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Opioid receptor densities analyzed across seasons in the POM and VTA of the dark-eyed junco, *Junco hyemalis*

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ABSTRACT

The motivation of songbirds to sing is influenced by two brain regions, the medial preoptic area (POM) and ventral tegmental area (VTA), which are located outside the song control system itself. These areas receive opioidergic innervation. Furthermore, the opioid enkephalin has been proposed to play a role in the reward for singing. In order to determine whether seasonal changes in song output relate to seasonal changes in opioid receptor (OR) densities in the POM and VTA, we measured the densities of μ, δ, and κ subtypes in these brain regions in adult male dark-eyed juncos (*Junco hyemalis*) sampled in spring (singing), summer (singing and breeding), and fall (no singing). Receptor densities in the rostral and caudal portions of the POM were measured separately because these subregions are thought to predominantly influence appetitive and consumatory sexual behaviors, respectively. δ ORs were generally denser than μ or κ in both parts of the POM and μ ORs were denser than the other subtypes in the VTA. Densities of μ ORs in the POM were higher in the summer than in spring or fall, although this difference was statistically significant only for cPOM ($p = 0.002$). In rPOM, κ OR densities tended to be higher in spring and summer than fall, although this pattern did not reach statistical significance ($p = 0.057$). In contrast, κ OR densities were lowest in the VTA during the summer compared to spring and fall, although this pattern did not reach statistical significance, either ($p = 0.094$). Results obtained for cPOM μ ORs suggest a heightened reward potential for sexual behavior during the breeding season.

1. Introduction

Animal behaviors that are rewarded are said to be motivated because of the desire of the individual to receive the reward. In songbirds, song is a motivated behavior that is influenced by brain regions including the ventral tegmental area (VTA) and the medial preoptic nucleus (POM) (Riters and Ball, 1999; Riters and Alger, 2004; Riters et al., 2005; Alger and Riters, 2006; Alger et al., 2009). The VTA is the origin of reward pathways in vertebrates and so is generally involved in all rewarded behaviors through various projections (Fields et al., 2007), whereas the POM is more specifically involved in sexually motivated behaviors. The VTA has similar connectivity and electrophysiological properties in birds and mammals (Durstewitz et al., 1999; Montagnese et al., 2004). In songbirds, in addition to the projections to basal ganglia and limbic areas found in mammals and non-oscine birds (Durstewitz et al., 1999), the VTA is connected by dopaminergic neurons to specialized brain regions that are part of the song control system (RA, HVC, and Area X; Lewis et al., 1981; Appeltants et al., 2000, 2002; Hara et al., 2007). By contrast, the POM does not project directly to the song system. However, it projects to the periaqueductal central gray, the dorsomedial nucleus intercollicularis, and the locus coeruleus, which all project to the song system (reviewed in Balthazart and Ball, 2007; Riters and Alger, 2004). The male songbird POM also projects to the VTA (Riters and Alger, 2004). The POM is divided into rostral (rPOM) and caudal (cPOM) regions. Studies suggest that the rPOM is more involved in the control of appetitive sexual behavior (ASB) than the cPOM, whereas the cPOM is more involved in the control of consumatory sexual behavior (CSB) than the rPOM (Balthazart and Ball, 2007). Supporting this contention, cPOM lesions in the male Japanese quail (*Corturnix japonica*) inhibit CSB and rPOM lesions decrease ASB (Balthazart et al., 1998). Additionally, the quail cPOM expresses more immediate early gene activity following copulation than the rPOM (Meddle et al., 1997). In the male house sparrow (*Passer domesticus*), birds vocalizing at the highest rate also had the highest level of immediate early gene activity in the rPOM (Riters et al., 2004). Song that immediately precedes

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copulation in birds is followed by a reward when the female responds, but song in the absence of a female is not associated with a clear external reward (Riters, 2009). In this case, internal reward systems, including those regulated by opioids, are likely to play an important role in the regulation of ASBs.

Several studies have measured sexually motivated behaviors after pharmacological manipulation with opioid receptor agonists or antagonists. Blocking opioid receptors with naloxone in the VTA of male rats increases ASB (reviewed by van Furth and van Ree, 1996). Administering naloxone to the preoptic area in male Japanese quail increases sexual behaviors toward females whereas administration of a δ receptor-specific agonist decreases these behaviors (Kotegawa et al., 1997). Infusing a δ receptor agonist in the female rat POM inhibits lordosis, a female CSB (Sinchak et al., 2004). In one study on European starlings (*Sturnus vulgaris*), opioid receptor blockade with 10 mg/kg naloxone increased the song rate of males singing directed song (Riters et al., 2005), particularly in those birds that normally had lower song rates (Riters, 2009). Although this finding was not replicated in a second study by the same investigators (Schroeder and Riters, 2006), this second study observed a decrease in sexually motivated singing in response to peripheral treatment with the μ opioid receptor-specific agonist fentanyl. Collectively, these studies suggest that opioid administration leads to satiety and reduces motivated behavior whereas opioid receptor antagonist treatment increases motivated behavior, as if the animal continues to try to receive an expected reward (Riters, 2009). Conversely, a recent study on zebra finches (*Taenopygia guttata*) found that systemic administration of a low (2.5 mg/kg), but not high (10 mg/kg), dose of naloxone decreases directed song rate and also affects spectral and temporal components of songs (Khurshid et al., 2010). The authors suggest that the dose–response profile reflects differential effects on the three opioid receptor subtypes at low vs. high dose, and that the opposite finding to that in European Starlings may be due to a species difference or to the fact that zebra finches with low song rates were excluded from their study.

As is the case of many seasonal songbirds, male dark-eyed juncos (*Junco hyemalis*) sing mostly during the breeding season. In high latitude populations of this species, males arrive already singing in early May and they establish breeding territories before females arrive (Corbitt and Deviche, pers. obs.). Males continue to sing during the summer breeding season but rarely thereafter, when they prepare to migrate south. A previous study identified opioid receptors in brain regions of the junco song system including the HVC, RA, and Area X, which control song learning and production, and found no seasonal differences in receptor densities (Gulledge and Deviche, 1995). Given the involvement of VTA and POM opioid receptors in motivation or reward, we hypothesized that birds express opioid receptors in these regions and receptors would be present at higher densities during than outside the breeding season. To test this hypothesis, we compared POM and VTA opioid receptor densities in male dark-eyed juncos sampled during (singing) and outside (non-singing) their reproductive season.

2. Methods

The methods used for bird collection, brain preparation, and autoradiography labeling are described by Gulledge and Deviche (1995), who did not measure opioid receptor densities in brain regions that control motivation and reward. Briefly, adult male dark-eyed juncos were collected in the Fairbanks, Alaska (65°N, 148°W) area (a) in mid-spring ("spring"; May 2–10; $n = 13$), shortly after arrival on breeding grounds; (b) in late spring ("summer"; June 8–9; $n = 12$), during the breeding season and when birds sang at a high rate; (c) during fall migration ("fall"; September 22–23; $n = 8$), when testes had regressed and birds were not singing any longer. Spring and fall males were caught in seed-baited traps and summer males were captured using Japanese mist nets and conspecific song playbacks. Birds were decapitated quickly after capture and brains were removed immediately, immersed in Freon, and stored at −70 °C. The testes of each bird were weighed to the nearest mg and cloacal protuberance widths, which correlate with testis sizes, were measured to the nearest mm. Fall birds had completely regressed testes, so their testis masses were not measured. All activities were preapproved by the University of Alaska Fairbanks Institutional Animal Care and Use Committee, the Alaska Fish and Game Department, and the US Fish and Wildlife Service.

Brains were sectioned coronally and sections used for *in vitro* autoradiography as described by Gulledge and Deviche (1995). For this, frozen coronal, 30-μm-thick sections were cut into 6 series using a cryostat at −15 °C. Sections were collected on gelatin-coated microscope slides, dehydrated overnight at 4 °C, and stored at −70 °C. Before radiolabeling, sections were preincubated with buffer at pH 7.40 to dissociate and eliminate receptor-bound endogenous ligands (Gulledge and Deviche, 1995). To label μ receptors, pairs of slides with adjacent sections (one slide for total binding, TB, and the other slide for non-specific binding, NSB) were incubated in a solution containing [Tyr-3,5-^{3}H(N)]-[D-Ala^2, N-Me-Phe^4, Gly-ol^5]-enkephalin (^{3}H-DAMGO) for 60 min. For δ receptors, slide pairs were incubated overnight with [Tyr-3, 5-^{3}H]-[D-Pen^2, pCl-Phe^4, D-Pen^5]-enkephalin (^{3}H-pCl-DPDPE). κ receptors were labeled by incubating slide pairs with [9-^{3}H(N)]-ethylketocyclazocine (^{3}H-EKC) for 60 min. Optimum incubation conditions and ligand binding specificities in dark-eyed Junco brains were previously determined (Deviche et al., 1993). Non-specific binding was measured in the presence of excess concentrations of three unlabelled ligands with high, non-selective affinity for the three receptor subclasses. To assure that radiolabeled ligands occupied similar proportions of their respective receptors, each ligand was used at a concentration equal to twice its dissociation constant (^{3}H-DAMGO, 11 nM; ^{3}H-pCl-DPDPE, 0.48 nM; ^{3}H-EKC, 2.48 nM; Deviche et al., 1993). After incubation, slides were rinsed, dried, placed in X-ray cassettes that each contained a set of tritiated plastic standards, and exposed to radioactivity-sensitive film for 10–12 weeks. Films were developed and images of interest were digitized under standardized light conditions in preparation for image analysis.

To identify the brain regions under study, sections were stained for Nissl substance using Cresyl violet. Brain regions were delineated based on anatomical landmarks (Fig. 1) and followed designations illustrated in Riters et al. (2005), Heimovics and Riters (2007), and Reiner et al. (2004). The rPOM is along the midline and located ventral to the septum and rostral to the anterior commissure. For the cPOM, the anterior commissure was the best indicator of the region, which is immediately ventral to it at the midline. The VTA is located immediately lateral to the third cranial nerve. Optical density measurements from films were taken in an area located near the center of the brain regions under study. These densities were determined using Image-Pro Plus (Media Cybernetics, Inc.) image analysis software. For each brain image, measurements were taken from the left and right hemispheres. Corresponding NSB images were aligned based on section edges, as most NSB images were not contrasted enough to distinguish landmarks within sections. The NSB measurement was subtracted from the corresponding TB measurement to calculate specific binding (SB). Specific binding values were then converted to μCi/g standard using equations derived from the optical density values of the standards on each film, and values obtained for the right and left hemispheres of each brain were averaged. For two images, only one side of the brain was measured because of missing tissue on the other side. Average SB values were used for statistical analysis.

Seasonal difference in testis mass was analyzed by Student's *t*-test. Seasonal differences in cloacal protuberance and for each of the three classes of opioid receptors for each region were analyzed with a one-way analysis of variance (ANOVA) using season as the independent factor and with post hoc pairwise multiple comparisons (Dunn's method) when warranted. Power analyses, with alpha set at 0.05, were also performed. Data sets that either were not normally distributed (δ rPOM) or were heteroscedastic (μ cPOM) were analyzed with a Kruskal–Wallis ANOVA on ranks.

3. Results

Average testis mass in the summer was nearly double that in the spring ($p < 0.001$; Table 1). Testes in the fall were regressed

Table 1
Morphology measurements.

Morphological measurement	Spring	Summer	Fall	*p*-Value
Cloacal protuberance width (mm)	4.0 ± 0.1 (13)	6.9 ± 0.2 (12)	3.7 ± 0.1 (8)	<0.001
Testes mass (mg)	162.5 ± 11.9 (13)	324.9 ± 21.5 (12)	–	<0.001

The testis mass was not taken in the fall birds because they are completely regressed then (<10 mg). The data is shown as the means ± standard error. The *n* values (number of birds) are shown in parentheses. *Note:* These measures also appear in Fig. 1 of Gulledge and Deviche (1995).