relationships between song and cFOS labeling within social behavior brain regions in male European starlings (*Sturnus vulgaris*). Horm. Behav. 50, 726–735. https://doi.org/10.1016/j.yhbeh.2006.06.013.Breeding.

Heimovics, S.A., Cornil, C.A., Ball, G.F., Riters, L.V., 2009. D1-like dopamine receptor density in nuclei involved in social behavior correlates with song in a context-dependent fashion in male European starlings. Neuroscience 159, 962–973. https://doi.org/10.1016/j.neuroscience.2009.01.042.

Hellier, V., Brock, O., Candlish, M., Desroziers, E., Aoki, M., Mayer, C., Piet, R., Herbison, A., Colledge, W.H., Prévot, V., Boehm, U., Bakker, J., 2018. Female sexual behavior in mice is controlled by kisspeptin neurons. Nat. Commun. 9. https://doi.org/10.1038/s41467-017-02797-2.

Hughes, A.M., Everitt, B.J., Herbert, J., 1987. Selective effects of beta-endorphin infused into the hypothalamus, preoptic area and bed nucleus of the stria terminalis on the sexual and ingestive behaviour of male rats. Neuroscience 23, 1063–1073.

Hull, E.M., Du, J., Lorrain, D.S., Matuszewich, L., 1995. Extracellular dopamine in the medial preoptic area: implications for sexual motivation and hormonal control of copulation. J. Neurosci. 15, 7465–7471.

Juhasz, J.R., Hasbi, A., Rashid, A.J., So, C.H., George, S.R., O'Dowd, B.F., 2008. Mu-opioid receptor heterooligomer formation with the dopamine D1 receptor as directly visualized in living cells. Eur. J. Pharmacol. 581, 235–243. https://doi.org/10.1016/j.ejphar.2007.11.060.

Kelm, C.A., Forbes-Lorman, R.M., Auger, C.J., Riters, L.V., 2011. Mu-opioid receptor densities are depleted in regions implicated in agonistic and sexual behavior in male European starlings (*Sturnus vulgaris*) defending nest sites and courting females. Behav. Brain Res. 219, 15–22. https://doi.org/10.1016/j.bbr.2010.12.003.

Kelm-Nelson, C.A., Stevenson, S.A., Cordes, M.A., Riters, L.V., 2013. Modulation of male song by naloxone in the medial preoptic nucleus. Behav. Neurosci. 127, 451–457. https://doi.org/10.1037/a0032329.

Kessel, B., 1957. A study of the breeding biology of the European starling (*Sturnus vulgaris* L.) in North America. Am. Midl. Nat. 58, 257–331.

Kleitz-Nelson, H.K., Cornil, C. a, Balthazart, J., Ball, G.F., 2010a. Differential effects of central injections of D1 and D2 receptor agonists and antagonists on male sexual behavior in Japanese quail. Eur. J. Neurosci. 32, 118–129. https://doi.org/10.1111/j.1460-9568.2010.07257.x.

Kleitz-Nelson, H.K., Dominguez, J.M., Ball, G.F., 2010b. Dopamine release in the medial preoptic area is related to hormonal action and sexual motivation. Behav. Neurosci. 124, 773–779. https://doi.org/10.1037/a0021490.

Kleitz-Nelson, H.K., Dominguez, J.M., Cornil, Charlotte A., Ball, G.F., 2010c. Is sexual motivational state linked to dopamine release in the medial preoptic area? Behav. Neurosci. 124, 300–304. https://doi.org/10.1037/a0018767.

Kotegawa, T., Abe, T., Tsutsui, K., 1997. Inhibitory role of opioid peptides in the regulation of aggressive and sexual behaviors in male Japanese quails. J. Exp. Zool. 277, 146–154.

Kriegsfeld, L.J., Ubuka, T., Bentley, G.E., Tsutsui, K., 2015. Seasonal control of gonadotropin-inhibitory hormone (GnIH) in birds and mammals. Front. Neuroendocrinol. 37, 65–75. https://doi.org/10.1016/j.yfrne.2014.12.001.

Markowski, V.P., Eaton, R.C., Lumley, L.A., Moses, J., Hull, E.M., 1994. A D1 agonist in the MPOA facilitates copulation in male rats. Pharmacol. Biochem. Behav. 47, 483–486. https://doi.org/10.1016/0091-3057(94)90147-3.

Matragrano, L.L., LeBlanc, M.M., Chitrapu, A., Blanton, Z.E., Maney, D.L., 2013. Testosterone alters genomic responses to song and monoaminergic innervation of auditory areas in a seasonally breeding songbird. Dev. Neurobiol. 73, 455–468. https://doi.org/10.1002/dneu.22072.

Matuszewich, L., Ormsby, J.L., Moses, J., Lorrain, D.S., Hull, E.M., 1995. Effects of morphiceptin in the medial preoptic area on male sexual behavior. Psychopharmacology 122, 330–335.

Merullo, D.P., Spool, J.A., Zhao, C., Riters, L.V., 2018. Co-localization patterns of neurotensin receptor 1 and tyrosine hydroxylase in brain regions involved in motivation and social behavior in male European starlings. J. Chem. Neuroanat. 89, 1–10. https://doi.org/10.1016/j.jchemneu.2018.01.002.

Ng, G.Y.K., Mouillac, B., George, S.R., Caron, M., Dennis, M., Bouvier, M., Dowd, B.F.O., 1994. Desensitization, phosphorylation and palmitoylation of the human dopamine D1 receptor. Eur. J. Pharmacol. 267, 7–19.

Panzica, G.C., Viglietti-Panzica, C., Calagni, M., Anselmetti, G.C., Schumacher, M., Balthazart, J., 1987. Sexual differentiation and hormonal control of the sexually dimorphic medial preoptic nucleus in the quail. Brain Res. https://doi.org/10.1016/0006-8993(87)91496-X.

Panzica, G., Viglietti-Panzica, C., Sanchez, F., Sante, P., Balthazart, J., 1991. Effects of testosterone on a selected neuronal population within the preoptic sexually dimorphic nucleus of the Japanese quail. J. Comp. Neurol. 303, 443–456. https://doi.org/10.1002/cne.903030310.

Panzica, G.C., Viglietti-Panzica, C., Balthazart, J., 1996. The sexually dimorphic medial preoptic nucleus of quail: a key brain area mediating steroid action on male sexual behavior. Front. Neuroendocrinol. 17, 51–125. https://doi.org/10.1006/frne.1996.0002.

Parra-Gámez, L., García-Hidalgo, A., Paredes, R.G., 2013. Infusion of endomorphin-1 (EM-1) in the MPOA and the Me modulate sexual and socio-sexual behavior in the male rat. Brain Res. 1517, 36–43. https://doi.org/10.1016/j.brainres.2013.04.004.

Pehek, E.A., Warner, R.K., Bazzett, T.J., Bitran, D., Band, L.C., Eaton, R.C., Hull, E.M., 1988. Microinjection of cis-flupenthixol, a dopamine antagonist, into the medial preoptic area impairs sexual behavior of male rats. Brain Res. 443, 70–76. https://doi.org/10.1016/0006-8993(88)91599-5.

Pinxten, R., De Ridder, E., Balthazart, J., Eens, M., 2002. Context-dependent effects of castration and testosterone treatment on song in male European starlings. Horm. Behav. 42, 307–318. https://doi.org/10.1006/hbeh.2002.1824.

R Core Team, 2017. R: A Language and Environment for Statistical Computing.

Riters, L.V., Ball, G.F., 1999. Lesions to the medial preoptic area affect singing in the male European starling (*Sturnus vulgaris*). Horm. Behav. 36, 276–286. https://doi.org/10.1006/hbeh.1999.1549.

Riters, L.V., Eens, M., Pinxten, R., Duffy, D.L., Balthazart, J., Ball, G.F., 2000. Seasonal changes in courtship song and the medial preoptic area in male European starlings (*Sturnus vulgaris*). Horm. Behav. 38, 250–261. https://doi.org/10.1006/hbeh.2000.1623.

Riters, L.V., Pawlisch, B.A., Kelm-Nelson, C.A., Stevenson, S.A., 2014a. Inverted-U shaped effects of D1 dopamine receptor stimulation in the medial preoptic nucleus on sexually motivated song in male European starlings. Eur. J. Neurosci. 39, 650–662. https://doi.org/10.1111/ejn.12429.

Riters, L.V., Stevenson, S.A., DeVries, M.S., Cordes, M.A., 2014b. Reward associated with singing behavior correlates with opioid-related gene expression in the medial preoptic nucleus in male European starlings. PLoS One 9, 1–18. https://doi.org/10.1371/journal.pone.0115285.

Riters, L.V., Spool, J.A., Merullo, D.P., Hahn, A.H., 2017. Song practice as a rewarding form of play in songbirds. Behav. Process. https://doi.org/10.1016/j.beproc.2017.10.002.

Rothmond, D.A., Weickert, C.S., Webster, M.J., 2012. Developmental changes in human dopamine neurotransmission: cortical receptors and terminators. BMC Neurosci. 13, 18. https://doi.org/10.1186/1471-2202-13-18.

RStudio Team, 2017. RStudio: Integrated Development for R.

Ruscio, M.G., Adkins-Regan, E., 2004. Immediate early gene expression associated with induction of brooding behavior in Japanese quail. Horm. Behav. 46, 19–29. https://doi.org/10.1016/j.yhbeh.2004.02.002.

Scaletta, L.L., Hull, E.M., 1990. Systemic or intracranial apomorphine increases copulation in long-term castrated male rats. Pharmacol. Biochem. Behav. 37, 471–475. https://doi.org/10.1016/0091-3057(90)90015-A.

Schnell, S.A., You, S., El Halawani, M.E., 1999. D1 and D2 dopamine receptor messenger ribonucleic acid in brain and pituitary during the reproductive cycle of the Turkey hen. Biol. Reprod. 60, 1378–1383. https://doi.org/10.1095/biolreprod60.6.1378.

Shevchouk, O.T., Ghorbanpoor, S., Ball, G.F., Cornil, C.A., Balthazart, J., 2017. Testosterone-induced neuroendocrine changes in the medial preoptic area precede song activation and plasticity in song control nuclei of female canaries. Eur. J. Neurosci. 45, 886–900. https://doi.org/10.1111/ejn.13530.

Spool, J.A., Stevenson, S.A., Angyal, C.S., Riters, L.V., 2016. Contributions of testosterone and territory ownership to sexually-motivated behaviors and mRNA expression in the medial preoptic area of male European starlings. Horm. Behav. 86, 36–44. https://doi.org/10.1016/j.yhbeh.2016.09.004.

Stevenson, T.J., Replogle, K., Drnevich, J., Clayton, D.F., Ball, G.F., 2012. High throughput analysis reveals dissociable gene expression profiles in two independent

neural systems involved in the regulation of social behavior. BMC Neurosci. 13, 126. https://doi.org/10.1186/1471-2202-13-126.

Tao, Y.M., Yu, C., Wang, W.S., Hou, Y.Y., Xu, X.J., Chi, Z.Q., Ding, Y.Q., Wang, Y.J., Liu, J.G., 2017. Heteromers of μ opioid and dopamine D1receptors modulate opioid-induced locomotor sensitization in a dopamine-independent manner. Br. J. Pharmacol. 174, 2842–2861. https://doi.org/10.1111/bph.13908.

Taziaux, M., Cornil, C.A., Dejace, C., Arckens, L., Ball, G.F., Balthazart, J., 2006. Neuroanatomical specificity in the expression of the immediate early gene c-fos following expression of appetitive and consummatory male sexual behaviour in Japanese quail. Eur. J. Neurosci. 23, 1869–1887. https://doi.org/10.1111/j.1460-9568.2006.04719.x.

Tóth, Z.E., Mezey, É., 2007. Simultaneous visualization of multiple antigens with tyramide signal amplification using antibodies from the same species. J. Histochem. Cytochem. 55, 545–554. https://doi.org/10.1369/jhc.6A7134.2007.

van Furth, W.R., van Emst, M.G., van Ree, J.M., 1995. Opioids and sexual behavior of male rats: involvement of the medial preoptic area. Behav. Neurosci. 109, 123–134.

Wickham, H., 2009. ggplot2: Elegant Graphics for Data Analysis.

Wickham, H., Francois, R., Henry, L., Muller, K., 2017. dplyr: A Grammar of Data Manipulation.

Wilke, C.O., 2017. cowplot: Streamlined Plot Theme and Plot Annotations for "ggplot2.".

Wingfield, J., Kenagy, G., 1991. Neural regulation of reproductive cycles. In: Schreibman, M., Jones, R. (Eds.), Vertebrate Endocrinology: Fundamentals and Biomedical Implications. Academic Press, New York, pp. 181–241.

Woods, J.K., Deviche, P., Corbitt, C., 2010. Opioid receptor densities analyzed across seasons in the POM and VTA of the dark-eyed junco, *Junco hyemalis*. J. Chem. Neuroanat. 40, 123–129. https://doi.org/10.1016/j.jchemneu.2010.05.002.