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Neural endocannabinoid CB_1 receptor expression, social status, and behavior in male European starlings

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Abstract

Many species modify behavior in response to changes in resource availability or social status; however, the neural mechanisms underlying these modifications are not well understood. Prior work in male starlings demonstrates that status-appropriate changes in behavior involve brain regions that regulate social behavior and vocal production. Endocannabinoids are ubiquitously distributed neuromodulators that are proposed to play a role in adjusting behavior to match social status. As an initial step to provide insight into this hypothesis we observed flocks of male starlings in outdoor aviaries during the breeding season. We used quantitative real-time PCR to measure expression of endocannabinoid CB_1 receptors in brain regions involved in social behavior and motivation (lateral septum [LS], ventral tegmental area [VTA], medial preoptic nucleus [POM]) and vocal behavior (Area X and robust nucleus of the arcopallium; RA). Males with nesting sites sang to females and displaced other males more than males without nesting sites. They also had higher levels of CB_1 receptor expression in LS and RA. CB_1 expression in LS correlated positively with agonistic behaviors. CB_1 expression in RA correlated positively with singing behavior. CB_1 in VTA also correlated positively with singing when only singing birds were considered. These correlations nicely map onto the well-established role of LS in agonistic behavior and the known role of RA in song production and VTA in motivation and song production. Studies are now needed to precisely characterize the role of CB_1 receptors in these regions in the production of status-appropriate social behaviors.

Keywords

cannabinoids; social behavior; dominance; songbird; motivation; territoriality

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1. Introduction

Many species modify the production of vocal, sexual, and agonistic behaviors in response to changes in social status or territory ownership (e.g., mammals (Oyegbile and Marler, 2005); fish (Burmeister et al., 2005; Maruska and Fernald, 2010); birds (Gwinner et al., 1987); reptiles; (Korzan et al., 2002)); however, the neural mechanisms underlying these changes are still unclear. Endocannabinoids (ECBs), their receptors, and associated enzymes are widely distributed in the brains of vertebrates (e.g., mammals (Egertova et al., 2003; Felder et al., 1996; Herkenham et al., 1991); birds (Soderstrom and Johnson, 2000); amphibians (Hollis et al., 2006)), and recent studies implicate ECBs in social, agonistic, and sexually-motivated behaviors (e.g., (Fattore et al., 2010; Rodriguez-Arias et al., 2013; Rodriguez-Manzo and Canseco-Alba, 2015; Trezza et al., 2012)).

The ECBs (anandamide and 2-arachidonoylglycerol) are synthesized “on demand” and released from postsynaptic membranes to primarily influence presynaptic neurons through retrograde signaling. In the central nervous system they bind to presynaptic G-protein coupled receptors (CB_1 and to a lesser extent CB_2) to inhibit calcium influx and neurotransmitter release (reviewed in (Castillo et al., 2012; Wilson and Nicoll, 2002)). It has been proposed that by rapidly gating neurotransmitter release, CB_1 receptors may function to adjust behavior so that it is appropriate given changing environmental or social factors (Soderstrom, 2009). The present study was designed as an initial step to begin to provide insight into this hypothesis.

Male European starlings, *Sturnus vulgaris,* provide an excellent study species to address this topic. At the onset of the breeding season as testosterone (T) concentrations rise, male starlings without nesting territories avoid other males and appear to ignore females. However, once a male acquires a territory, T peaks and males begin to court females and socially dominate other males (Cordes et al., 2014; Gwinner et al., 1987; Kelm et al., 2011; Riters et al., 2000; Sartor and Ball, 2005). Past studies in starlings show that such “status-appropriate” behavioral changes are associated with differences in brain areas that across vertebrates are centrally involved in sexual motivation (i.e., the medial preoptic nucleus [often referred to as POM in birds]), agonistically-motivated behavior (i.e., the lateral septum [LS]), and the motivation to engage multiple behaviors (i.e., the ventral tegmental area [VTA]) (Balthazart and Ball, 2007; Blanchard et al., 1977; Goodson et al., 1999; O'Connell and Hofmann, 2011; Ramirez et al., 1988; Riters, 2012; Will et al., 2014). Similar brain regions are also implicated in status-appropriate behavioral differences in fish, lizards, rodents, and primates (e.g., (Albers, 2012; Fuxjager et al., 2010; Korzan et al., 2006; Maruska, 2015; Noonan et al., 2014; O'Connell and Hofmann, 2011)), indicating well-conserved mechanisms.

Past studies show that the promoter region of the CB_1 receptor gene *Cnr1* has androgen and estrogen binding sites, and both estrogens and androgens can alter CB_1 expression (Gonzalez et al., 2000; Lee et al., 2013; Notarnicola et al., 2008; Proto et al., 2012). T is critical for male starlings (and other species) to initiate territorial acquisition and associated behaviors (Ball et al., 2002; Pinxten et al., 2002). In male starlings, androgen receptor markers in POM are denser in males with nest sites and correlate positively with female-

directed courtship song (Cordes et al., 2014; Cordes et al., 2015). It is thus possible that T may directly or indirectly (via its metabolite estradiol) modify CB_1 receptor expression to adjust status-appropriate behaviors.

If CB_1 receptors influence status-appropriate behavior in starlings this may be reflected in CB_1 receptor mRNA expression levels. Thus, as a first step we chose to focus on CB_1 receptor expression. Here we observed flocks of male starlings in outdoor aviaries during the breeding season. Brains were collected and quantitative real-time polymerase chain reaction (qPCR) was used to measure expression of ECB CB_1 receptors in POM, LS, and VTA in males with and without nesting sites. We additionally looked at CB_1 receptor expression in two brain regions that are unique to songbirds; one that is selectively involved in vocal motor production (the robust nucleus of the arcopallium; RA) and one that is involved in vocal learning and contextual adjustment of song (Area X) (Bottjer et al., 1984; Nottebohm et al., 1976; Scharff and Nottebohm, 1991; Sohrabji et al., 1990). These regions are rich in CB_1 receptor expression (Soderstrom and Johnson, 2000), and it is thought that CB_1 receptors play a role in vocal motor production in RA and a role in auditory learning in Area X (Soderstrom and Tian, 2006). Finally, we measured T to gain insight into a possible role for circulating T (or its metabolites) in modifying CB_1 expression.

2. Results

Nest site possession and behavior

Results of a MANOVA revealed that behavior (square root transformed for analyses) differed significantly in males that acquired and occupied nest boxes (n=10) compared to males that did not (n =10; main effect for nest box possession: $F_{1,18} = 43.20$, p = 0.000004). Fisher LSD posthoc tests indicate that males with nest boxes sang at higher rates (p = 0.00007; effect size Cohen's $d = 1.40$; Figure 1A) and displaced other males more often (p = 0.000004; effect size Cohen's $d = 2.82$; Figure 1B) than males without nest boxes (Confidence intervals [CIs] Song rate: males with nest boxes CI −95% = 2.67, +95% = 4.97; males without nest boxes CI −95 = 0.45, +95 = 2.73; Displacement: males with nest boxes CI −95% = 2.56, +95% = 4.49; males without nest boxes CI −95 = 0.40, +95 = 1.00). No differences were found between males with and without nest boxes for feeding (p = 0.537; effect size Cohen's $d = 0.29$; males with nest boxes CI −95% = 1.83, +95% = 2.93; males without nest boxes CI −95 = 1.33, +95 = 2.88) or drinking behaviors (p = 0.377; effect size Cohen's $d = 0.90$; males with nest boxes CI −95% = 1.39, +95% = 1.79; males without nest boxes CI −95 = 0.80, +95 = 1.59). A separate t-test on time spent singing also revealed that males with nest boxes spent significantly more time singing than males without boxes ($t_{18} = 3.29$, p = 0.004; effect size Cohen's $d = 1.47$; males with nest boxes CI −95% = 10.29, +95% = 20.45; males without nest boxes CI −95 = −0.47, +95 = 10.01).

Nest site possession and CB_1 expression

Compared to males without nest boxes, males that acquired and occupied nest boxes had significantly higher CB_1 expression in RA (Table 1; Figure 1C) and LS (Table 1; Figure 1D). The sample size was n = 10 for each brain region, except that for RA one outlier was removed for a male with a nest box with a CB_1 value that was more than 2 standard

deviations below the mean. No significant differences were found for Area X, VTA or POM ($p > 0.270$ in all cases; Table 1). Results for RA and LS were not significant when Bonferroni corrected for the 5 separate t-tests run for each brain region ($p = 0.032$ and 0.039, respectively which are above the corrected p value of 0.01).

Behaviors and CB_1 expression

A standard multiple regression analysis in which CB_1 expression in RA was entered as the predictor variable and time singing, displacements, and feeding were entered as independent variables was significant (Table 2) with both time singing (Table 2; Figure 1E) and feeding (Table 2; Figure 2) relating significantly to CB_1 expression in RA. Displacement did not contribute significantly (Table 2). (Note: the lowest CB_1 value that was identified as an outlier in the t-test described above was not found to be a statistical outlier when included in regression analyses. Its inclusion or removal had no effects on statistical significance. This point is therefore included.) The result of the same analysis for LS was also significant (Table 2) with displacements relating significantly to CB_1 expression in LS (Table 2; Figure 1F). Time singing and feeding did not contribute significantly (Table 2).

For VTA, the overall multiple regression model was not significant (Table 2); although feeding did relate significantly to CB_1 in VTA (Table 2). Examination of scatterplots suggested that time spent singing related to CB_1 in VTA (but not any other brain region) when only singers were considered. This observation was significant when analyzed using simple regression ($r = 0.64$, $p = 0.035$; Figure 3). No significant models or contributing variables were identified in analyses of CB_1 expression in either POM ($p = 0.66$) or Area X ($p = 0.27$) (Table 2).

Testosterone concentrations and CB_1 expression

T-tests indicated that although testosterone concentrations tended to be greater in males with nest boxes compared to those without nest boxes, this was not statistically significant ($p > 0.24$; Table 1). For reference, these values are in the range of spring condition males with fully recrudesced gonads (Cordes et al., 2014; Gwinner et al., 2002; Stevenson et al., 2009). Results of correlation analyses revealed no significant correlations between testosterone concentrations and CB_1 expression in any brain region (RA: $r = 0.06$, $p = 0.814$; LS: $r = -0.08$, $p = 0.727$; VTA: $r = -0.17$, $p = 0.504$; POM: $r = 0.28$, $p = 0.226$; Area X: $r = -0.37$, $p = 0.106$).

3. Discussion

The results presented here show strong associations between status-appropriate behaviors and CB_1 receptor expression in brain regions involved in social motivation (LS and VTA) and vocal control (RA). Based on past studies demonstrating cannabinoids to modify production of sociosexual behaviors (e.g., (Fattore et al., 2010; Rodriguez-Arias et al., 2013; Rodriguez-Manzo and Canseco-Alba, 2015)), we interpret our results as consistent with a causal role for CB_1 in these regions in modifying social behavior. However, our data are correlational and also support the non-mutually exclusive possibility that agonistic and

sexual behavior modify CB_1 expression (or that an unidentified third variable may be involved).

Nest site possession, CB_1 expression, and sociosexual behaviors

As expected, males that successfully acquired nesting territories sang more than males without nest sites, and they socially dominated other males (as indicated by displacement behavior). Males with nest boxes had higher CB_1 receptor mRNA expression in LS and RA. These results were nonsignificant when Bonferroni corrected. However, the effect sizes were strong and confidence intervals were relatively narrow, which we suggest may reflect a biologically important effect (as proposed by (Garamszegi, 2006)).

CB_1 expression in LS correlated positively and strongly with agonistic behavior, whereas CB_1 expression in RA correlated positively with singing behavior. This distinction nicely maps onto the well-established role of LS in agonistic behavior and the known role of RA in song production (Blanchard et al., 1977; Goodson et al., 1999; Heimovics et al., 2009; Nottebohm et al., 1976; Ramirez et al., 1988). The positive correlation between CB_1 expression in RA and singing behavior is also consistent with a proposed role for CB_1 in RA in vocal motor production (Soderstrom and Tian, 2006). The positive correlation that we identify between CB_1 expression in LS is novel. Some studies in mammals show that cannabinoid administration decreases agonistic behavior (e.g., (Miczek, 1978; Rodriguez-Arias et al., 2013)); however, other studies demonstrate that administration of cannabinoids can induce agonistic behavior, particularly in animals exposed to stressful conditions (e.g., (Carlini, 1977; Fujiwara and Ueki, 1979)). Furthermore, effects can be biphasic with high doses inhibiting agonistic behavior and low doses stimulating agonistic behavior in low-aggressive mice (Sulcova et al., 1998). Studies that directly manipulate cannabinoids in LS are now needed to determine the role of CB_1 in LS in agonistic behavior. Finally, whether acquiring a nest box is inducing the changes in gene expression or alternatively birds with a particular pattern of gene expression are more likely to become nest box owners must be examined in future experimental studies.

Our results also offer support for a role for CB_1 receptors in VTA in singing behavior. When only singing birds (perhaps the most highly sexually-motivated birds) were considered, CB_1 mRNA expression in VTA correlated positively with time spent singing. Studies using electrophysiology and immediate early gene expression demonstrate a central role for VTA in female-directed song (Goodson et al., 2009; Hara et al., 2007; Heimovics and Riters, 2005; Huang and Hessler, 2008; Yanagihara and Hessler, 2006). Measures of dopamine and opioids as well as GABA and glutamate neuron activity in VTA also relate positively to sexually-motivated singing behavior (Goodson et al., 2009; Hara et al., 2007; Heimovics and Riters, 2008; Heimovics et al., 2011; Huang and Hessler, 2008). Studies in mammals show that in VTA, CB_1 receptors inhibit glutamate release (Melis et al., 2004) and inhibit GABA- and opioid-mediated inhibition of dopaminergic VTA neurons (Johnson and North, 1992; Wang and Lupica, 2014), which is proposed to disinhibit activity in VTA dopamine neurons to facilitate production of motivated behaviors (Oleson et al., 2012). Studies are now needed to test the prediction that CB_1 inhibition of opioid and GABA neurons facilitates dopamine release and female-directed song.

Expression of CB_1 receptors in RA and feeding behavior

We found a positive correlation between CB_1 expression in RA and feeding behavior. This finding is consistent with results of a study in zebra finches that showed that limiting access to food increases ECB levels in tissue samples that included RA (as well as other regions (Soderstrom et al., 2004)). Perhaps birds that fed more in the present study were relatively more food deprived (and hungry) than birds that fed less, which may increase CB_1 expression in RA. However, past studies in zebra finches suggest that CB_1 receptors have opposing effects on feeding and singing (stimulatory and inhibitory, respectively) (Soderstrom et al., 2004; Soderstrom, 2009). Thus the finding that CB1 expression in RA correlates positively with both feeding and singing in the present study is difficult to resolve. Our results are certainly consistent with the idea that CB_1 receptors, feeding and singing are interrelated; however additional study is required to fully interpret these somewhat contradictory results.

Expression of CB_1 receptors and testosterone

In male starlings, T rises just before and after a male has acquired a nesting site (Gwinner et al., 1987; Gwinner and Gwinner, 1994). As reviewed in the introduction, both androgens and estrogens can modulate CB_1 receptor expression (Gonzalez et al., 2000; Lee et al., 2013; Notarnicola et al., 2008; Proto et al., 2012); however in this study we did not find any correlations between circulating T and CB_1 expression. Changes in local production of neurosteroids in the brain are also associated with singing and social behavior in songbirds (reviewed in (Heimovics et al., 2015; Remage-Healey et al., 2010)). It is thus possible that local interactions between steroids produced by the brain influenced CB_1 expression in some brain regions. The influence of T on CB_1 expression and behavior requires further study.

Interpretational limitations

The qPCR method stands out for yielding highly quantifiable information; however, as with any technique there are limitations. We interpret our results to suggest that high CB_1 mRNA expression reflects high ECB receptor synthesis and increased tissue sensitivity to ECBs. This is plausible and supported by existing past studies (as detailed above); however, there are important caveats. First, we do not have information about translational and post-translational regulation of CB_1 receptors, including availability of mRNA for translation or post-translational receptor migration and turnover. Second, with the existing study design, we do not know whether preexisting CB_1 differences in the brain drive behavioral differences or whether behavior or other variables influence CB_1 expression. We suggest that our data reflect constitutive, stable individual differences in CB_1 expression and behavior; however, at least one study demonstrated that changes in CB_1 mRNA in the goldfish brain can be detected 1 hour after presentation of a stressor (Palermo et al., 2013). If these changes can occur even earlier, it may be that behavior in the present study led to rapid transcriptional changes in CB_1 expression. Future studies using measures and manipulations of CB_1 as well as CB_2 receptors, ECBs, synthetic and degradative enzymes are now needed to thoroughly characterize ECB regulation of behavior.

4. Experimental procedure

Study species, capture, and housing

Male starlings were captured during 2009–2010 in baited fly-in traps on a local farm in Madison, WI. Males were banded with stainless steel identification bands and unique combinations of plastic color bands to aid behavioral observations. Individuals were housed in single sex cages (91 cm × 47cm × 47 cm; 5 birds/cage) in the University of Wisconsin's Department of Zoology indoor animal facility and subjected to artificial photoperiods of 18h light (L):6h dark (D) for 6 weeks, followed by 6 weeks of 8L:16D to induce photosensitivity before the onset of the experiment. All procedures and protocols adhered to the guidelines of the *National Institutes of Health Guide for the Care and Use of Laboratory Animals* and a protocol approved by the University of Wisconsin Institutional Animal Care and Use Committee.

Behavioral observations

Outdoor aviaries (2.13 m × 2.4 m × 1.98 m) exposed to natural light (approximately 13L: 11D) were used to conduct behavioral observations. All aviaries (5) were equipped with appropriate enrichment for starlings (nest boxes, perches, nesting material, baths) and food and water were provided *ad libitum*. Twenty-five male starlings were randomly distributed into the aviaries (5 birds / aviary) and allowed to acclimate for 7-days prior to behavioral observations. By the end of the study 5 birds were removed from aviaries for various reasons (e.g., concerns that injury would result from agonistic interactions) leaving 20 birds. After the acclimation period, the behavior of each male in response to a 20 minute exposure to a female stimulus bird was recorded for 4 consecutive days. A single observer recorded behavioral measures, which included number of songs produced per 20 minute observation period (song rate), time spent singing (sec), and bouts (performance of behavior separated by at least 2 seconds from prior behavior) of non-specific behaviors eating and drinking. The number of displacements (times a male approached another male [landed within approx. 4cm] followed by that male's departure) were also recorded. Each male was also categorized as a nest box owner or non-owner as determined by a male spending a majority of his time near a nest box opening or entering the box. At the end of each behavioral observation, the stimulus female was removed from the aviary and returned to the indoor housing facility (with similar light cycle length as outdoor aviaries). A single female was rotated through all aviaries on a single day and a new female was used each day (4 females total). The sum of behavior for each individual across the 4 test days was used for analyses (transformed when appropriate as detailed below).

Quantitative real-time polymerase chain reaction preparation and analyses

After the last behavioral observation (on day 4), brains were collected via rapid decapitation, flash frozen in Isopentane (C5H12) surrounded by dry ice, and stored at −80°C. Brains were sectioned coronally at −15°C into 200μm sections with a cryostat and placed onto slides. For LS, VTA, RA and Area X 2 punches were collected (one from each hemisphere) and for POM a single central punch was taken using a Stoelting brain punch set (#57401; punch sizes and locations are shown in Figure 4). Tissue punches were stored in 0.5 ml microcentrifuge tubes at −80°C until RNA extraction. All tissue punches were homogenized

with an electric Dremel tool and RNA was extracted with a Bio-Rad Aurum Total RNA Fatty and Fibrous Tissue Kit (Catalog No. 732-6830; Bio-Rad, Hercules, CA). The concentration of RNA was measured with a Nanodrop system (Thermo Scientific, Wilmington, DE) and RNA integrity verified with Agilent 2100 BioAnalyzer and Agilent RNA 6000 Pico Kit (Agilent Technologies, Santa Clara, CA) on a subset of samples. The RNA (100ng) was then converted into single stranded cDNA using Invitrogen SuperScript III First-Strand Synthesis System (Catalog No. 18080-051; Life Technologies, Carlsbad, CA). Following cDNA conversion, relative gene expression for CB_1 was determined for all brain regions as a normalized ratio to reference genes as described below using qPCR analysis. (As part of separate studies, tissue from this set of males was also used to examine androgen receptor, opioid-related, dopamine-related and neurotensin-related mRNA (Cordes et al., 2015; DeVries et al., 2015; Merullo et al., 2015)).

Primers for CB_1 were designed (NCBI Gene Database, Primer-Blast) using the chicken (*Gallus gallus*) genome for qPCR analysis (Accession number: NM_001038652.1; Forward sequence: GGTCTTCTGTGGACTTAGGG; Reverse sequence: CTCCTCTATTCCTTTGTTGCTC; Product (bp) = 30). The qPCR reaction product was sequenced using Sanger sequencing with both forward and reverse primers at the University of Wisconsin Biotechnology Center (Sequence: TAATTTTTCCCCCTCCTTTTCTTTCACATGCATATGAGACTAACAGCAACAAAGGA ATAGAGGAGA). Using NCBI BLAST this sequence matches the intended target. Two reference genes (glyceraldehyde-3-phosphate dehydrogenase [GAPDH]; hypoxanthine-guanine phosophoribosyltransferase [HPRT]) were also analyzed to normalize mRNA levels across samples. These reference genes were selected because they have been reported to be stable in starling neural tissue and suitable for use in calculating a normalization factor in studies of testicular growth, season-, context- and sex differences (Bentley et al., 2013a; Bentley et al., 2013b). The primers used for references genes were previously shown to match the intended targets (using Sanger sequencing) in starlings (Riters et al., 2014).

Samples were prepared for analysis in qPCR reaction tubes containing sample cDNA, nuclease free water, forward and reverse primers (5μM; University of Wisconsin), and SsoFast EvaGreen Supermix (Catalog No. 172-5201; Bio-Rad, Hercules, CA). Five standards were run with each plate of samples (1:10 serial dilution, starting concentration at 500ng/μl), along with a negative control (nuclease free water substituted for cDNA). Standards and samples were run in triplicate on each plate and all plates were read with the BioRad CFX96 Touch Real-Time PCR Detection System (Catalog No. 185-5195; Bio-Rad, Hercules, CA). Each qPCR run consisted of the following: an initiation step at 95°C for 30s, followed by 40 cycles of 95°C for 5s, a 30s annealing phase, a 20s elongation phase at 72°C, and a melt curve from 60°C to 88°C, 0.5 degrees for each 5s step. Plates were read following each elongation and melt curve step. Criteria for inclusion in the dataset were: run efficiencies between 90–110%, an R^2 of at least 0.990, and a melt curve displaying a single peak indicative of primer specificity.

Testosterone

Trunk blood samples were collected at the time of sacrifice to assay T. Plasma T was measured using a commercial grade competitive enzyme immunoassay (Cayman Chemical, Ann Arbor, MI, USA, Catalog # 582701) and has been reported for these same birds (Cordes et al., 2015).

Statistical analyses

The Pfaffl Method (Pfaffl, 2001) was used to determine relative levels of gene expression (detailed in (Cordes et al., 2015)) for statistical analyses. This method is comparable to the commonly used 2 delta-delta Ct method (Livak and Schmittgen, 2001) but allows input of efficiencies for each individual run, rather than assuming 100% efficiency (Pfaffl, 2001). Following this method, the mean cycle threshold (Ct; amplification threshold = 200 RFU) values of all samples were transformed. The geometric mean of the Ct values for the two reference genes for each region was used to transform the Ct values for each gene analyzed to a normalized ratio. Outliers were identified using studentized residuals >2. All data were analyzed with Statistica software (StatSoft 2014, Tulsa, OK).

A MANOVA was used to examine differences in behavior (song rate, displacements, feeding, and drinking) between males with and without nesting sites. A separate t-test was run on time spent singing because values were so much higher (even when transformed) than those for other variables that its inclusion washed out all other findings. Behavioral measures were square root transformed to improve homogeneity of variance. Separate t-tests were used to analyze CB_1 expression in each brain region in males with and without nesting sites. A single MANOVA was not used for CB_1 measures because data points were missing in some brain regions which would cause these individuals to be dropped from all regions. Residuals were examined and outliers identified if they were + 2 sd from the mean and their inclusion or removal influenced results. A standard Bonferroni corrected p value of 0.01 was used to reduce Type 1 error. This caused all p values to fall short of significance, which may be a result of Bonferroni reducing statistical power and raising Type II error (Nakagawa, 2004). To assist in interpretation of these and all other results (as recently recommended by the American Statistical Association (Wasserstein and Lazar, 2016)) we also report confidence intervals and effect sizes using Cohen's *d* values to provide insight into the strength of the difference between categorical variables with 0.20, 0.50, and 0.80 considered loose benchmarks for small, medium, and large effect sizes) (Cohen, 1988).

Standard multiple regression analyses were run to examine statistical associations between CB_1 expression in each brain region and time spent singing, displacements, and feeding behavior. Nearly identical results to those obtained for time spent singing were obtained when we examined song rate. We report only the former results to avoid redundancy. In separate analyses CB_1 expression in each brain region was entered as predictor variables and the three behavioral measures were entered as independent variables. VTA was missing 3 CB_1 measures due to technical problems, reducing the sample size to 17. For all other regions n = 20. To provide insight into the possibility that T might alter CB_1 expression, we ran correlation analyses between T concentrations and CB_1 expression in each brain region. For each analysis residuals were examined and outliers identified using standard residual > 2

standard deviations in the population. Standardized Betas are reported for multiple regression analyses as indices of effect sizes (with 0.20, 0.50, and 0.80 also considered loose benchmarks for small, medium, and large effect sizes). For correlations, r values of r = 0.10, 0.30, and 0.50 have been suggested to be indicative of small, medium, and large effects, respectively (Cohen, 1988).

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Abbreviations

ECB	endocannabinoid
LS	lateral septum
POM	medial preoptic nucleus
qPCR	quantitative real-time polymerase chain reaction
RA	robust nucleus of the arcopallium
T	testosterone
VTA	ventral tegmental area

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Highlights

- Dominant male starlings had higher CB_1 expression in LS and RA than subordinates
- CB_1 mRNA expression in LS correlated positively with agonistic behavior
- CB_1 mRNA expression in RA and VTA correlated positively with song
- CB_1 mRNA expression in RA also correlated positively with feeding behavior
- Endocannabinoid signaling may adjust behavior to match social status

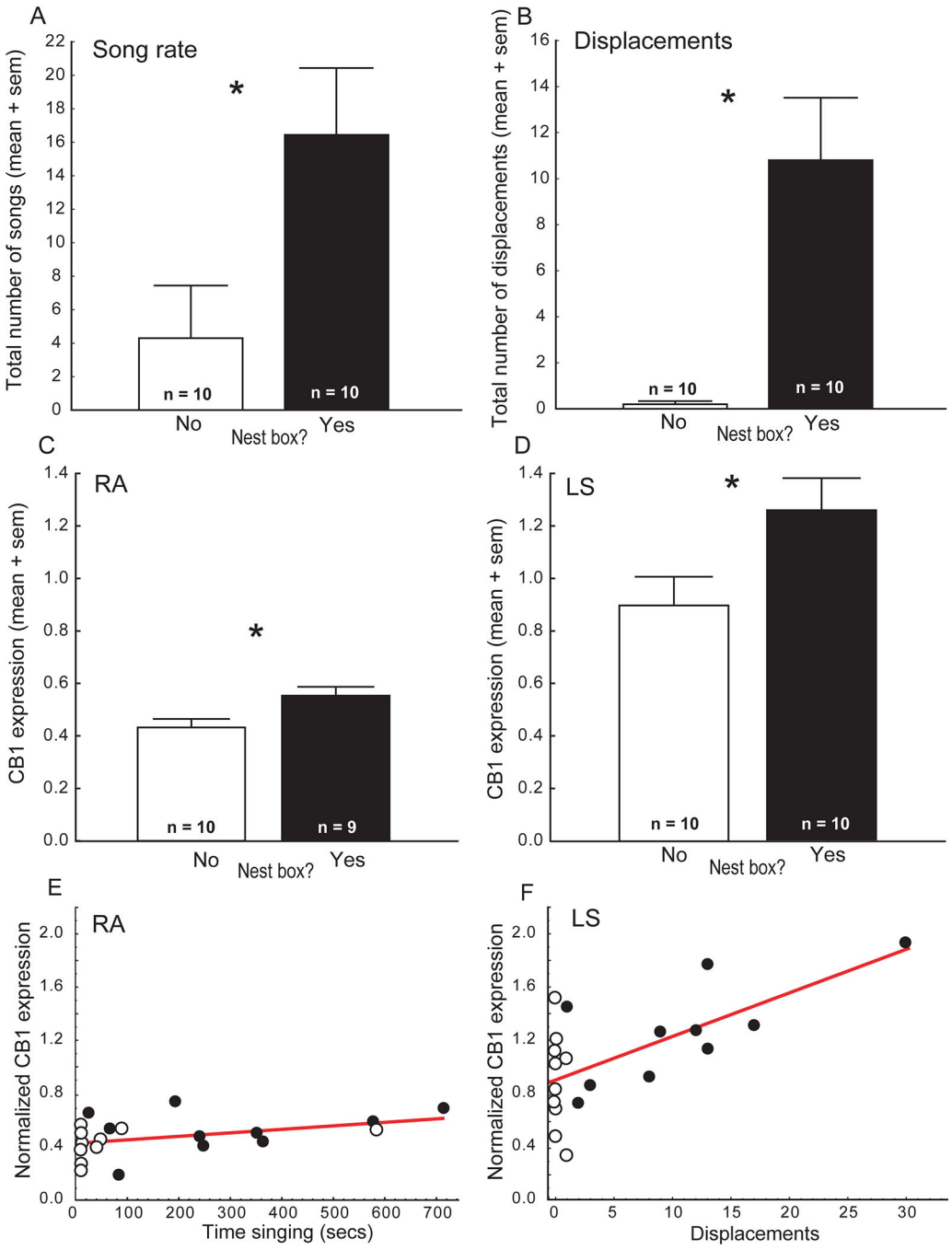

Figure 1.

Territory ownership, behavior and CB_1 expression. Mean total song rate (i.e., number of songs produced during 4, 20 min observation periods) (A) and displacement behavior (B) for males with (filled bars) or without (open bars) nest sites. Data presented show the sum of behaviors for each male produced during 4, 20 min observation days divided by the total number of males in each group. Raw data are shown here. Data were square root transformed for analysis. Mean CB_1 expression levels in RA (C) and LS (D) for males with (filled bars) or without (open bars) nest sites. * indicates $p < 0.05$, but not significant at a

Bonferroni corrected $p < 0.01$, see discussion and methods for discussion of effect sizes. Scatterplots illustrating relationships between CB_1 expression levels in RA and time spent singing (E; total number of seconds a male spent singing during 4, 20 min observation periods) and CB_1 expression levels in LS and the number of times males displaced other males (F; total number of times a male displaced another male during 4, 20 min observation periods). Each point represents data for an individual male. Filled points indicate males with nest sites; open point indicate males without nest sites. Regression line indicates $p < 0.05$.

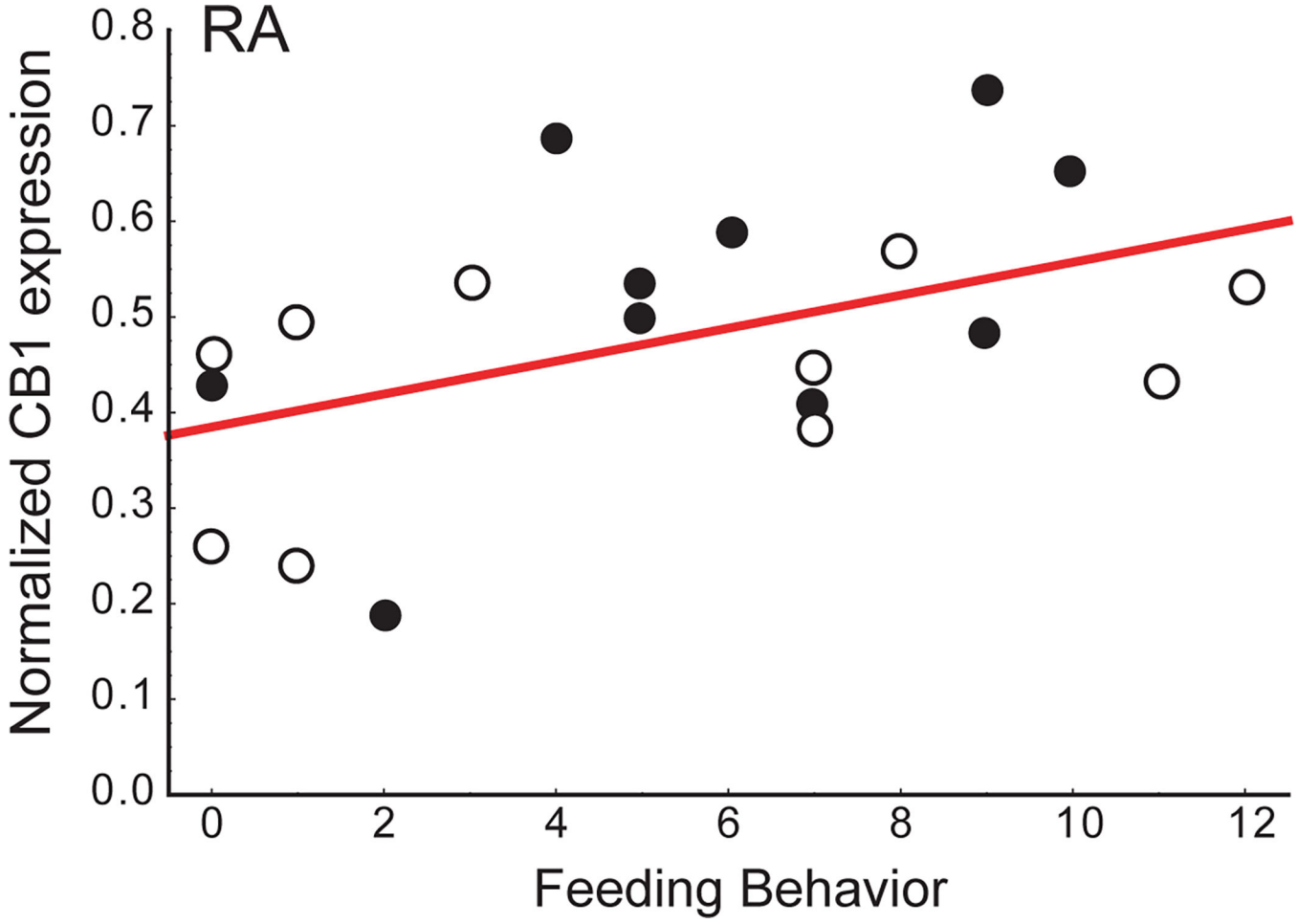

Figure 2.

Scatterplots illustrating relationships between feeding behavior and CB_1 expression levels in RA. Each point represents data for an individual male. Filled points indicate males with nest sites; open point indicate males without nest sites. Regression line indicates $p < 0.05$.

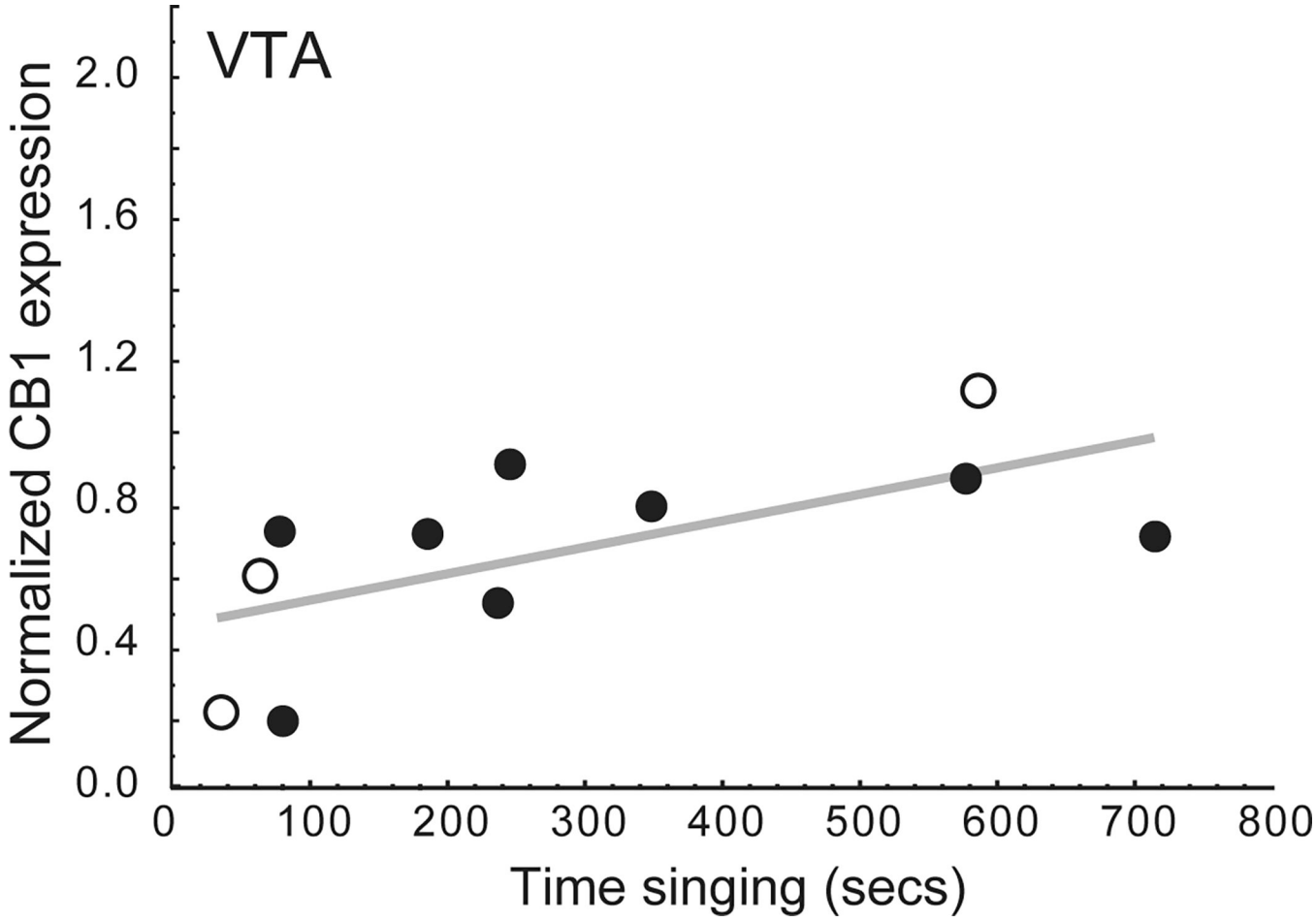

Figure 3.

CB_1 expression levels in VTA and time spent singing. Each point represents data for an individual male. Filled points indicate males with nest sites; open point indicate males without nest sites. Regression line indicates $p < 0.05$. Light regression line indicates that this was only significant when singing males were considered (see text for details).

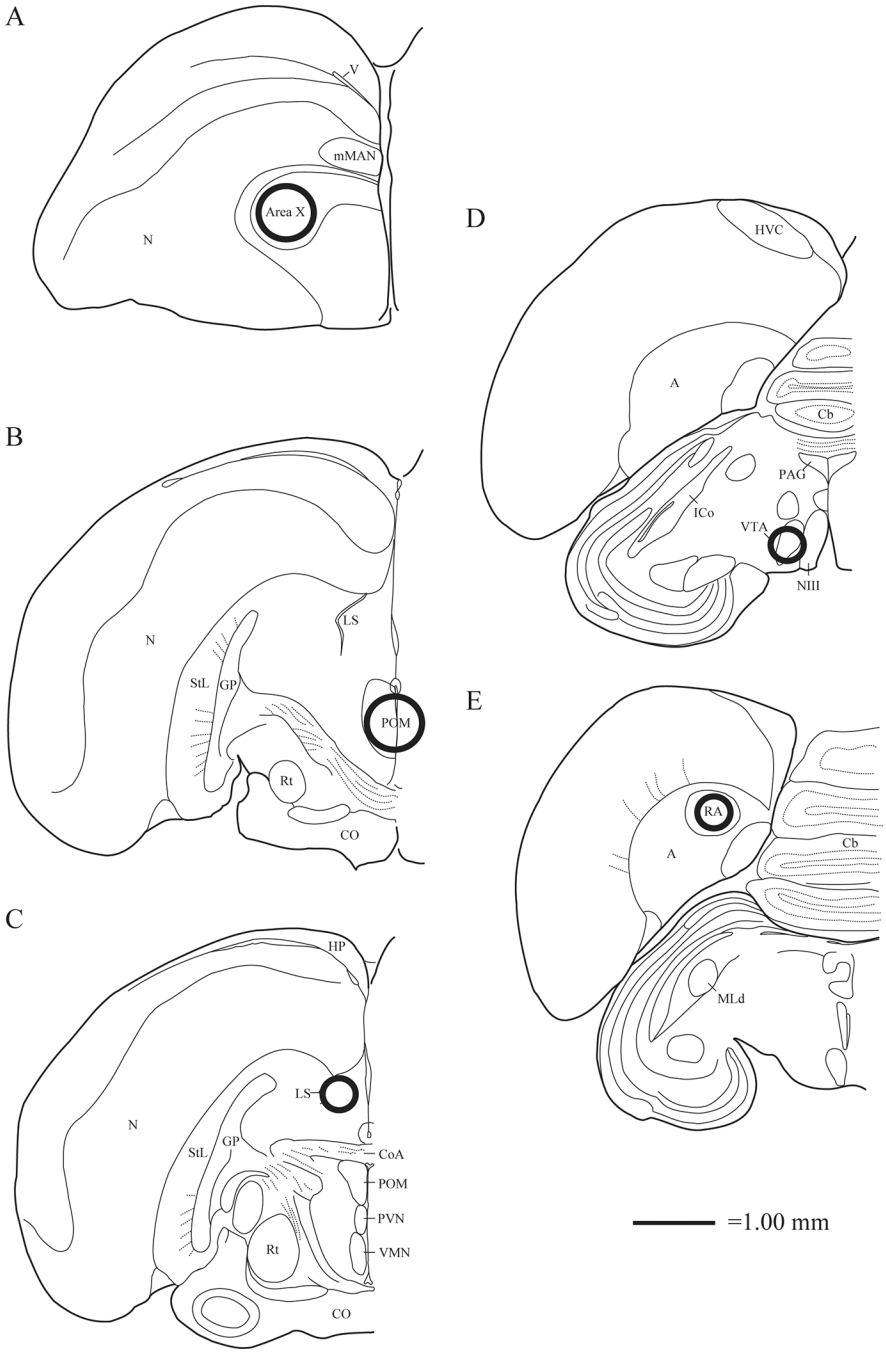

Figure 4.

Location of tissue punches illustrated in one, coronal hemisphere of the starling brain. Punches were taken bilaterally, except in POM for which a single central punch was collected. Sections progress rostrally to caudally from A – E. Approximate punch sizes and locations are represented by circles centered in Area X and POM (1.25 mm diameter), LS, VTA, and RA (0.75 mm diameter). Abbreviations listed alphabetically: A = arcopallium; Cb = cerebellum; CO = optic chiasm; CoA = anterior commissure; GP = globus pallidus; HP = hippocampus; HVC = used as a proper name; ICo = nucleus intercollicularis; MLd =

mesencephalicus lateralis, dorsalis mMAN = medial portion of the magnocellular nucleus; N = nidopallium; NIII = 3rd cranial nerve; PAG = periaqueductal gray; PVN = periventricular nucleus; Rt = nucleus rotundus; StL = striatum laterale; V = ventricle; VMN = ventromedial nucleus of the hypothalamus.

Table 1

CB1 and testosterone: t-test results for comparisons of males with and without nest boxes

RA CB1 expression	mean	sd	CI −95	CI +95	t (df)	p	Effect size (Cohen's *d*)
no box (n = 10)	0.43	0.11	0.36	0.51	2.34 (17)	***0.032***	1.07
box (n = 9)	0.56	0.12	0.47	0.65			
LS CB1 expression							
no box (n = 10)	0.90	0.35	0.64	1.15	2.23 (18)	***0.039***	1.00
box (n = 10)	1.26	0.39	0.99	1.54			
Area X CB1 expression							
no box (n = 10)	1.08	0.45	0.76	1.4	0.01 (18)	0.990	0.01
box (n = 10)	1.08	0.31	0.85	1.3			
VTA CB1 expression							
no box (n = 9)	0.92	0.59	0.47	1.38	1.11 (15)	0.285	0.55
box (n = 8)	0.68	0.23	0.48	0.87			
POM CB1 expression							
no box (n = 10)	1.22	0.62	0.78	1.67	1.13 (18)	0.273	0.51
box (n = 10)	1.51	0.50	1.15	1.87			
Testosterone (pg/ml)							
no box (n = 10)	912.98	972.43	217.34	1608.62	1.21 (18)	0.242	0.54
box (n = 10)	1469.36	1082.66	694.87	2243.85			

Table 2

CB1 and behavior: Multiple regression results

RA (dependent variable)	IV	Beta	std error of Beta	t (df)	p
adj R2 = 0.40, p = 0.011	time singing	0.56	0.19	2.91 (16)	***0.010***
n = 20	displacements	−0.22	0.19	1.11 (16)	0.282
	feeding	0.50	0.18	2.77 (16)	***0.014***
LS (dependent variable)	IV	Beta	std error of Beta	t (df)	p
adj R2 =0.34, p = 0.021	time singing	−0.14	0.20	0.69 (16)	0.499
n = 20	displacements	0.71	0.20	3.54 (16)	***0.003***
	feeding	0.08	0.19	0.42 (16)	0.679
VTA (dependent variable)	IV	Beta	std error of Beta	t (df)	p
adj R2 = 0.20, p = 0.12	time singing	0.02	0.24	0.09 (13)	0.930
n = 17	displacements	−0.22	0.24	0.96 (13)	0.356
	feeding	−0.54	0.22	2.42 (13)	0.031
POM (dependent variable)	IV	Beta	std error of Beta	t (df)	p
adj R2 = −0.08, p = 0.656	time singing	−0.18	0.26	0.71 (16)	0.485
n = 20	displacements	0.21	0.26	0.79 (16)	0.439
	feeding	−0.20	0.24	0.81 (16)	0.427
Area × (dependent variable)	IV	Beta	std error of Beta	t (df)	p
adj R2 = −0.06, p = 0.272	time singing	0.08	0.24	0.35 (16)	0.727
n = 20	displacements	0.02	0.24	0.10 (16)	0.919

RA (dependent variable)	IV	Beta	std error of Beta	t (df)	p
	feeding	−0.43	0.23	1.92 (16)	0.073