Published in final edited form as:
Behav Brain Res. 2011 May 16; 219(1): 15–22. doi:10.1016/j.bbr.2010.12.003.

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

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Abstract

Social status and resource availability can strongly influence individual behavioral responses to conspecifics. In European starlings, males that acquire nest sites sing in response to females and dominate other males. Males without nest sites sing, but not to females, and they do not interact agonistically with other males. Little is known about the neural regulation of status- or resource-appropriate behavioral responses to conspecifics. Opioid neuropeptides are implicated in birdsong and agonistic behavior, suggesting that opioids may underlie differences in the production of these behaviors in males with and without nest sites. Here, we examined densities of immunolabeled mu-opioid receptors in groups of male starlings. Males that defended nest boxes dominated other males and sang at higher rates when presented with a female than males without nest boxes, independent of testosterone concentrations. Multiple regression analyses showed nest box ownership (not agonistic behavior or singing) predicted the optical density of receptor labeling in the medial bed nucleus of stria terminalis, paraventricular nucleus, ventral tegmental area and the medial preoptic nucleus. Compared to males without nest boxes, males with nest boxes had *lower* densities of immunolabeled mu-opioid receptors in these regions. Singing additionally predicted the area covered by labeling in the ventral tegmental area. The results suggest that elevated opioid activity in these regions suppresses courtship and agonistic behavioral responses to conspecifics in males without nest boxes. The findings are consistent with a dynamic role for opioid receptors in adjusting social behavior so that it is appropriate given the resources available to an individual.

Keywords

birdsong; motivation; vocal communication; socially appropriate behavior; dominance; territoriality

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1. Introduction

Across vertebrates both social status and the resources available to an individual can strongly influence the way in which an individual responds to conspecifics. For example, in male European starlings (*Sturnus vulgaris*), the acquisition and defense of a nest site (or nest box in aviary studies) is a crucial first step for initiating mating behavior [1]. Male starlings with nest boxes sing high rates of song in response to females (Riters et al., 2000). In contrast, male starlings that do not successfully obtain nest boxes often sing, but not in response to a female [2]. Males with nest boxes additionally displace other males from food sources and perches more often than males without nest boxes, suggesting nest box owners socially dominate males without boxes [3]. Thus, the distinct behavioral responses to conspecifics observed in males with and without nest boxes are established early in the breeding season through competition over limited available nest sites. Little is known about how social status or resource possession alters the brain to ensure that an individual produces status- or resource-appropriate behavioral responses to conspecifics. In the present study we begin to explore a possible role for opioids in social behavior associated with nest box possession in flocks of male starlings.

Data implicate opioids in the regulation of birdsong and suggest that the effects of opioids on song differ depending upon whether song is directed towards a female (as in nest box owners) or undirected (as in males without nest boxes). In male starlings with nest boxes, opioids acting at the mu-opioid receptor inhibit courtship song produced in response to a female ([4,5], but see Khurshid et al., 2009 for different effects in zebra finches). Furthermore, male starlings that sang at naturally high or low rates responded differently to opioid receptor pharmacological treatments. For example, treatment with the opioid receptor antagonist naloxone increased song production in low singers, but did not have a dramatic effect on high singers [6]. This suggests differences in mu-opioid receptor density or binding affinity may underlie individual differences in males singing at high and low rates in response to a female (i.e., in males with and without nest boxes respectively).

Studies using immunolabeling for immediate early genes (IEGs) identify differences in neuronal activity in males with compared to males without nest boxes. Specifically, males with nest boxes had higher numbers of IEG labeled cells in regions implicated in both agonistic behavior and birdsong, including the medial preoptic nucleus (POM; see Table 1 for abbreviations), medial bed nucleus of the stria terminalis (BSTm), ventral tegmental area (VTA), and mesencephalic central gray (CG) [7–11]. Each of these regions contains opioids or opioid receptors [5,12–15]. In male starlings, immunolabeling for the opioid met-enkephalin in the POM and VTA correlated with male song production, however for POM this correlation existed only for song that was *not* produced in response to a female (with a similar trend observed for VTA) [5]. These results link opioids in the POM and VTA specifically to the type of song produced by males without nest boxes. The results of opioid-receptor pharmacological manipulations in male zebra finches also support the idea that opioids more strongly influence undirected song compared to female-directed song [15].

Altogether, the results of pharmacology studies and met-enkephalin immunolabeling suggest that the effects of opioids in part acting within POM and VTA may differ in males singing at high rates in response to a female (i.e., males with nest boxes) compared to those singing low rates of undirected song (i.e., males without nest boxes). To test this, we examined individual differences in densities of immunolabeled mu-opioid receptors in flocks of male starlings in aviaries with nest boxes. If the inhibitory role of opioids in female-directed song in starlings is mediated by opioids in POM and VTA, then we predict that mu-opioid receptor densities will be lower in these and possibly other regions in males that successfully acquire nest boxes compared to those that fail to obtain a nest box.

2. Material and Methods

2.1 Animals

In January of 2008, 19 adult male and 10 adult female starlings were captured on a single farm west of Madison, Wisconsin using baited fly-in traps. After capture, birds were immediately housed indoors in stainless steel, single sex cages (91 cm×47 cm×47 cm) within the University of Wisconsin's Department of Zoology indoor animal facilities. Food (Purina Mills Start and Grow Sunfresh Recipe, 61S3-IGH-G) and water were provided ad libitum. Each animal was assigned a numbered and colored leg band for identification. All procedures and protocols were in adherence with the Food and Drug Administration's Guide and in accordance with the University of Wisconsin-Madison Research Animal Resource Committee (RARC). All procedures were approved by the University of Wisconsin Institutional Animal Care and Use Committee.

Prior to study initiation, a hackle feather was removed from the breast of each bird and the length of iridescence was measured using calibrated calipers. Previous research [16] shows that males with hackle feathers ranging from 11–15mm are older than one year of age. Based on hackle feather length, all birds in the present study were estimated to be over one year of age at the start of the experiment.

2.2 Light cycle and hormone manipulations

Prior to study initiation, birds were housed indoors and placed on artificial photoperiods of 18L: 6D for six weeks, followed by a photoperiod of 6L: 18D for an additional six weeks. These photoperiod manipulations induce photosensitivity, a condition in which when given T implants male starlings with nest boxes display high rates of courtship song and behaviors in response to a female [17,18]. Each male test subject received two subcutaneous implants of testosterone (T) (as detailed below). Each stimulus female also received two subcutaneous implants of 17β-estradiol, to maximize female sexual interest in males in the laboratory.

Hormones were implanted seventeen days prior to the first day of behavioral testing. All birds were lightly anesthetized using isoflurane gas anesthesia and a small pocket-like incision was made in the skin over the breast muscle. The incision site was sutured closed (Ethilon nylon suture, 13mm, 3.8c, #698G). Males received two, 14-mm lengths of silastic tubing (i.d., 1.47-mm; o.d. 1.96-mm; Dow Corning, Midland, MI) packed for 10-mm with crystalline testosterone proprionate (Sigma-Aldrich, St. Louis, MO). Females received two, 17-mm lengths of silastic tubing packed for 13-mm with 17β-estradiol (Sigma-Aldrich). After recovering on a heated pad, male test subjects were placed in outdoor aviaries on natural day length and allowed to acclimate to study conditions. Female stimulus birds were returned to their home cages.

2.3 Housing and acclimation

Animals were randomly assigned to one of five outdoor aviaries (4 birds per aviary; 2.13m × 2.4m × 1.98m per aviary) and allowed to habituate prior to the beginning of behavioral testing. Each aviary contained four nest boxes and branches for perching. Food and water were provided ad libitum. Each aviary was visually, but not acoustically isolated from the others by the use of camouflage blinds. Each day during the week prior to the first day of behavioral observations, green nest materials (green grass clippings and leaves) and a novel female were introduced into each aviary to allow males to habituate to the study procedures as well as the presence of an observer. During this time the observer additionally, practiced observing each male starling. Starlings display high site fidelity and behavioral data can reliably be collected from multiple birds in a single aviary simultaneously [17,19,20].

2.4 Behavioral observations and study procedures

Over five consecutive days, each aviary was provided with nest material and one stimulus female (a novel female each day) was released into the aviary. Five minutes after the introduction of the female, each aviary was observed for 20min by a single observer concealed by dark green camouflage. The order of observations across aviaries was randomized each day, with observations performed between 8:00 and 13:00. Starling song is complex and consists of at least four distinct components including introductory whistles, complex phrases, click series, and high-frequency phrases [21]. During each observation period the *total number of complete song bouts* (i.e., bouts containing each of the 4 components) was recorded. A distinct bout was defined as an event separated from the next event by at least two seconds. Furthermore, *nest box directed behaviors* were recorded, including the number of times males entered and exited nest boxes, collected nesting material and looked in the nest box. Additionally, measures of *agonistic behavior* (including chasing and displacements from perches or food dishes) as well as bouts of wing waving, feeding, and drinking were also recorded.

2.5 Tissue collection and processing

Immediately after the last twenty-minute observation period, all males in a group were sacrificed via rapid decapitation. Each male was checked to confirm the presence of hormone implants. Brains were removed by dissection, fixed and agitated in a 5% acrolein solution overnight, rinsed and placed in a 30% sucrose cryoprotectant for 4 days, rapidly frozen with crushed dry ice, and stored at − 80 °C until sectioning. Using a cryostat, brains were cut in the coronal plane in three, 40μm series (each series contained every third section) and stored in anti-freeze cryoprotectant (PBS, polyvinylpyrrolidone, sucrose and ethylene glycol mixture) until processing. Storage in a cyroprotectant allows for the long term storage of free floating sections to reduce the loss of antigenicity [22]. Series one was used for mu-opioid receptor immunohistochemistry described here.

2.6 Blood sampling and serum analysis

As part of a separate study not reported here, a single sample of no more than 200uL of blood was collected via venipuncture of the ulnar vein of each male once during the five days of testing. A terminal trunk blood sample was taken immediately after sacrifice for use in the present study.

To insure that T concentrations were elevated by the implants, T plasma metabolites in the terminal blood sample were measured with a commercial grade competitive assay immunoassay (EIA; Cayman Chemical, Ann Arbor, MI, USA, Catalog No. 582701). Samples were run in duplicate, using the manufacture's protocol at a dilution of 1:8 (determined in pilot studies as the optimal concentration) in buffer solution and visualized at 405nm with a BioTek 800 plate reader (#7331000, ELv800™, BioTek Instruments, Inc. Winooski, VT, USA). Sensitivities of the commercial EIA according to the manufacture's specifications indicate a limit of detection: 80% B/B_0: 6 pg/ml and sensitivity: 50% B/B_0: 32 pg/ml. The assay is specific (cross reactivity to 5 α-DHT is 27.4%, and to 17- β -estradiol <. 01 %). All samples were run within a single assay. The intraassay CV was 12.39%.

2.7 Antibody specificity tests

Brain tissue was processed using immunohistochemistry for the mu-opioid receptor. Antibody specificity was verified using multiple techniques. First, a preadsorption test (tissue was incubated in mu-opioid receptor antibody (Abcam, ab10275) with the mu-opioid receptor peptide (1:50; Abcam, ab46988)) revealed no labeling; additionally, in pilot studies that omitted the primary antibody no labeling was detected. Secondly, a Western blot

analysis was run to confirm specificity. Snap frozen dissected brain blocks from the hypothalamus were homogenized after adding 300 μ L ice-cold lysis buffer consisting of 50 mM Tris-HCl, 1% Na-deoxycholate, 0.25% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, protease inhibitor cocktail (P-8340, as directed; Sigma, St. Louis, Missouri, USA) and phosphatase inhibitor cocktail (P-2850, as directed, Sigma, St. Louis, Missouri, USA). In order to remove cellular debris and nuclei, the samples were centrifuged at 14000 r.p.m. for 10min at 4°C. The supernatant was collected and protein concentration determined by a BCA protein assay (Cat# 23225, Thermo Fisher Scientific, Rockford, IL, USA) . Fifteen micrograms of total protein from each animal were gel electrophoresed using a precast SDS-PAGE 4–20% Tris Glycine gel (Cat# 58645, Cambrex Bio Science Rockland, Inc., Rockland, Maine, USA) and transferred to a polyvinyl difluoride membrane (Immobilon-P; Cat# IPVH20200 Millipore, Bedford, Massachusetts, USA). Membranes were blocked for one hour in 0.1 M TBS containing 5% nonfat dry milk with constant agitation at room temperature. Membranes were then incubated overnight at 4°C with agitation in TBS containing 0.05% Tween-20 (TBS-T) and 2% nonfat dry milk with primary antibody (for mu-opioid receptor, Ab10275 [Abcam, 1:5000]) overnight at 4°C. The next day, the membranes were given three quick washes and then four five minute washes in TBS-T. Following washes, membranes were incubated in a goat anti-rabbit horseradish peroxidase-linked secondary antibody (Cat# 7074, Cell Signaling Technology, Inc., Beverly, Massachusetts, USA, 1:3000) and horseradish peroxidase conjugated anti-biotin antibody (Cat# 7075, Cell Signaling Technology, Inc., Beverly, Massachusetts, USA, 1:5000) for 30min at room temperature with agitation and then washed three times for 5min each with TBS-T and twice for 5min each with TBS. Immunoreactive bands were detected using a chemiluminisense kit (Ca# 7003, Cell Signaling Technology, Inc., Beverly, Massachusetts, USA) and exposed to X-ray film (Cat #178 8207, Eastman Kodak Co., Rochester, New York, USA).

Western immunoblot indicated a dark band at 48kDa, the molecular weight for the mu-opioid receptor protein [14,23]. Two additional bands were observed at approximately 50 and 160 kDa. These bands were lighter and may represent splice variants [24,25] or dimeric forms of the mu-opioid receptor due to glycosylation [26].

2.8 Immunohistochemistry

Immunohistochemistry on tissue from all males (n = 19) was run in a single batch and background labeling was observed to be similar across all sections. Sections were rinsed in phosphate buffered saline (PBS) for 30 min, incubated in 0.5% sodium borohydride solution for 15 min, rinsed in PBS for 20 min, incubated in 0.5% hydrogen peroxide solution for 10 min, rinsed in PBS for 20 min, incubated in 20% normal goat serum (NGS (made in PBS with 0.2% triton (PBS-T))) solution for 1 h, and then incubated in 2% NGS (made in PBS-T) primary solution overnight at room temperature (rabbit anti-mu-opioid receptor at 1:5000 (Abcam, ab10275)). Sections were then rinsed in PBS-T for 30 min and incubated in 2% NGS (made in PBS-T) biotinylated secondary solution for 90 min at room temperature (goat anti-rabbit at 1:1000 (Vector Laboratories, Burlingame, CA)). Sections were then rinsed in PBS-T for 30 min, incubated in AB solution (Vectastain Elite ABC, Vector Laboratories) for 1 h, rinsed in PBS-T for 30 min, and the avidin–biotin complex was visualized using 3,3′-Diaminobenzidine (DAB) tablets (Sigma Aldrich, St. Louis, MO, USA). Sections were float mounted onto gel-coated slides, dehydrated in a series of alcohols, and cover slipped.

2.9.1 Quantification

Using a Spot Camera (Diagnostic Instruments, Inc., Sterling Heights, MI, USA) connecting a Nikon microscope to a computer to acquire images of brain sections and using METAVUE (Fryer Company, Inc., Huntley, IL, USA) software, mean optical density and

pixel area for mu-opioid receptor labeling were quantified bilaterally on three serial sections for each bird in regions implicated in reward, agonistic, and sexual behavior (POM, VTA, CG, LS, and BSTm). The locations of these nuclei were based on Heimovics and Riters (2007). Additionally, we took bilateral measures of labeling in the paraventricular nucleus (PVN) of the hypothalamus from three serial sections containing the anterior commissure. This region was included because it was rich in mu-opioid receptor labeling, and past work implicates PVN in sociosexual behaviors [27]. Labeling was not observed at high levels in areas in the nidopallium or arcopallium, thus measurements were not made in song control regions.

For measures of optical density and pixel area, a single computer generated threshold selected material that a blind observer agreed represented labeling. A separate threshold was generated for each brain region of interest. The METAVUE autoscale function was used to calculate the correct exposure of each image as a percentage of the total range of light, which further reduced the variation in background among individuals. Optical density is a measure of how much light is transmitted through the tissue, providing an index of labeling density. Pixel area (pixels highlighting fibers using the computer generated threshold) provides a measure of the approximate area covered by labeled receptors. Measures in POM, VTA, CG, PVN, LS, and BSTm were made within boxes or ovals (Figure 1; Table 2) centered within each region of interest, and measures were generated by the METAVUE software in each section in both hemispheres for each bird. In cases of tissue damage, labeling was quantified either on a fourth section or the individual was dropped from analysis for affected brain areas.

2.9.2 Statistical analysis

Data were analyzed using Statistica 6.0 software (Stat Soft Inc., Tulsa, OK). Males were defined as either nest box owners or non-owners. Specifically, if a male was observed entering and exiting the same nest box(es) multiple times (more than 5 times) on more than one day, he was designated as a nest box owner. Birds that did not display any aspect of song (intro whistles, fragments, or full song) during the behavioral observation period were dropped from analysis (n=3, non-owners).

A multivariate analysis of variance (MANOVA) was run with total song, nest box directed behaviors, total agonistic behavior, and the sum of feeding plus drinking behavior entered as repeated measures dependent variables and nest box ownership as a categorical between subjects independent variable (males with nest boxes, n=7; males without nest boxes, n=9). Separate MANOVAs were run with mu-opioid receptor optical density and pixel area in POM, VTA, CG, BSTm, LS, and PVN entered as repeated measures dependent variables and nest box ownership as a categorical between subjects independent variable. For the MANOVAs on mu-opioid receptors, a mean substitution (mean of all other animals in the group) was used to replace missing data. Specifically, data were substituted for: one male with no box for the POM, one male with a nest box for LS, and three males with and one without a box for BSTm. Because four replacements were required for BSTm (tissue folded or tore from the ventricle in this area) the MANOVA was run with and without this region included. The results were similar; therefore, the BSTm is included in the analysis. Finally, because mu-opioid receptors differed in males with and without nest boxes we ran multiple regression analyses to determine what best explained variance in mu-opioid receptor optical density and pixel area. Specifically, in separate multiple regression analyses a brain region was entered as the dependent variable and total number of songs, total number of agonistic interactions, and whether or not a male possessed a nest box were entered as predictor variables. Multiple regression analyses were run on the original data set for each region with no mean replacement. Influential statistical outliers were removed from analyses, resulting in removal of one data point from BSTm pixel area measures. For multiple regression

analyses backward and forward analyses were performed. In all cases except for BSTm results were identical. For BSTm backward regression resulted in the same (though not identical) significant effects. Results of the backward analysis are provided because this model best explained the data based on the highest adjusted R^2, lowest standard error, and the best residual plots.

3. Results

3.1 Behavior

Sixteen males were used for behavioral analysis. Seven males obtained one or more nest boxes and nine failed to acquire a nest box. The results of a MANOVA revealed an overall effect for nest box ownership ($F_{1, 14} = 45.59$, $p = 0.000009$). Fischer's post hoc analyses indicated that compared to males without nest boxes, nest box owners responded to the introduction of a female with significantly higher rates of total song ($p = 0.0005$; Figure 2A), displayed significantly more nest box directed behaviors ($p = 0.000009$; Figure 2B), and higher levels of agonistic behavior (the sum of all chases and displacements; $p = 0.011$; Figure 2C). Bouts of wing waving were only performed by nest box owners. Feeding and drinking behavior were not significantly different between males with or without nest boxes ($p>0.05$).

3.2 Mu-opioid receptor labeling and nest box ownership

The results of a MANOVA revealed an overall effect for nest box ownership on both the optical density ($F_{1,14} = 8.68$, $p = 0.01$) and pixel area covered by labeled receptors ($F_{1,14} = 8.26$, $p = 0.011$). Specifically, mu-opioid receptor labeling area and density were significantly lower in males with compared to males without nest boxes (optical density shown in Figure 3; pixel area means in Table 3).

3.3 Mu-opioid receptor labeling and behavior

Results of multiple regression analyses revealed nest box ownership (and not total songs or agonistic interactions) to contribute significantly to variance in mu-opioid receptor optical density in BSTm, PVN, POM, and VTA (Figure 3 and 4; BSTm: adj $R^2 = 0.28$, N = 12, nest box beta = 0.587, sd of beta = 0.26, $t_{10} = 2.29$, $p = 0.045$; PVN: adj $R^2 = 0.24$, N = 16, nest box beta = 0.541, sd of beta = 0.22, $t_{14} = 2.40$, $p = 0.031$; POM: adj $R^2 = 0.40$, N = 15, nest box beta = 0.668, sd of beta = 0.21, $t_{13} = 3.24$, $p = 0.006$; VTA: adj $R^2 = 0.27$, N = 16, nest box beta = 0.566, sd of beta = 0.22, $t_{14} = 2.56$, $p = 0.022$). No variables contributed significantly to variance in LS or CG.

For measures of the pixel area covered by mu-opioid receptor labeling, results of multiple regression analyses revealed nest box ownership (and not total songs or agonistic interactions) to contribute significantly to variance in mu-opioid receptor pixel area in BSTm and POM (BSTm: adj $R^2 = 0.39$, N = 11, nest box beta = 0.670, sd of beta = 0.25, $t_9 = 2.71$, $p = 0.024$; POM: adj $R^2 = 0.26$, N = 15, nest box beta = 0.557, sd of beta = 0.23, $t_{13} = 2.71$, $p = 0.031$; results of analyses of pixel area were similar to optical density and are thus not presented graphically but appear in Table 3). For VTA pixel area the total number of songs (rather than nest box ownership or agonistic behavior) contributed significantly (negatively) to variance in mu-opioid receptor pixel area (VTA: adj $R^2 = 0.24$, N = 16, song beta = −0.540, sd of beta = 0.22, $t_{14} = 2.40$, $p = 0.031$; Table 3; Figure 5). No variables were found to contribute significantly to variance in pixel area in LS, CG or PVN.

3.4 Testosterone

An independent t test revealed no significant differences in T concentrations between males with or without nest boxes (with nest box: Mean= 0.08pg/ml, sd=0.04, without nest box: Mean=0.10 pg/ml, sd =0.07, $p > 0.05$).

4. Discussion

Consistent with past studies, males with and without nest boxes differed dramatically with respect to their behavioral responses to conspecifics [3,4,7,28]. Males with nest boxes sang at high rates and accompanied song with wing waves (a courtship display); whereas, males without nest boxes sang at low rates and did not wing wave. Males with nest boxes also chased and displaced other males from perches or feeding sites more frequently than males without nest boxes indicating that they were socially dominant.

4.1 Mu-opioid receptor densities were lower in POM and VTA in males with compared to without nest boxes

As predicted based on the extreme differences in singing behavior in males with and without nest boxes, as well as past studies implicating opioids in the POM and VTA in male song [5], nest box ownership significantly explained variance in mu-opioid receptor labeling in both POM and VTA. Specifically, male starlings with nest boxes had lower densities of mu-opioid receptors in these regions than males without nest boxes. This finding is similar to a study in rats in which social defeat (similar to a failure to acquire a nest box) resulted in increased mu-opioid receptor mRNA in VTA [29,30]. In addition to finding mu-opioid receptor optical density to be lower in VTA in males with nest boxes, multiple regression analysis also revealed song to best explain variance in mu-opioid receptor pixel area. This latter finding reflected the fact that males without nest boxes sang at the lowest rates in response to a female and had the greatest area covered by mu-opioid receptor labeling.

In starlings, pharmacological manipulations demonstrate an inhibitory role for opioids in female-directed song [4]. Furthermore, opioids in the avian POM inhibit neuronal firing [31], as well as suppress agonistic, and sexual behaviors in Japanese quail [32]. Given the inhibitory role of opioids in POM, the high densities of mu-opioid receptors in this region in males without nest boxes may make these regions more sensitive to opioid input thereby inhibiting inappropriate social responses to conspecifics. In contrast, the low opioid receptor density in males with nest boxes may render the POM less sensitive to opioids, thereby disinhibiting behavioral responses to female and male conspecifics.

In songbirds, opioids in VTA also act at mu-opioid receptors to inhibit neuronal firing [33]; however unlike opioids in the POM, in rat studies opioids in VTA *increase* sexually-motivated behavior [34]. This effect is mediated by opioid inhibition of GABA receptors, which causes a disinhibition of activity in VTA dopamine neurons [35,36]. However, in a recent study elevated firing rates in VTA neurons and dopamine release were found in association with social defeat in male rats [37]. Thus, the finding that males that failed to acquire nest boxes have elevated densities of mu-opioid receptors in VTA may reflect a role for opioid stimulation of dopamine release associated with social defeat.

Previously, in male starlings immunolabeling density for the opioid met-enkephalin in POM correlated positively with undirected but not female-directed song (with a similar trend observed for VTA; $p = 0.06$) [5]. The limited range of singing observed in males without nest boxes in the present study prevented us from running a similar analysis; however, the present finding that males without nest boxes (i.e., males that sing undirected but not directed song) have elevated densities of mu-opioid receptors in POM and VTA is consistent with the met-enkephalin study and with a study in male zebra finches showing opioid

blockade to more potently suppress undirected as opposed to directed song [15]. We have previously proposed that the production of undirected song is intrinsically motivated and reinforced by opioid release in POM and VTA [6]. The present data showing opioids to be elevated in the group of males singing only undirected song are consistent with this hypothesis.

Here, we report a negative correlation between the area covered by mu-opioid receptor pixel area in VTA and song when males with and without nest boxes were combined. This may reflect an inhibitory role for mu receptors in VTA in song in response to a female in males without nest boxes. However it is somewhat in contrast with past data showing labeling for the opioid met-enkephalin to relate positively to male song production when males singing to attract a female in spring and males singing undirected songs in fall were combined [5]. Interpretation of these differences is difficult because met-enkephalin labeling can reflect stored or released enkephalin and because of the dynamic relationships that exist between receptors and peptides (e.g., receptors can be up-regulated in the presence of low levels of opioid release). Thus, the reason that song relates positively to met-enkephalin labeling but negatively to mu-opioid receptor labeling must be examined in future work using direct measures of opioid release or opioid receptor manipulations in the VTA.

4.2 Possible roles for opioids in BSTm and PVN in males with and without nest boxes

The present results also suggest two novel regions in which opioids may regulate song and agonistic interactions, the BSTm and PVN. BSTm is implicated in sexual and agonistic behaviors in birds as in mammals [11,38–42]. Immediate early gene responses in BSTm differ in male starlings with and without nest boxes and depending on whether males are singing to attract a female or singing undirected song [7,9]. Across vertebrates, the PVN has been implicated in agonistic behaviors and responses to stressors, including social stressors such as opposite sex conspecific intruders [43–47]. Little is known about the role of opioids in BSTm or PVN in social behavior, however opioids in BSTm can inhibit neuronal firing [48] and male sexual behavior in rodents [49]. Therefore, the elevated mu-opioid receptor activity in BSTm in males without nest boxes may serve to inhibit sexual behavior. Opioids in the PVN have been found to modify physiological responses to stress in rodents [50]. Accordingly, the differences observed in mu-opioid receptors in PVN in the present study may reflect opioid regulation of differential stress responses to social challenges in males with and without nest boxes.

4.3 What do differences in receptor densities reflect?

Reductions in mu-opioid receptor density measures may reflect a decrease in receptor numbers or reduced opioid receptor activation. When bound and activated mu-opioid receptors internalize, which is reflected as an increase in optical density [51–54]. Thus, opioid receptors may be more active in males without nest boxes in POM, VTA, BSTm and PVN. In POM and BSTm the area (pixel area) covered by labeled receptors was also reduced in nest box owners. The differences in pixel area may reflect differences in the sensitivity of a region to opioids; with the larger the area covered by receptors indicating a possible greater sensitivity of the region to opioids.

Interestingly, only nest box ownership (and not agonistic or singing behavior) explained variance in mu receptor measures in POM, PVN, and BSTm. This suggests owning a nest box rather than exhibiting associated behaviors either causes a reduction in opioid receptors in these areas, or conversely that only males with lower densities of mu receptors are motivated to own a nest box. Whether the differences in mu-opioid receptor densities are the cause or consequence of acquiring a nest box is not clear from the present study. This issue must be resolved with studies in which males are assigned individually to aviaries with or

without nest boxes or by examining effects of site specific pharmacological manipulations of opioid receptors on behavior of males with and without nest boxes.

4.4 Testosterone and differences in males with and without nest boxes

T is linked to nest box ownership [2,3,28], as well as song in response to a female [2,3,20]. In the present study males received implants of T, and serum concentrations of T did not differ in males with or without nest boxes. This suggests that differences in behavior and mu-opioid receptors observed in males with and without nest boxes were not determined by differences in serum T, but perhaps by resource possession alone or by the production of behaviors associated with nest box ownership. However, a recent study in territorial California mice (*Peromyscus californicus*) demonstrates that winning an agonistic interaction (which can be considered similar to "winning" a nest box in starlings) results in increased expression of androgen receptors in BSTm and VTA [55]. Thus, it may be that acquisition of a nest box results in increased sensitivity of neural tissue to androgens which then modifies mu-opioid receptors and behavior (independent of serum T concentrations). This possibility must be addressed in future studies.

4.5 Concluding remarks

The results of the present study suggest that the acquisition of a nest box may cause a reduction in opioid receptor activity within the BSTm, PVN, POM, and VTA of male starlings to promote agonistic responses to males and sexually motivated vocal responses to females. The results are consistent with past studies suggesting a context-dependent role for opioids in birdsong. Moreover, they suggest an important, testosterone-independent, role for opioids in modifying social behavior so that it is appropriate given the resources available to an individual.

Research Highlights

- Male starlings with nest boxes displayed agonistic and courtship behaviors
- Male starlings without nest boxes displayed little agonistic or courtship behavior
- Mu-opioid receptors in several regions were densest in males without nest boxes
- Mu- receptors may inhibit responses to conspecifics in males without nest boxes
- Differences in males with and without nest boxes were not dependent on testosterone

Acknowledgments

The data presented in this paper are based upon work supported by grants from R01 MH080225 to LVR. We gratefully acknowledge Kate Skogen, and Chris Elliot for animal care taking; Sharon Stevenson for help with tissue processing and Bill Feeny for help with illustrations.

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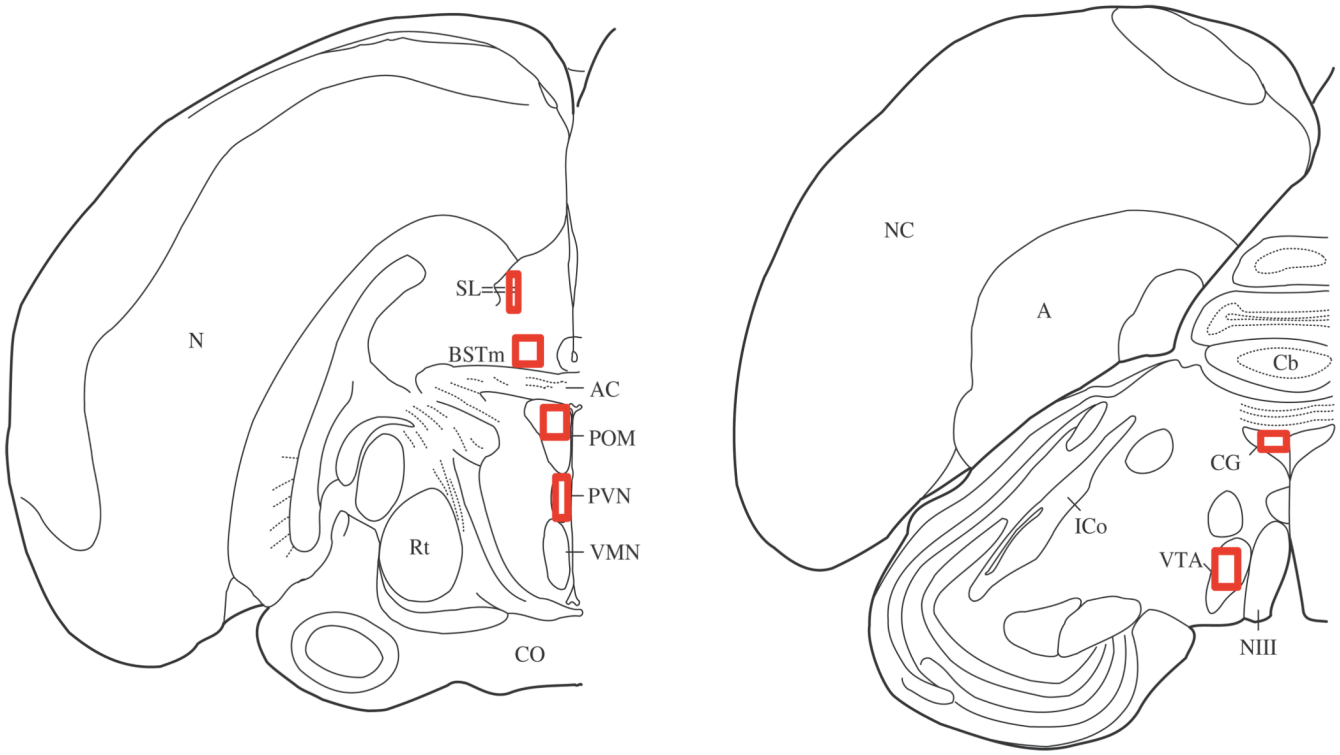

Figure 1.

Brain nuclei measurement locations. Boxes indicate approximate areas in which optical density and pixel area of mu-opioid receptor labeling was quantified. *Abbreviations*: A, arcopallium; BSTm, medial bed nucleus of the stria terminalis; Cb, cerebellum; AC, anterior commissure; CO, optic chiasm; CG, medial central gray; ICo, nucleus intercollicularis; NIII, third cranial nerve; N, nidopallium; NC, caudal nidopallium; POM, medial preoptic nucleus; PVN, paraventricular nucleus of the hypothalamus; Rt, nucleus rotundus; LS, lateral septum, VMN, ventromedial nucleus; VTA, ventral tegmental area.

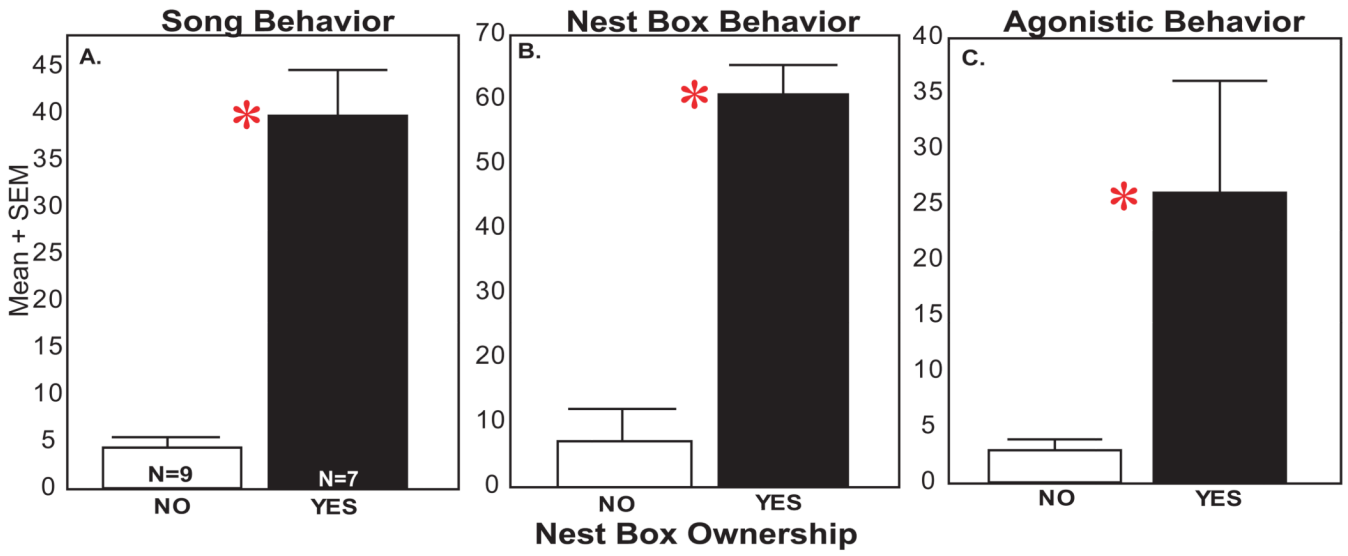

Figure 2.

Difference in behaviors between males with and without nest boxes. Mean number of behaviors in nest box owners, in black boxes, and non-owners in white for (A) Song, (B) Nest box specific behaviors (looking in the nest box, entering the nest box, collecting nest material), and (C) measures of agonistic behavior (chasing and displacements of conspecific males). Asterisk indicates significant differences between nest box owners and non-owners ($p < 0.05$).

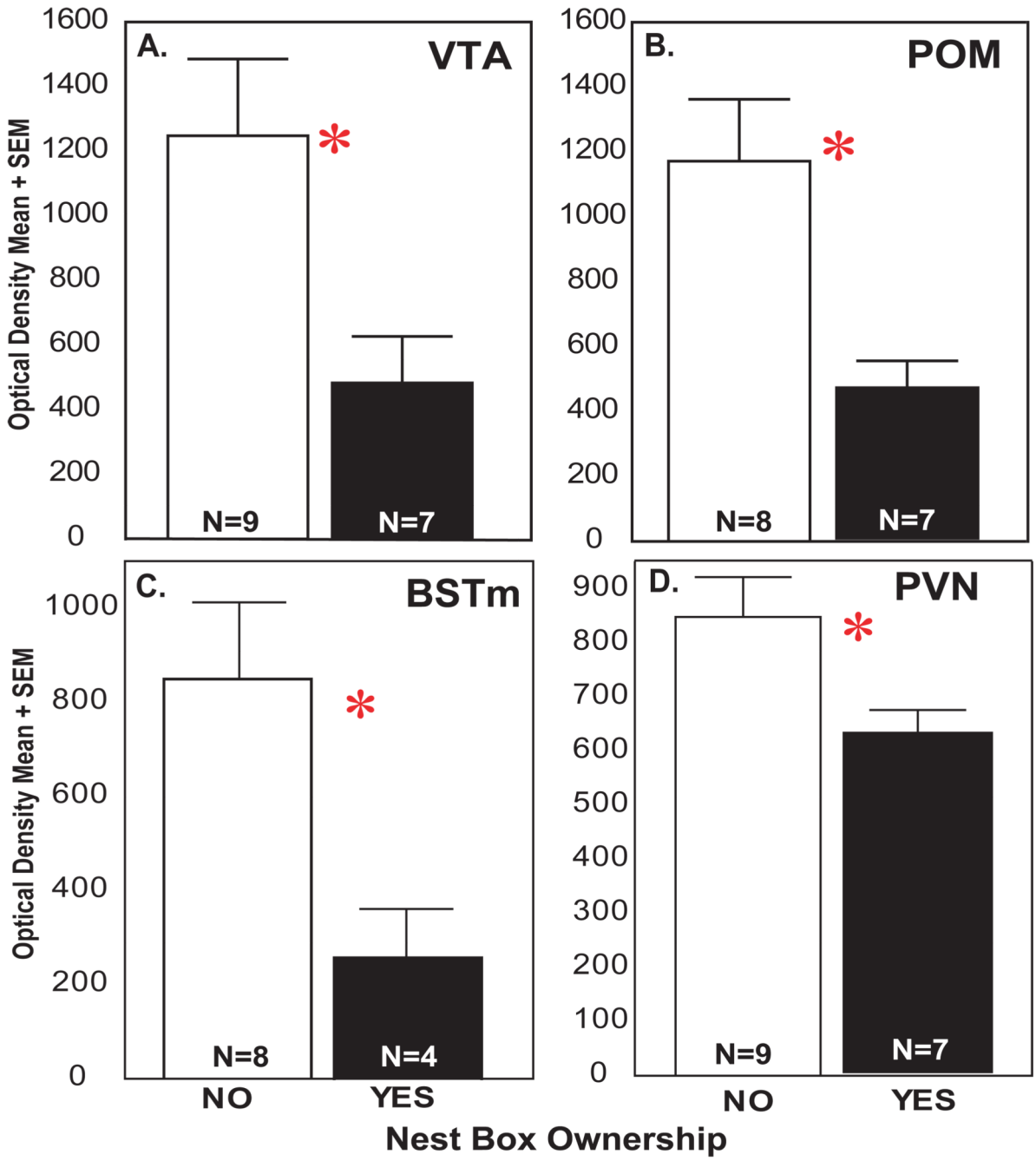

Figure 3.

Differences in mu-opioid receptor labeling in brain regions in males with and without nest boxes. Mean optical density of mu-opioid receptor labeling in (A) VTA, (B) POM, (C) BSTm, (D) PVN. Black bars represent males with nest boxes; white bars, males without nest boxes. Asterisk indicates a significant contribution of nest box ownership to mu-opioid receptor labeling density based on multiple regression analysis ($p < 0.05$).

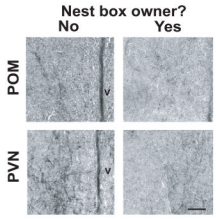

Figure 4.

Photomicrographs illustrating mu-opioid receptor labeling. Representative images include POM (top) and PVN (bottom) in males without (left) and with (right) nest boxes. Scale bar at the bottom right box is approximately .005mm.

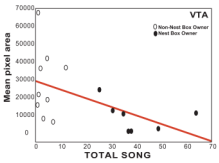

Figure 5.
Scatterplot showing the relationship between pixel area measurements in VTA and song. Each point represents one individual, darkened circles represent males with nest boxes, open circles represent males without nest boxes. Presence of the regression line indicates a significant relationship ($p<0.05$).

Table 1

List of Abbreviations

Abbreviation	Full Name
BSTm	Bed nucleus of the stria terminalis, medial part
CG	Midbrain central gray
LS	Lateral septum
POM	Medial preoptic nucleus
PVN	Paraventricular nucleus
T	Testosterone
VTA	Ventral tegmental area

Table 2

Box measurements

Nucleus	Shape	Size (mm)
BSTm	Rectangle	Area= 0.67 × 0.37
CG	Oval	Diameter= 0.60
POM	Rectangle	Area= 0.43 × 0.44
PVN	Rectangle	Area= 0.22 × 0.42
LS	Rectangle	Area= 0.36 × 0.54
VTA	Rectangle	Area= 0.38 × 0.53

Table 3

Pixel Area Measurements (without mean data substitution)

Brain Region	Nest box owners	Owners Pixel Area Mean +/− SEM	Nest box Non-owners	Non-Owners Pixel Area Mean +/− SEM
VTA*	7	9103.66 +/− 3206.408	9	28010.14 +/− 6502.517
POM**	7	4976.16 +/− 1366.120	8	11528.34 +/− 2227.134
BSTm**	4	4859.77 +/− 1415.464	8	16397.77 +/− 5684.518
PVN	7	18342.13 +/− 4061.620	9	19848.47 +/− 2138.746
LS	6	7429.17 +/− 1527.734	9	12099.39 +/− 2271.638
CG	7	17085.27 +/− 5289.357	9	26433.04 +/− 5474.350

* indicates a significant contribution of singing behavior to mu-opioid receptor labeling density based on multiple regression analysis ($p < 0.05$).

** indicates a significant contribution of nest box ownership to mu-opioid receptor labeling density based on multiple regression analysis ($p < 0.05$).