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Modulation of male song by naloxone in the medial preoptic nucleus

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Abstract

Studies in songbirds implicate opioid neuropeptides in singing behavior, however past results are contradictory. In starlings, the effect of opioid manipulations on sexually-motivated courtship song differed in birds naturally singing at low compared to high rates, and mu-opioid receptors were denser in several regions, including the medial preoptic nucleus (POM) in low singing males. In the present study we found that low singing male starlings also had significantly higher enkephalin (ENK) immunolabeling densities in the POM than high singers. We then blocked opioid receptor activity in the POM with naloxone injections and found that this increased both song rate and song bout length in low singers, suggesting that high densities of mu receptors and ENK in the POM actively suppress song in these males. In contrast to its effects on low singers, naloxone in the POM of high singers dose dependently decreased song rate and tended to reduce song bout length. This suggests that at least some level of opioid activity in POM is necessary for song production. Our results are the first to demonstrate that direct administration of opioids into the POM influences sexually-motivated song and that effects differ depending on an individual's initial rate of song and associated density of ENK. We suggest that differential effects seen in past studies of opioids and song may in part be explained by differences in the natural song rate of subjects and accompanying differences in enkephalin activity and neural substrate sensitivity to opioids in POM.

Keywords

opioids; vocal communication; birdsong; motivation; medial preoptic area

1. INTRODUCTION

Little is known about the neurochemical mechanisms regulating differences observed across individuals in the motivation to communicate. In songbirds and other vertebrates studies implicate opioid neuropeptides in vocal production (Carden, Hernandez, & Hofer, 1996; Panksepp, Herman, Vilberg, Bishop, & DeEsquinazi, 1980; Schroeder & Ritters, 2006); however, the results are contradictory. For example, in male European starlings peripheral treatment with the opioid receptor antagonist naloxone increased sexually-motivated song production (Schroeder & Ritters, 2006); whereas in male zebra finches, naloxone treatment reduced the number of female-directed songs (Khurshid, Jayaprakash, Hameed, Mohanasundaram, & Iyengar, 2010). Interestingly, responses to opioid receptor manipulations in male starlings appeared to depend on whether males were singing naturally at low or high rates (Ritters, 2010). Specifically, the mu receptor agonist fentanyl uniformly abolished song in all high singers. In contrast, low singers did not respond uniformly to

fentanyl, with some increasing and others decreasing song production. Naloxone uniformly increased song production in low singers, but resulted in a less uniform response in high singers (Riters, 2010). This suggested that individual differences may exist in opioid receptor densities or binding affinity in individuals communicating at low or high rates; a hypothesis supported by a study showing that densities of immunolabeled mu-opioid receptors are higher in low compared to high singing males (Kelm, Forbes-Lorman, Auger, & Riters, 2011).

In birds as well as mammals, endogenous opioids, including enkephalin opioids, in the POM inhibit male sexual behavior (Kotegawa, Abe, & Tsutsui, 1997; Van Furth, Van Emst, & Van Ree, 1995; Van Ree *et al.*, 2000). This suggests that enkephalin activity may also be elevated in the POM of males singing low compared to high rates of sexually-motivated courtship song. High densities of mu receptors have been found in the POM of low singing males and may act to suppress sexually-motivated song. If true, mu-opioid receptor blockade in POM should facilitate song in these males. In contrast, the lower densities of mu receptors in POM in high singing males may facilitate sexually motivated song by reducing region sensitivity to opioids. Thus, mu receptor blockade in high singing males may act to further increase singing behavior. However, it may be that at least some level of mu receptor activity is needed to maintain song even in high singing males. Here, we tested these predictions.

2. EXPERIMENTAL METHODS

All procedures and protocols are in adherence with the *National Institutes of Health Guide for the Care and Use of Laboratory Animals* (DHEW Publication 80-23, Revised 1985, Office of Science and Health Reports, DRR/NIH, Bethesda, MD 20205) and approved by the University of Wisconsin-Madison Institutional Animal Care and Use Committee.

2.1 Experiment 1: Song and enkephalin (ENK) labeling

2.1.1 Animals and housing—Twenty adult male European starlings (*Sturnus vulgaris*) and five females were trapped on the same farm in Madison, WI (2008-2010). Birds were assigned leg bands for identification and were randomly assigned to single-sex indoor communal aviaries (47cm H×47cm W×91cm L) containing multiple perches and nest boxes.

2.1.2 Light cycle and hormone manipulations—Manipulations of photoperiod and testosterone implants were used to place males into a breeding-like physiological state (Dawson, King, Bentley, & Ball, 2001; Kelm *et al.*, 2011). Two weeks prior to study initiation each male received testosterone implants as in Kelm *et al.* (2011), and each stimulus female received two subcutaneous implants of 17 β -estradiol as in Riters, Oleson and Auger (2007) to facilitate sexually motivated behaviors in the laboratory.

2.1.3 Habituation—After two weeks, an observer screened aviaries to identify singing males. Nest material (consisting of green grass, leaves, and straw) and a stimulus female were introduced into male aviaries. Only males observed to sing in response to a female during screening were used in the study. We have found that when housed in pairs males tend to sing at much higher rates to females than when housed singly (unpublished observation). Thus, selected males were placed in pairs in new aviaries equipped with a one-way observation mirror (same dimensions as the community rooms). Pairs were housed with one nest box, multiple perches, food and water.

2.1.4 Behavioral testing—Each aviary was provided with nest material and a novel stimulus female. During a single 20 min observation period between 0900 and 1300, the

total number of full song bouts (i.e., bouts containing each of the 4 components of starling song (Eens, 1997; Eens, Pinxten, & Verheyen, 1990)) and the *mean song bout length (total seconds of full song/number of songs)* were measured in all males by a single observer. Furthermore, *nest box directed behaviors* were recorded, including the sum of times males entered nest boxes, collected nesting material, looked into the nest box and landed on the perch of the nest box. Measures of *agonistic behavior* (chasing and displacements from perches or food dishes) and *general behavior* (bouts of feeding or beak wiping) were also recorded.

2.1.5 ENK immunolabeling—Approximately 90 min after the start of the observation period all males, $n=20$, were sacrificed via rapid decapitation. This is a window in which labeling for the ENK protein was found to increase in association with singing in area X (a song control region) in male zebra finches (Wada *et al.*, 2006).

After decapitation, the presence of the hormone implants was confirmed. Brains were fixed in a 5% acrolein solution cryoprotected, frozen and sectioned in the coronal plane in three, 40 μm series (each series contained every third section) and stored in anti-freeze until processing as detailed in Kelm *et al.* (2011). Series one was used for ENK labeling. Immunohistochemistry on tissue from all males was run in a single batch following a prior protocol (Kelm *et al.*, 2011), except that the primary antibody was rabbit anti-ENK at 1:2500 (Immunostar, #20065, previously manufactured by IncStar, Sillwater, MN) and secondary antibody was goat anti-rabbit at 1:2500 (Vector Laboratories, Burlingame, CA, USA). To ensure antibody specificity, a pilot study was run where primary antibody was omitted, and tissue failed to show labeling. This antibody has also been validated in starling brain tissue using preadsorption tests (Ball, Absil, & Balthazart, 1995), and in male zebra finches specificity was validated using Western immunoblots (Wada *et al.*, 2006). In a more recent paper, pretreatment with leu-enkephalin also partially blocked immunoreactive staining (Stevenson, Calabrese, & Ball, 2012), therefore we refer to immunolabeling generally as enkephalin (ENK).

Using a Spot Camera (Diagnostic Instruments, Inc., Sterling Heights, MI, USA) connecting a Nikon microscope to a computer, images of brain sections were analyzed using METAVUE (Fryer Company, Inc., Huntley, IL, USA) software. Specifically, the average *total pixel area* covered by ENK receptor labeling was quantified bilaterally using methods detailed in Kelm *et al.* (2011) on three serial sections for each bird within a box centered in the POM (Area= 0.496×0.440 ; Fig. I).

2.2 Experiment 2: POM cannula and pharmacological manipulations

For this study, males were captured, housed, and hormonally treated following an identical protocol to that used for the birds in Experiment 1, except that in each aviary one focal male per pair, $n=22$ out of 44 individuals, was randomly selected to receive a cannula guide targeting the POM.

2.2.1 POM cannula—Males were deeply anesthetized with isofluorane/oxygen gas and stereotactically fitted with a permanent unilateral, 26 gauge cannula guide (#C315G/SPC, Plastics One, Inc., Roanoke, VA). The stereotaxic apparatus (Kopf Instruments 995) was set with the beak approximately 45° below the plane of the ear bars. Ear bars were placed in the most rostral (anterior) ear position possible. The point between the ear-bars was used as the anterior-posterior zero point. A small incision was made through the skin, exposing the skull, and the cannula guide tip lowered to the skull at the center of the mid vein. The mid vein can be seen quite clearly through the skull immediately upon exposure. Both the lateral and vertical zeros were established at this position. The anterior-posterior target coordinate

was placed 0.8mm posterior to the stereotaxic zero. The lateral target was placed 0.6mm from skull zero, either to the right or left hemisphere (birds were randomly assigned to receive a cannula in either the right or left hemisphere). The apparatus was adjusted to a 2° angle to the right or left of center so that the cannula was implanted obliquely. A hole was drilled and the vertical target was lowered -5.0mm from skull zero. Each cannula was secured to the skull with screws using dental cement (clear Ortho-Jet powder combined with acrylic liquid; Lang Dental Manufacturing Company, Inc. Wheeling, IL). Birds were allowed three days to recover from surgery, confirmed by presence of singing behavior in response to a novel female conspecific. The day before the observation period, a mock injection was performed to familiarize males to the injection procedure.

2.2.2 Pharmacological manipulations and testing—On each test day, males were anesthetized with isoflurane/oxygen gas and either vehicle or a dose of the general opioid receptor antagonist (Naloxone (Sigma, #N165, molecular weight= 327.27 g/mol) low dose = 0.20 nmol (.065ug in 0.03uL), intermediate dose = 2.0 nmol (0.65ug in 0.03uL), high dose = 20.0 nmol (6.54ug in 0.03uL), dissolved in 0.9% saline (Ricca Chemical, #721016), which served as vehicle) was infused (using a Latin squares design).

The dose was infused using a 33-gauge cannula that was attached to a Hamilton syringe, with PE50 tubing (Plastics One). The volume and rate of the infusion were controlled by an 11 Plus pump (Harvard Apparatus; Holliston, MA). The cannula extends 2mm below the cannula guide when inserted. The infusion rate was set at 0.100 µl/min until 0.30 µl of drug was infused. Infusions were verified by following movement of an air bubble in the tubing, and the injection cannula remained in place for 180 sec following each injection. The cannula was left within the guide for five min to allow the drug to diffuse out from the cannula tip. Males were allowed fifteen min to recover in the aviary before observations began. Behavioral observations of the experimental male began 15 min after injection and followed protocols identical to that described for Experiment 1. The observer was blind to the treatment condition of the focal male.

2.2.3 Cannula verification, histological confirmation of injection sites—After behavioral testing on the last day, males were infused with Chicago Blue 6B (Fisher Scientific) while under anesthesia and then rapidly decapitated. The brains were dissected out of the skull, frozen immediately with dry ice, and stored at -80°C. The brains were sectioned at 50 µm using a cryostat and thaw mounted on to gel-coated slides. Alternate sections were either coverslipped without stain (to avoid washing away the blue dye) or Nissl stained, dehydrated in a series of alcohols, and coverslipped (to highlight the POM and other landmarks). Cannula placement was determined using a microscope based on perforations within POM and the location of blue dye by 2 independent observers blind to the behavior of the males during the study.

3. RESULTS

3.1 Experiment 1: Song and enkephalin (ENK) labeling

Data were analyzed using Statistica 6.0 software (Stat Soft Inc., Tulsa, OK). The assumptions of parametric statistics were tested using Lilliefors tests for normality and Levene's test for homogeneity of variance. In cases in which assumptions were not met, data were square root transformed.

Twenty males were used in the analysis. Males were categorized as either high or low singers based on a median split of full song bouts on the testing day (mean low singers=0.10, SD=0.32, mean high singers=7.44, SD=4.53). A Student's t-test revealed that ENK labeling density in POM was significantly lower in males that sang at high rates

compared to males that sang at low rates (Figure I A-D). Immunolabeling did not correlate with behavioral measures ($p>0.05$).

3.2 Experiment 2: POM cannula and pharmacological manipulations Results of analyses of males with cannulae targeting POM

Cannulae were centered in the POM in 16 males (Figure II A-D). A residual plot analysis was performed and one male for whom data were greater than 2 standard deviations of the mean for full song production on the saline administration day (full song= 12) was removed from the analysis. Animals used in analysis were defined as either low or high singers (male categorization) based on their full song production the day of the vehicle (saline) injection (median for birds combined=2.50, mean=3.150, SD=3.498; mean for high singers=6.62, SD=2.67; mean for low singers=0.38, SD=0.74).

Mean Song Bout Length—For the measure of mean song bout length there was a significant interaction between dose of drug and male categorization ($F_{(3,42)}=3.29$, $p=0.03$). There was no significant main effect of male categorization ($F_{(1,14)}=2.48$, $p=0.14$) or dose ($F_{(3,42)}=0.77$, $p=0.52$). Post hoc contrasts examining the effects of doses of naloxone on the mean song bout length in low singers demonstrated a significant increase in song bout length at the high dose compared to saline ($p=0.008$; Figure II, E).

Full Song Production—Repeated measures ANOVA showed significant main effects of male categorization ($F_{(1,14)}=9.50$, $p=0.0081$) and there was a significant interaction between dose of drug and male categorization ($F_{(3,42)}=4.18$, $p=0.011$). There was no significant main effect of dose ($F_{(3,42)}=0.40$, $p=0.75$). Post hoc contrasts demonstrated that there were significantly more full song bouts produced after a low dose of naloxone compared to saline vehicle administration for males singing at low rates (low dose $p=0.03$; Figure II, F). There were no significant effects at the medium or high doses for males singing at low rates. In contrast, naloxone decreased the number of full song bouts produced by high singers. Specifically, there were fewer full songs produced after medium and high doses of naloxone, compared to saline, for males singing at high rates (medium dose $p=0.019$, high dose $p=0.014$; Figure II, F).

Results of analysis of negative control animals—Four males had cannulae that were positioned outside the POM (Figure IIA, C) and were analyzed here as controls (Song Bout Length: mean_{saline}=20.4, SD=24.10, mean_{low}=9.87, SD=19.75, mean_{medium}=41.92, SD=8.40, mean_{high}=19.57, SD=23.28; Full Song Bouts: mean_{saline}=1.75 SD, 2.06, mean_{low}=2.25, SD=4.5, mean_{medium}=5.75, SD=4.11, mean_{high}=2.25, SD=2.25). Repeated measures ANOVA demonstrated no significant effect of dose on mean song bout length ($F_{(3,9)}=2.25$, $p=0.14$) or full song bouts ($F_{(3,9)}=2.34$, $p=0.15$). One of these animals fell in the range of high singers and 3 fell in the range of low singers. When separated according to low and high still there were still no consistent effects or trends for drug treatments.

Nest Box Directed Behaviors, Agonistic Behaviors and General Behaviors—

For nest box directed behaviors there was a significant main effect for male categorization ($F_{(1,14)}=6.55$, $p=0.022$), in which high singers performed more nest box directed behaviors than low singers. But, there was not a significant main effect for dose ($F_{(3,42)}=1.20$, $p=0.32$) or interaction between male categorization and dose ($F_{(3,42)}=2.04$, $p=0.12$). No significant main effects or interactions were identified for agonistic behaviors, feeding or beak wiping. There were also no significant findings in the negative control birds (cannula misses).

4. DISCUSSION

The POM plays a central role in facilitating sexually-motivated male song (Alger, Maasch, & Ritters, 2009; Alger & Ritters, 2006; Ritters & Ball, 1999). We found that low singing males had substantially higher densities of ENK labeling in the POM compared to high singing males, a finding consistent with past data showing that low singing males had higher densities of mu-opioid receptors in the POM (Kelm *et al.*, 2011). Enkephalin opioids in the POM suppress male sexual behavior in Japanese quail (Kotegawa *et al.*, 1997), thus it may be that high levels of opioid activity in the POM of low singers suppress sexually-motivated song production. This prediction was strongly supported by the present data showing that blocking opioid receptors in POM with naloxone caused low singing males to sing at higher rates and to sing longer songs (a feature of song found to be attractive to female starlings (Eens *et al.*, 1990; Eens, Pinxten, & Verheyen, 1991)). These findings suggest that under natural conditions high mu-opioid receptor activity and ENK release may suppress sexually motivated singing behavior to match environmental conditions, for example in males that do not have access to nesting sites (Kelm *et al.*, 2011) or have reduced fitness (i.e. high parasitic load), as seen in rodents (Kavaliers, Colwell, & Choleris, 1998).

The relatively lower densities of ENK labeling and mu-opioid receptor labeling observed in high singing males suggest that low levels of mu-opioid receptor activity may be necessary to facilitate singing behavior and that targeted opioid receptor blockade in the POM would further increase sexually-motivated singing behavior in high singing males. In contrast to this prediction, we found that naloxone reduced song rate in males that sang at high rates. A possible explanation for this finding relates to the well-known inverted-U shaped relationship between opioids and behavior (Harrigan & Downs, 1978; Katz & Cooper, 1989; Lukas, Brady, & Griffiths, 1986; Martin, Kim, Harris, & Smith, 1997; Van Ree, Gerrits, & Vanderschuren, 1999). Specifically, opioid-dependent behaviors tend to be observed at high rates when opioids are released at intermediate levels, and to be produced at low rates when opioid levels are extremely low or extremely high. It is possible in the present study that the further reduction by naloxone of already low opioid activity in the POM (based on the measures of ENK and mu receptors) of high singers reduced opioid activity to an extreme low level resulting in song suppression. In contrast, the same treatment in low singers may have lowered extremely high opioid levels down to an intermediate range resulting in song facilitation.

Another contrasting interpretation is that in high singers song is linked to elevated use of ENK. Immunolabeling measures do not reveal whether ENK is stored or released and changes in ENK protein induced by singing can be observed within 1-2 hours (Wada *et al.*, 2006). It is thus possible that high singing is linked to high ENK use (resulting in protein depletion in POM) and the high ENK release results in receptor down regulation (reflected in lower densities of mu receptors in these males) (Kelm *et al.*, 2011). The finding that naloxone in the POM suppressed singing in high singers is consistent with this hypothesis. Future studies are needed to explore further the possibility that opioids differentially modify singing behavior in low and high singers.

Although naloxone differentially affected singing behavior in low compared to high singers, drug treatment did not affect nest box directed, agonistic, feeding, or beak wiping behaviors. This indicates that deficits are specific to singing behavior and not likely caused by general naloxone-induced impairments of behavior. Furthermore, effects on song were only observed in males in which the cannula was centered in the POM, indicating that these effects are specific to opioid receptors in the POM.

Relationship to past studies on opioids and sexually-motivated song

The present data may provide insight into previous conflicting results in studies using peripheral manipulations of opioid receptors. Specifically, naloxone significantly increased sexually-motivated singing behavior in one study in male starlings (Riters *et al.*, 2005), decreased sexually-motivated song in male zebra finches (Khurshid *et al.*, 2010), and in another study in starlings had no significant effect on song (Schroeder & Riters, 2006). In the zebra finch study (Khurshid *et al.*, 2010), males were screened and the study included only the very highest singing males. The finding that naloxone suppressed singing behavior in high singers in this study is thus consistent with the present data showing naloxone to suppress singing only in high singing males. When data from the low and high singing male starlings from the Riters *et al.*, 2005 study were examined separately, naloxone was found to uniformly increase sexually-motivated song in low singers, but to less uniformly affect song production in high singing males (Riters, 2012). This past finding is thus consistent with the present data showing naloxone to increase singing only in low singing males.

The relationships between ENK labeling and singing behavior reported here also differ somewhat from a past study in male starlings in which ENK immunolabeling density did not differ in the low compared to high singers (Riters *et al.*, 2005). The results of the two studies cannot be compared directly as the earlier study focused on a correlated but different measure of song (i.e., the proportion of minutes at which a male sang). However, it is possible that the birds in the past study produced more uniformly high rates of song in response to a female given that they were housed under more natural conditions in outdoor aviaries in flocks without cannula. Thus, it may be that a more restricted range of singing masked a significant relationship between song production and ENK fiber density. Although this possibility must be tested, both our past and present data indicate that in future studies of opioids and behavior it is necessary to consider the range of singing behavior in a study population.

Future directions and concluding remarks

Overall, our results are consistent with past studies suggesting that opioid neuropeptides influence an individual's motivation to communicate. Several additional brain regions have been implicated in motivation, reward, and song, including the ventral tegmental area and periaqueductal gray (Kelm *et al.*, 2011). Furthermore, the songbird POM is rich in mu, kappa, and delta opioid receptors (Woods, Deviche, & Corbitt, 2010). Future studies examining additional opioid sensitive brain regions as well as specific opioid receptor subtypes is necessary to elucidate further the role of opioids in vocal communication.

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Figure I.

Differences in ENK immunolabeling in POM. A) Mean square root of the total pixel area +SEM labeled by ENK in POM in males that sang low (black bar) or high (white bar) rates of female-directed song. Asterisk indicates significant result of a Student's t-test ($p < 0.05$). Sample size is indicated at the bottom of each bar. B) Photomicrograph showing ENK labeling (dense uniform fiber label with sparsely labeled cell bodies) in a coronal brain section (2X) with the boundaries of POM indicated by white arrows. The boxed area indicates the approximate region in which ENK was measured. Photomicrographs illustrating mu-opioid receptor labeling in POM in C) low and D) high singing males at a higher magnification (20X). Scale bar indicated to the right of each photomicrograph.

Figure II.

Schematic of cannula placement and effects of naloxone on singing behaviors. Illustration of one hemisphere of a coronal brain section containing A) intermediate (i) POM and B) caudal (c) POM. Black and gray circles represent areas in which the cannula tip ended within the boundaries of POM, with gray circles located in a portion of POM located approximately 50microns rostral to the black circles and open circles indicating cannulae placed outside the boundaries of POM. Photomicrograph taken at 2X of the cannula tip in C) iPOM and D) cPOM; tissue injected with Chicago Blue dye. Dashed ovals represent approximate boundaries of POM. Scale bar at the lower right corner of panel D is 2 μ m. The square root of E) mean song bout length (+SEM) and F) the mean number of full song bouts after

treatment with vehicle (saline), a low (0.20 nmol), medium (2.0 nmol), and high (20.0 nmol) dose of naloxone, respectively, in male starlings grouped based on a median split into low (black bars) and high (white bars) singers. Asterisks indicate significant p values ($p < 0.05$) resulting from Fisher's post-hoc analyses. Abbreviations: SL, lateral septum; M, mesopallium; N, nidopallium; CO, optic chiasm; LHy, region lateralis hypothalami; CoA, anterior commissure; PVN, paraventricular nucleus of the hypothalamus; v, ventricle.