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Song-associated reward correlates with endocannabinoid-related gene expression in male European starlings (*Sturnus vulgaris*)

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Abstract

Vocal communication is required for successful social interactions in numerous species. During the breeding season, songbirds produce songs that are reinforced by behavioral consequences (e.g., copulation). However, some songbirds also produce songs not obviously directed at other individuals. The consequences maintaining or reinforcing these songs are less obvious and the neural mechanisms associated with undirected communication are not well-understood. Previous studies indicate that undirected singing is intrinsically rewarding and mediated by opioid or dopaminergic systems; however, endocannabinoids are also involved in regulating reward and singing behavior. We used a conditioned place preference paradigm to examine song-associated reward in European starlings and quantitative real-time PCR to measure expression of endocannabinoid-related neural markers (CB_1, FABP7, FABP5, FAAH, DAGLα), in brain regions involved in social behavior, reward and motivation (ventral tegmental area [VTA], periaqueductal gray [PAG], and medial preoptic nucleus [POM]), and a song control region (Area X). Our results indicate that starlings producing high rates of song developed a conditioned place preference, suggesting that undirected song is associated with a positive affective state. We found a significant positive relationship between song-associated reward and CB_1 receptors in VTA and a significant negative relationship between song-associated reward and CB_1 in PAG. There was a significant positive relationship between reward and the cannabinoid transporter FABP7 in POM and a significant negative relationship between reward and FABP7 in PAG. In Area X, FABP5 and DAGLα correlated positively with singing. These results suggest a role for endocannabinoid signaling in vocal production and reward associated with undirected communication.

Keywords

conditioned place preference; endocannabinoid; reward; songbird; vocal communication

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Animals produce vocalizations in a variety of contexts. Often there are obvious behavioral consequences that occur after a vocalization is produced, resulting in reinforcement of the vocal behavior. Examples of behavioral outcomes that can follow a vocalization include: attracting a mate, deterring a predator, or scaring off a rival. In each of these examples, the vocal behavior is reinforced (i.e., a mate is obtained; a predator or competitor stays away) and the vocalization is more likely to be produced again in the future. In other contexts, the function of vocalizing, or the reinforcer maintaining the vocal behavior, is less clear. For example, some songbirds produce songs that are not obviously directed at other individuals and appear to be ignored by potential recipients (Dunn and Zann, 1996). This type of song was first termed 'undirected song' in zebra finches (*Taeniopygia guttata*) (Sossinka and Böhner, 1980; Dunn and Zann 1996), with similar forms of communication observed in other species (e.g., Bengalese finches, *Lonchura striata domestica*, Dunning et al., 2014; European starlings, *Sturnus vulgaris*, Riters et al., 2000). Although the precise functions of undirected song may vary across species, this type of communication is facilitated by the presence of conspecifics (Jesse and Riebel, 2012); for example, starlings produce high rates of song during the nonbreeding season while in large affiliative flocks (Feare, 1984). This communication is proposed to maintain social groups (Hausberger et al., 1995; Eens, 1997) and is important for song learning and maintenance (Adret-Hausberger et al., 1990; Böhner et al., 1990; Chaiken et al., 1994; Kao et al., 2005). Females may use male undirected song to assess potential mates (Holveck and Riebel, 2007), but, unlike female-directed song, undirected songs are not accompanied by courtship displays or followed by mating (Morris, 1954; Sossinka and Böhner, 1980). In other words, there is not an obvious external reinforcer that follows this form of undirected song (in contrast to female-directed song, which may be reinforced by copulation). It has been proposed that because there is no immediate, obvious external reinforcer for undirected song, producing this type of song may be facilitated or maintained by intrinsic reward mechanisms (Riters, 2010, 2011, 2012).

Consistent with the idea that the reward mechanisms associated with female-directed and undirected song vary, there is evidence in male starlings and zebra finches that producing undirected song may be intrinsically rewarding and associated with a positive affective state, while song directed at conspecifics (e.g., female-directed song) is more tightly linked to the reinforcement provided by the receiver's behavior (Riters and Stevenson, 2012). Using a conditioned place preference paradigm, male zebra finches and male starlings developed a conditioned place preference associated with producing song that was not directed at other individuals (i.e., undirected song), while males did not develop a conditioned place preference associated with producing directed song.

Across vertebrates, opioid neuropeptides are strongly implicated in reward and several studies demonstrate opioid neuropeptides to be tightly coupled to undirected singing behavior (Riters et al., 2005; Kelm-Nelson et al., 2012) and to reward associated with undirected singing behavior (Riters et al. 2014). There is also evidence that dopamine may be involved in singing in this context (Heimovics et al., 2009; Merullo et al., 2016), and it is likely that other neurochemical systems also play a role. The endocannabinoid system is a likely candidate for potentiating reward processes, as endocannabinoids interact with other neurochemical systems that modulate motivation and reward, including the opioid and dopaminergic systems (for review see Solinas et al., 2007; 2008).

Endocannabinoids, their receptors and enzymes are distributed across the brains of vertebrates (Elphick, 2012) and are involved in a variety of rewarding behaviors (for review see Fattore et al., 2010). The best characterized endogenous ligands of the endocannabinoid system are anandamide and 2-arachidonylglycerol (2-AG). Both anandamide and 2-AG are hydrophobic, synthesized "on demand," and transported via intracellular carriers, such as fatty acid-binding proteins (FABP; Sanson et al., 2013). Anandamide and 2-AG, which primarily signal in a retrograde manner, are released from the postsynaptic membrane and interact with cannabinoid receptors on presynaptic neurons, including the cannabinoid receptor CB_1. CB_1 receptors are G-protein coupled receptors abundant in the CNS (for review see Wilson and Nicoll, 2002; Castillo et al. 2012) and activation of CB_1 receptors suppresses neurotransmitter release (Kano et al., 2009). Given the role of endocannabinoids in reward and the relationship with other reward- and undirected song-related neurotransmitters (i.e., dopamine, opioids), the endocannabinoid system may also have a role in undirected singing.

The objective of this study was to examine the extent to which mRNA expression for endocannabinoid-related markers is associated with singing behavior and reward. In order to examine song-associated reward, we observed male European starlings singing undirected song while in nonbreeding flocks and used a conditioned place preference (CPP) task to measure reward state associated with song production (as in Riters and Stevenson 2012; Riters et al. 2014). Following the CPP procedure, we collected neural tissue and used quantitative real-time PCR (qPCR) to measure expression levels of mRNA encoding CB_1 receptors, two endocannabinoid transporters (FABP7 and FABP5; Kaczocha et al., 2009), fatty acid amide hydrolase (FAAH; the degradative enzyme for anandamide), and diacylglycerol lipase-α (DAGLα; the synthesizing enzyme for 2-AG). We examined mRNA expression of these neural markers in brain regions involved in undirected song and reward, including medial preoptic area (POM) and two brain regions that are interconnected with POM: ventral tegmental area (VTA) and periaqueductal gray (PAG; Riters and Alger, 2004). We also examined Area X, a song control nucleus that is involved in song learning and receives direct projections from VTA (Lewis et al., 1981).

Experimental procedures

Subjects

Twenty-four male European starlings were captured on a farm in Madison, WI using baited fly-in traps. Following capture, starlings were brought to the facilities at the University of Wisconsin-Madison and were housed in colony rooms in stainless steel cages (91 cm × 47 cm × 47 cm) in same-sex groups with no more than five individuals per cage. Colored leg bands allowed identification of individual birds. Birds were housed indoors and placed on 18L:6D light cycle for at least six weeks. Starlings maintained under this photoperiod become photorefractory, during which birds have regressed gonads and are not in a reproductive state, which mimics fall nonbreeding conditions (Dawson et al., 2001).

During the experiment, males were housed in groups of four in indoor aviaries (3.5 m × 2.25 m × 2 m) containing four perches, a nest box, and a water bath. Birds were provided with ad libitum access to food and water. Birds were housed in the aviaries for at least two weeks

before the start of behavioral testing. All procedures were conducted in accordance with the guidelines in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and a protocol approved by the Institutional Animal Care and Use Committee at the University of Wisconsin-Madison.

Behavioral observations

Behavior was observed for 20 min prior to CPP conditioning (described below) by a single experimenter hidden behind a one-way mirror. We measured the number of songs produced and several non-vocal behaviors including: feeding bouts, drinking bouts, and preening bouts. A bout was defined as a behavior that was separated from a previous behavior by at least 2 s. Behavior was also observed for 20 min prior to sacrifice (at least one day following the final day of the CPP procedure).

Behavioral testing: Conditioned place preference

The CPP procedure consisted of three phases, each occurring on a separate day: habituation day, conditioning day, and test day. The CPP apparatus was a cage (118 cm × 59 cm × 59 cm) divided into two distinct compartments. Each compartment contained distinct visual cues (i.e., red or blue construction paper) to visually distinguish between the two sides of the cage.

On habituation day, a single bird was removed from its aviary (caught using a net) and placed into the CPP apparatus for 30 minutes in order to habituate the bird to the apparatus. The bird was allowed to freely explore both sides of the apparatus and the amount of time spent (in seconds) on each side of the apparatus was recorded. Following this habituation period, each bird was returned to its home aviary.

Conditioning was conducted on the following day. On conditioning day, once a bird was singing, it was observed for 20 min while in its home aviary and we measured the number of songs produced and other non-vocal behaviors (feeding bouts, drinking bouts, preening bouts; see *Behavioral observations*, above). Following the behavioral observations, each bird was quickly captured and placed singly into one side of the CPP apparatus for 30 min. An opaque barrier separated the two sides of the CPP apparatus, so a bird was restricted to one distinct side (i.e., the conditioned side) of the apparatus. The conditioned side of the apparatus was pseudo-randomly assigned and counterbalanced across birds, so that half of the birds were conditioned to the red side and half to the blue side. Following the conditioning period, each bird was returned to its home aviary.

The extent to which an individual developed a conditioned preference was tested the next day (i.e., CPP test day). On test day, a single bird was removed from its aviary and placed into the CPP apparatus. On this day, the opaque barrier in the apparatus was removed and the bird was allowed to freely move between the two distinct sides of the CPP apparatus for 30 min. Similar to habituation day, the total amount of time that a bird spent on each side of the apparatus on test day was recorded.

The rationale for this CPP procedure is that singing (or other behavior) is associated with a particular affective state before the birds are placed into the CPP apparatus. This affective

state is the unconditioned stimulus. This affective state is then paired with one side of the CPP apparatus. That side of the apparatus becomes the conditioned stimulus and is linked with the affective state associated with producing song. If song is associated with a positive (or rewarding) affective state, males should develop a CPP. We employ this CPP procedure because birds rarely produce song while in the CPP apparatus. This method has produced consistent results in previous studies examining song-associated reward in songbirds (Riters and Stevenson, 2012; Riters et al., 2014).

We removed four birds from the analyses that did not explore (i.e., spend at least 1min on each side) the CPP apparatus on the habituation day (these subjects were not habituated to both sides of the apparatus before conditioning day) and one bird that did not explore on test day. All other subjects visited each side of the apparatus on the habituation and test days. Three additional birds were removed due to external confounds during testing that interfered with the bird's exploration of the apparatus. We lost one brain during extraction (see below), resulting in $n = 15$. Final samples sizes for each statistical test are given in the results.

Tissue preparation for qPCR

Following the last behavioral observation, birds were rapidly decapitated. Brains were extracted, frozen using isopentane (Catalog No. 277258, Sigma, St. Louis, MO) cooled with dry ice, and stored at -80° C until further processing. One brain was lost during extraction. Coronal sections (200μm) were collected using a cryostat between -14° and -16° C and placed onto slides. Fine Science Tools Sample Corers (Item Nos. 18035-01 and 18035-02; Foster City, CA) were used to collect samples from target brain regions. A 1 mm diameter punch was taken from VTA (2 bilateral punches on 1 section), 2 mm diameter punches were taken from Area X (2 bilateral punches on 2 sections), POM (1 medial punch from 3 sections), and PAG (1 medial punch from 1 section). See Fig. 1 for approximate size and location of tissue punches. Tissue from each individual was placed in separate centrifuge tubes (one tube for each brain region) and stored at -80° C.

For RNA extraction, tissue was homogenized in a 1.7 mL cone-tipped tube with a cone-tipped Dremel tool and RNA was extracted with the Bio-Rad Aurum Total RNA Fatty and Fibrous Tissue Kit (Catalog No. 732-6830; Bio-Rad, Hercules, CA). PureZol was used to isolate RNA and RNA was treated with DNAse. Using 30 μL of nuclease free water, RNA was eluted and a NanoDrop system (Thermo Scientific, Wilmington, DE) was used to measure RNA concentration. 100 ng of RNA was converted into single-stranded cDNA using the Invitrogen SuperScript III First-Strand Synthesis System (Catalog No. 18080-051; Life Technologies, Carlsbad, CA). cDNA from surrounding sections were pooled and used as standard tissue for qPCR. Relative gene expression for CB_1, FABP7, FABP5, FAAH, and DAGLα was quantified using qPCR.

qPCR analysis

We used NCBI Primer Blast to design primers using zebra finch, European starling, or chicken (*Gallus gallus*) genome. Primers were prepared by the University of Wisconsin Biotechnology Center. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and hydroxymethylbilane synthase (HMBS) were used as reference genes for each brain region.

For some genes, we used forward and reverse primers that have been reported previously (GAPDH: Riters et al., 2014; HMBS: Merullo et al., 2015; CB_1: DeVries et al., 2016; FAAH: Dickens et al., 2015). See Table 1 for primer information. Sanger sequencing using the forward and reversed primers was conducted to sequence the qPCR reaction product at the University of Wisconsin Biotechnology Center and matched the intended targets using NCBI BLAST (Table 2).

For qPCR, we used the BioRad CFX96 Touch Real-Time PCR Detection System (Catalog No. 185-5195; Bio-Rad, Hercules, CA). To amplify cDNA, samples were mixed with Sso Fast Evagreen Supermix (Catalog No. 172-5204; Bio-Rad, Hercules, CA), nuclease-free H_2O, and forward and reverse primers. Along with each plate of samples, a run included five standards (1:10 serial dilution with a starting concentration of 500ng/μl; an exception was that the plate for FABP7 in Area X used standards with a 1:12 serial dilution) and a negative control consisting of nuclease free H_2O in place of cDNA. On each plate, samples and standards were run in triplicate. A run consisted of an initiation step at 95° C for 30 s, followed by 40 cycles of 95° C for 5 s, an annealing phase for 30 s at the annealing temperature (T_a) specific to the primer (see Table 1), followed by an elongation phase at 72° C for 30 s. Plates went through a melt curve from 65° C to 88° C, 0.5° C for each 5 s step. All runs followed the MIQE guidelines (Bustin et al., 2009) and had an efficiency between 90-110%, an R^2 of at least 0.990, and a melt curve with a single peak, which verified primer specificity.

We used the Pfaffl method to determine the relative levels of gene expression (for a detailed description see Cordes et al., 2015; Pfaffl, 2001). In brief, the amplification threshold was set at 200 RFU and the average number of cycles that crossed this threshold (Ct) was transformed for each sample. For each brain region, the geometric mean of the Ct values for the two reference genes was calculated and used to calculate the Ct values for each gene (i.e., CB_1, FABP7, FABP5, FAAH, or DAGLα) as a normalized ratio.

Statistical analysis

Our prediction was that song production would be associated with individual reward state and endocannabinoid-related expression. We were interested in the relationship between singing and constitutive mRNA expression in individuals. Consistent with past studies (e.g., Riters et al. 2014), a correlation analyses revealed that the song rate of a given individual positively correlated between observation days ($r = 0.57$, $r^2 = 0.33$, $p = 0.026$), demonstrating that an individual's propensity to sing is relatively constant. This suggests that mRNA expression on the day that tissue was collected is indicative of an individual's consistent tendency to produce song.

In order to examine the relationship between the absolute amount of time that the subject spent on the conditioned side of the apparatus on test and our behavioral measures we conducted correlation analyses. We conducted one correlation analyses with the number of songs the bird produced on conditioning day and we conducted a separate correlation analyses with bouts of non-vocal behaviors on conditioning day. We combined bouts of feeding, drinking and preening for our measure of non-vocal behavior, because overall birds produced few bouts of each behavior (range: 0-6 bouts). To account for multiple

comparisons we used a Bonferroni correction for our alpha value (α = 0.05/2 comparisons = 0.025).

We conducted multiple regression analyses with the absolute amount of time that the subject spent on the conditioned side of the apparatus on test day as the dependent variable. We included two behavioral measures from conditioning day as predictor variables: (1) number of songs and (2) bouts of non-vocal behaviors. We also included mRNA expression in VTA, PAG, and POM as predictor variables. We conducted a separate regression analysis for each neural marker (i.e., CB_1, FABP7, FABP5, FAAH, DAGLα). The neural markers were intercorrelated with one another in some brain regions, so inclusion in one regression analyses was not appropriate. See Table 3 for the predictor variables included in each regression analysis. In order to examine endocannabinoid-related expression in the song control nucleus, Area X, we first conducted a regression analysis with the absolute amount of time that the subject spent on the conditioned side of the apparatus on test day as the dependent variable. For the predictor variables, we included our two behavioral measures (number of songs and bouts of non-vocal behaviors), and expression in Area X for each neural marker (CB_1, FABP7, FABP5, FAAH, DAGLα). While, Area X may play a role in reinforcement related to vocal learning (e.g., Hoffman et al., 2016), it does not have a definitive role in reward processes comparable to other brain regions (e.g., PAG, POM, VTA; thus we would not predict a relationship between CPP and Area X mRNA expression, a prediction we confirmed below). However, Area X is critical for song learning (Sohrabji et al., 1990; Scharff and Nottebohm, 1991) and variability in song production (Leblois et al., 2010; Leblois and Perkel, 2012); therefore, we also conducted separate correlation analyses for each neural marker to examine the relationship between the number of songs produced and mRNA expression in Area X. We examined Area X because it is the nucleus involved in song learning, compared to other nuclei in the song control system (HVC and the robust nucleus of the arcopallium, RA), which are proposed to regulate the temporal structure of song production (Fee et al., 2004). In some instances, data were missing for a sample because it did not meet our inclusion criteria (e.g., a sample ran after 35 cycles), reducing our sample size. Below we note when sample size was reduced or when we excluded a brain region from the analysis because of low sample size. All statistics were conducted with Statistica (StatSoft, Inc., Tuslsa, OK).

Results

Relationship between CPP and behavioral measures

A correlation analysis revealed that the song rate of a given individual positively correlated with the amount of time on the conditioned side of the apparatus on test day ($r = 0.703$; $r^2 = 0.494$; $p = 0.0035$; $n = 15$; see Fig. 2), a result consistent with previous studies (Riters and Stevenson, 2012; Riters et al., 2014). A second correlation analysis revealed that bouts of non-vocal behaviors (feeding, drinking, preening) did not significantly correlate with the amount of time on the conditioned side of the apparatus on test day ($r = 0.506$; $r^2 = 0.256$; $p = 0.0542$; $n = 15$). To account for multiple comparisons we used a Bonferroni correction (α = 0.025).

Song-associated preference and CB_1

We conducted a multiple regression analysis with the amount of time on the conditioned side of the apparatus on test day as the dependent variable and number of songs, bouts of non-vocal behaviors, mRNA expression for the cannabinoid receptor CB_1 in VTA, CB_1 mRNA expression in PAG, and CB_1 mRNA expression in POM as predictor variables. We were missing mRNA expression in VTA for 1 bird, reducing the sample size to 14. The model explained a significant amount of the variance (*adjusted* $R^2 = 0.770$, $N = 14$, $p = 0.0030$). Number of songs produced (Beta = 0.398, SE = 0.172, $t(8) = 2.312$, $p = 0.0495$), CB_1 mRNA expression in VTA (Beta = 0.374, SE = 0.159, $t(8) = 2.351$, $p = 0.0466$), and CB_1 mRNA expression in PAG (Beta = -0.468, SE = 0.148, $t(8) = -3.176$, $p = 0.0131$) statistically explained variance in our measure of CPP. No other variables were significant. See Table 3; Fig. 3.

Song-associated preference and FABP7

We conducted a multiple regression analysis with the amount of time on the conditioned side of the apparatus on test day as the dependent variable and number of songs, bouts of non-vocal behaviors, mRNA expression for the endocannabinoid transporter FABP7 in VTA, FABP7 mRNA expression in PAG, and FABP7 mRNA expression in POM as predictor variables. We were missing mRNA expression in VTA for 2 birds, reducing the sample size to 13. The model explained a significant amount of the variance (*adjusted* $R^2 = 0.687$, $N = 13$, $p = 0.0160$). FABP7 mRNA expression in POM (Beta = 0.771, SE = 0.234, $t(7) = 3.289$, $p = 0.0133$), and FABP7 mRNA expression in PAG (Beta = -0.460, SE = 0.192, $t(7) = -2.399$, $p = 0.0476$) statistically explained variance in our measure of CPP. No other variables were significant. See Table 3; Fig. 4.

Song-associated preference and FABP5

We conducted a multiple regression analysis with the amount of time on the conditioned side of the apparatus on test day as the dependent variable and number of songs, bouts of non-vocal behaviors, mRNA expression for the endocannabinoid transporter FABP5 in VTA, FABP5 mRNA expression in PAG, and FABP5 mRNA expression in POM as predictor variables. We were missing mRNA expression in VTA for 2 birds, reducing the sample size to 13. The multiple regression analysis was not significant (*adjusted* $R^2 = 0.409$, $N = 13$, $p = 0.1172$) and there were no significant predictor variables (all ps ≥ 0.1366; see Table 3) for any of the brain regions measured.

Song-associated preference and FAAH

We conducted a multiple regression analysis with the amount of time on the conditioned side of the apparatus on test day as the dependent variable and number of songs, bouts of non-vocal behaviors, mRNA expression for the degradative enzyme FAAH in PAG, and FAAH mRNA expression in POM as predictor variables. FAAH mRNA expression in VTA was missing for 5 birds, likely because expression for this neural marker is low in this brain region, so we excluded VTA from the analysis. We were missing mRNA expression in POM for 1 bird and PAG for 2 birds, reducing the sample size to 12. The model explained a

significant amount of the variance (*adjusted* R^2 = 0.565, N = 12, p = 0.0394); however, none of the individual predictor variables were significant (all ps ≥ 0.0867; see Table 3).

Song-associated preference and DAGLα

We conducted a multiple regression analysis with the amount of time on the conditioned side of the apparatus on test day as the dependent variable and number of songs, bouts of non-vocal behaviors, mRNA expression for the synthesizing enzyme DAGLα in POM as predictor variables. DAGLα mRNA expression in VTA was missing for 11 birds and DAGLα mRNA expression in PAG was missing for 6 birds, so we excluded these brain regions from the analysis. The model explained a significant amount of the variance (*adjusted* R^2 = 0.385, N = 15, p = 0.0398). The number of songs produced (Beta = 0.611, SE = 0.252, $t(11)$ = 2.420, p = 0.0340) statistically explained variance in our measure of CPP. No other variables were significant. See Table 3.

Song production and endocannabinoid-related expression in Area X

We conducted a multiple regression analysis with the amount of time on the conditioned side of the apparatus on test day as the dependent variable and number of songs, bouts of non-vocal behaviors, CB_1, FABP7, FABP5, FAAH, and DAGLα mRNA expression in Area X as predictor variables. We were missing FABP7 mRNA expression for 1 bird, reducing the sample size to 14. As predicted, mRNA expression in Area X did not significantly predict our measure of reward (amount of time on the conditioned side of the apparatus on test day; all ps ≥ 0.2103). The number of songs produced (Beta = 0.98, SE = 0.36, $t(6)$ = 2.69, p = 0.0360) statistically explained variance in our measure of reward; however, the overall regression model was not significant (*adjusted* R^2 = 0.54, N = 14, p = 0.0877).

In order to examine the relationship between song production and endocannabinoid-related markers in Area X, we conducted correlation analyses. We used a Bonferroni correction for our alpha value (α = 0.05/5 comparisons = 0.01) to account for multiple comparisons. We were missing FABP7 mRNA expression for 1 bird, reducing the sample size to 14 for the analysis of FABP7. The number of songs produced positively correlated with DAGLα mRNA expression in Area X (r = 0.67; r^2 = 0.45; p = 0.006) and FABP5 expression in Area X (r = 0.64; r^2 = 0.41; p = 0.01; Fig. 5).

Discussion

Our results show that the affective state associated with the production of undirected song in male starlings is associated with cannabinoid-related gene expression in brain regions implicated in motivation and reward. We identified strong, linear relationships between song-associated reward (as measured via CPP) and genes involved in cannabinoid activity in brain regions implicated in motivation and reward (VTA, PAG, POM). It is possible that endocannabinoids in these regions induce a positive affective state that then facilitates singing behavior. Alternatively, but not mutually exclusively, singing behavior could influence endocannabinoid activity which then induces a positive affective state. Our data are correlational and it is possible that a third variable explains these relationships; however, given the role of endocannabinoids in reward we suggest that endocannabinoids may directly

link the act of song production with a positive affective state. Relationships between endocannabinoid markers and song were also identified for the song control region, Area X, which we interpret to suggest a role for cannabinoids in Area X in song learning as suggested previously in studies of zebra finches (Soderstrom and Johnson, 2003).

Undirected song is related to reward state

This is the fourth study to provide evidence for a positive linear relationship between undirected singing and reward state (measured via CPP; Riters and Stevenson, 2012; Riters et al., 2014). Our hypothesis regarding reward associated with undirected singing is that neurochemicals associated with reward are released prior to or during the act of singing. Given the nature of singing behavior, song production and the lack of song production cannot be equally paired with one side of the apparatus during the conditioning phase (discussed in detail in Riters and Stevenson 2012), so one side of the apparatus (i.e., the conditioned side) may be more familiar to the individual. However, we do not have compelling reasoning to explain why the familiar side of the apparatus would be more appealing to birds that sing higher rates of songs (compared to birds that sing fewer songs); therefore, we suggest that the more parsimonious explanation is that singing high rates of song is associated with a positive affective state and birds that sing high rates of song spend more time on the previously-conditioned side of the apparatus because it is associated with this positive affective state. In addition, we only included birds that explored both sides of the apparatus to ensure that neither side was completely novel to the individual. See Riters and Stevenson (2012) for a full discussion of the interpretational limitations of our CPP methodology.

Consistent with the notion that individual propensity to sing is constant, and the related neural expression is constitutive, we found evidence that individual song rate was consistent across days; similar findings of consistency in song production across days have been reported by previous studies (e.g., Naguib et al., 2010; Riters et al., 2014). The current results support the hypothesis that undirected singing is stimulated and/or reinforced by intrinsic reward processes, as birds that developed a song-associated preference sang more songs compared to birds that did not demonstrate a conditioned preference.

CB_1 expression in VTA and PAG relate to song-associated reward

There was a significant positive relationship between CPP (measured as the amount of time on test day spent on the conditioned side of the apparatus), the number of songs a bird produced, and relative CB_1 expression in VTA. VTA is a brain region strongly implicated in reward and motivation (Fields et al., 2007; O'Connell and Hofmann, 2011; Riters, 2012). In mammals, there is evidence that endocannabinoids in VTA regulate reward and motivational processes by influencing dopamine release (Oleson et al., 2012; Sagheddu et al., 2015). Specifically, in VTA, dopamine neurons release endocannabinoids, which then interact with CB_1 receptors on GABAergic and glutamatergic axon terminals and inhibit neurotransmitter release (Melis et al., 2004; Riegel & Lupica, 2004). In VTA, endocannabinoids reduce the GABAergic inhibition of dopaminergic neurons (Szabo et al., 2002) and this is a possible mechanism for dopaminergic neurons in VTA to fine-tune their own activity (Riegel and Lupica, 2004; Wang and Lupica, 2014) and mediate reward-related behavior (Oleson et al.,

2012). In addition, VTA is well-positioned to influence the motivation to produce vocalizations, as VTA projects directly to song control nuclei including Area X (Lewis et al., 1981; Appeltants et al., 2000). Consistent with the idea that CB_1 receptors in VTA play a role in the motivation associated with social communication, a previous study with male European starlings in breeding condition found that CB_1 receptors in VTA positively correlated with singing and males with nest sites produced more song, suggesting that singing was related to a bird's motivational state (i.e., males with nest sites are more sexually-motivated compared to males without nest sites; DeVries et al., 2016). Our results also indicate a positive relationship between CB_1 receptors in VTA and reward or motivation associated with singing.

We also found a significant negative relationship between CPP and relative CB_1 expression in PAG. This is consistent with prior studies suggesting a role for PAG in undirected song (Lynch et al., 2008; Kelm-Nelson and Riters, 2013). PAG has a role in reward (e.g., Olmstead and Franklin, 1997) and vocal communication in mammals (Jürgens, 1994). In songbirds there are projections from PAG to brain regions critical for song production (Appeltants et al., 2000) and PAG receives projections from other brain regions involved in motivation (such as POM; Riters and Alger, 2004). PAG is thus anatomically well-positioned to integrate information about motivational state and then send this information to brain regions critical for vocal production, resulting in an individual producing vocalizations indicative of its affective state, as proposed in studies in mammals (Jürgens and Pratt, 1979; Dubbeldam and den Boer-Visser, 2002; Dujardin & Jürgens, 2005).

In addition to reward, cannabinoids (Finn et al., 2003) and opioids (Jensen and Yaksh, 1986) in PAG induce analgesia in mammals and analgesia can thus serve as an indirect measure of opioid or cannabinoid release. Consistent with this, previous studies with male starlings found that undirected song was tightly coupled to analgesia (Kelm-Nelson et al., 2012) and mu opioid receptor (MOR) labeling in PAG related to undirected song, with high and low singers exhibiting lower receptor labeling (compared to intermediate singers), resulting in a curvilinear relationship between undirected song and MOR (Kelm-Nelson and Riters, 2013). The results of the current study indicate a negative linear relationship between CB_1 and song-associated reward. MOR and CB_1 receptors co-localize in PAG (Wilson-Poe et al., 2012), so it is possible that our results are highlighting the negative portion of the curve found by Kelm-Nelson and Riters (2013). However, double-labeling studies examining MOR and CB_1 receptors in PAG in males producing undirected song are needed.

FABP7 expression in PAG and POM relate to song-associated reward

We found significant relationships between CPP and FABP7 in PAG and FABP7 in POM. While our correlation analysis revealed a significant positive relationship between song rate and CPP, song rate was not a significant predictor variable in this regression analysis with FABP7, so it is possible that the relationship between FABP7 and CPP is not mediated by singing. FABPs are intracellular transporters of endocannabinoids (Kaczocha et al., 2009; Sanson et al., 2013); however, FABPs may also store endocannabinoid ligands, (Howlett et al., 2011), suggesting that endocannabinoids can accumulate within a cell. Our results indicate that individuals that are in a positive affective state may have fewer

endocannabinoid ligands in PAG compared to birds not in a positive affective state, which is consistent with the negative relationship we found between CPP and CB_1 expression in PAG.

Birds that developed a song-associated preference (compared to those that did not) had more FABP7 expression in POM. There are direct connections between POM and other brain regions in which endocannabinoids are involved in motivation and reward (e.g., VTA: Oleson et al., 2012; Sagheddu et al., 2015), including regions that the current study found significantly related to song-associated reward (i.e., VTA, PAG). POM also projects both directly and indirectly to song control regions (Riters and Alger, 2004). Opioids in POM relate to reward (Ågmo and Gómez, 1991; Ågmo and Gómez, 1993) and in POM are tightly coupled to song-associated CPP (Riters et al., 2014). The current results suggest that birds in a positive affective state may have more endocannabinoids in POM; however, more research is needed to examine the relationship between endocannabinoids in POM and reward.

DAGLα and FABP5 expression in Area X relate to undirected song

Area X, a song control nucleus in songbirds that is involved in song learning (Sohrabji et al., 1990; Scharff and Nottebohm, 1991) and song variability (Leblois et al., 2010; Leblois and Perkel, 2012) does not have a definitive role in reward processes comparable to other brain regions. Consistent with this, endocannabinoid-related genes in Area X did not relate to reward. We examined the relationship between undirected song production and endocannabinoid-related genes in Area X, because CB_1 is expressed in Area X (Soderstrom and Tian, 2006) and cannabinoid exposure during development alters songbird vocal production (Soderstrom and Johnson, 2003), leading to long-term impairment of the endocannabinoid system (Soderstrom et al., 2011). It has also been proposed that DAGLα and CB_1 in Area X have a role in vocal learning (Soderstrom and Tian, 2006; Soderstrom and Wilson, 2013). Our results are consistent with this previous research suggesting that the endocannabinoid system has a role in songbird vocal development and production; we found significant positive correlations between how much song a bird produced and FABP5 (an intracellular transporter) and DAGLα (the synthesizing enzyme for 2-AG) expression in Area X. As adults, European starlings continue to learn and add elements into their songs. Singing outside of the breeding season (such as the undirected singing examined in the current study) may be important for this song modification and practice.

We did not find a significant relationship between singing and CB_1 expression in Area X, a result that is consistent with a previous study examining CB_1 receptor expression in male starlings producing breeding song (DeVries et al., 2016). Taken together, these results suggest that in adult starlings there are similar levels of relative CB_1 receptor expression in Area X regardless of how much a bird sings. However, other endocannabinoid-related genes responsible for the production and transport of endocannabinoids have higher levels in birds that sing more undirected song, suggesting that high singers may have more endocannabinoid ligands in Area X. Area X indirectly projects to VTA via the ventral pallidum and dopaminergic neurons in VTA project back to Area X, forming an anatomical loop (Gale et al., 2008). This could be a mechanism for endocannabinoids produced in Area X to mediate reward-related behaviors by interacting with CB_1 receptors in VTA, thus

motivating song production. Consistent with this idea, not only do the current results indicate that an endocannabinoid synthesizing enzyme (DAGLα) in Area X positively correlates with singing, but the results also indicate that CB_1 receptor expression in VTA positively correlates with song-associated reward.

Conclusions

Endocannabinoids have been implicated in the reward processes associated with numerous behaviors including eating, social interactions, and social play (reviewed in Fattore et al., 2010), and the current results indicate a role for endocannabinoids in undirected communication associated with a positive affective state. Previous studies have provided evidence linking dopamine (Heimovics et al., 2009; Merullo et al., 2016) and opioids (Khurshid et al., 2010; Riters et al., 2014) with undirected communication. Given that the endocannabinoid system interacts with these and other neurochemical systems to modulate motivation and reward (Solinas et al., 2007; 2008), future work should examine how these neural systems interact in the reward processes associated with vocal communication. It should be noted that in the current study we quantified mRNA expression and not the final protein product. Therefore the final site of action for the translated protein may be different from the regions in which we quantified mRNA expression. Additionally, the current results are correlational, but a critical first step in examining the extent to which endocannabinoid-related genes relate to song-associated reward. Additional studies are now needed to determine the causal role of endocannabinoids in song production and song-associated reward.

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Abbreviations

2-AG	2-arachidonylglycerol
CPP	conditioned place preference
DAGLα	diacylglycerol lipase-α
FAAH	fatty acid amide hydrolase
FABP	fatty acid-binding protein
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
HMBS	hydroxymethylbilane synthase
PAG	periaqueductal gray
POM	medial preoptic area
qPCR	quantitative real-time polymerase chain reaction
VTA	ventral tegmental area

- Male starlings producing a high rate of undirected song developed a song-associated conditioned place preference
- CB_1 mRNA expression in VTA and FABP7 mRNA expression in POM positively correlated with song-associated reward
- CB_1 and FABP7 mRNA expression in PAG negatively correlated with song-associated reward
- DAGLα and FAPB5 mRNA expression in Area X positively correlated with singing
- Endocannabinoid signaling may play a role in the reward associated with undirected communication

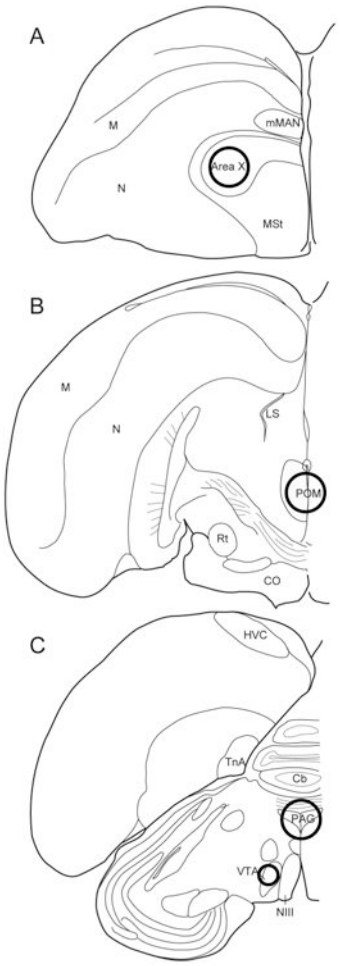

Figure 1.

Coronal sections showing approximate sizes and locations of tissue punches in (A) Area X (2 mm diameter), (B) POM (2 mm diameter), and (C) VTA (1 mm diameter) and PAG (2 mm diameter). Bilateral tissue punches were collected, except for POM and PAG (for each of these regions, a single central punch was taken). Abbreviations: Cb: cerebellum; CO: optic chiasm; HVC: letter-based proper name; LS: lateral septum; M: mesopallium; mMAN: medial magnocellular nucleus; MSt: medial striatum; N: nidopallium; NIII: third cranial nerve; Rt: nucleus rotundus; TnA: nucleus taenia of the amygdala

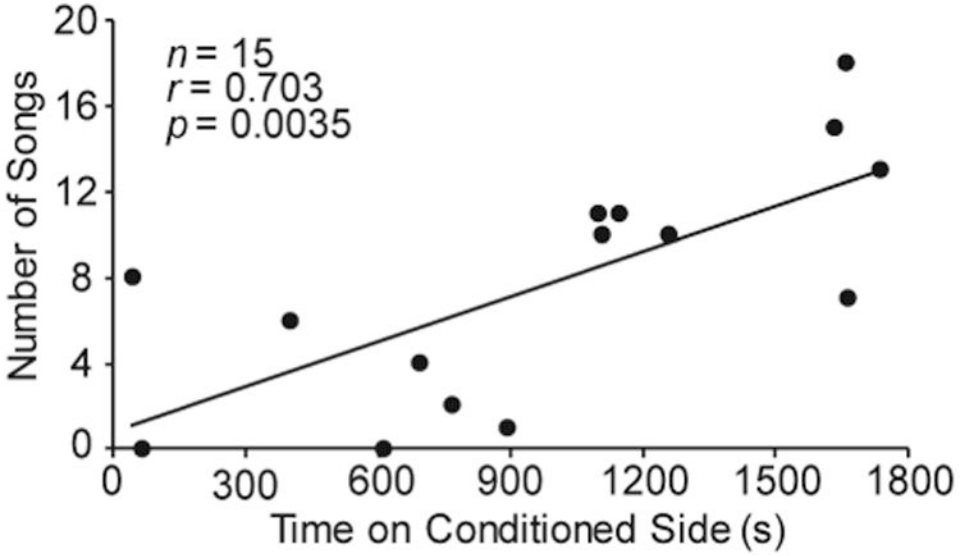

Figure 2.

Correlation between the number of songs produced and CPP (measured as the amount of time (s) on test day spent on the conditioned side of the apparatus; $n = 15$). Line indicates a significant correlation ($p \leq 0.025$).

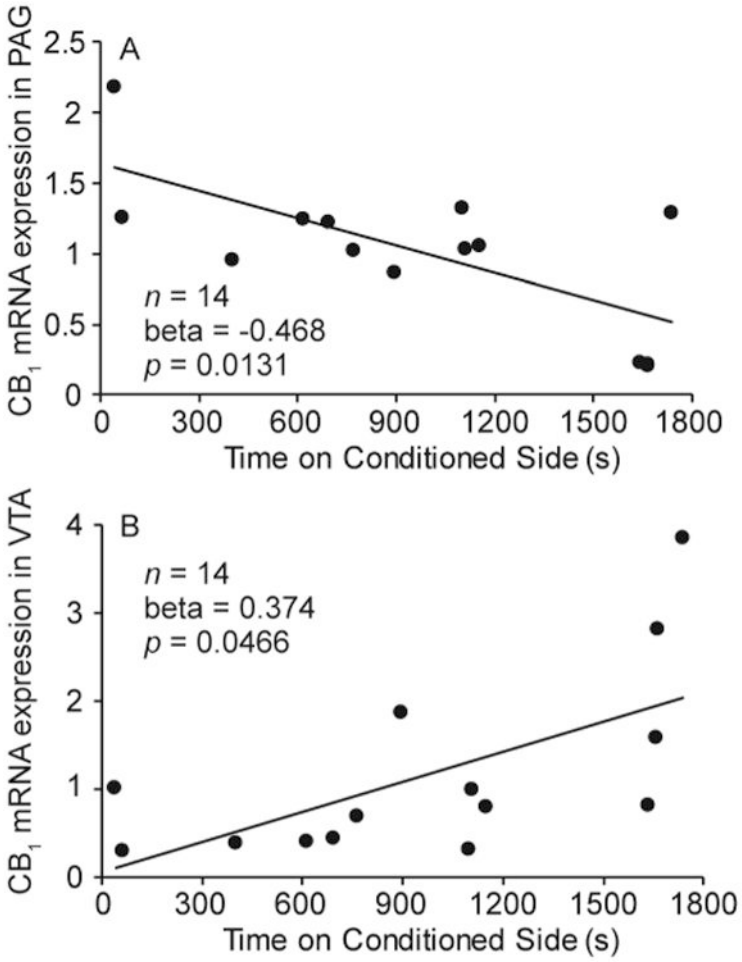

Figure 3.

Relationship between CPP (measured as the amount of time (s) on test day spent on the conditioned side of the apparatus) and CB_1 mRNA expression in (A) PAG and (B) VTA. Beta and p values were determined with a multiple regression model ($n = 14$). Linear regression lines indicate a significant relationship ($p \leq 0.05$).

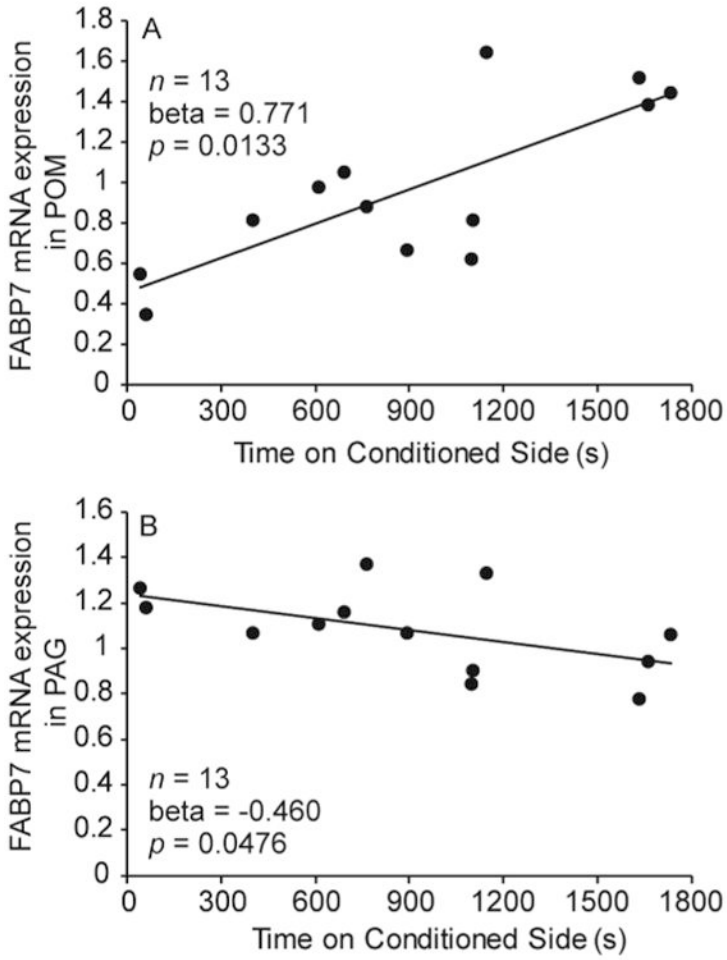

Figure 4.

Relationship between CPP (measured as the amount of time (s) on test day spent on the conditioned side of the apparatus) and FABP7 mRNA expression in (A) POM and (B) PAG. Beta and *p* values were determined with a multiple regression model ($n = 13$). Linear regression lines indicate a significant relationship ($p \leq 0.05$).

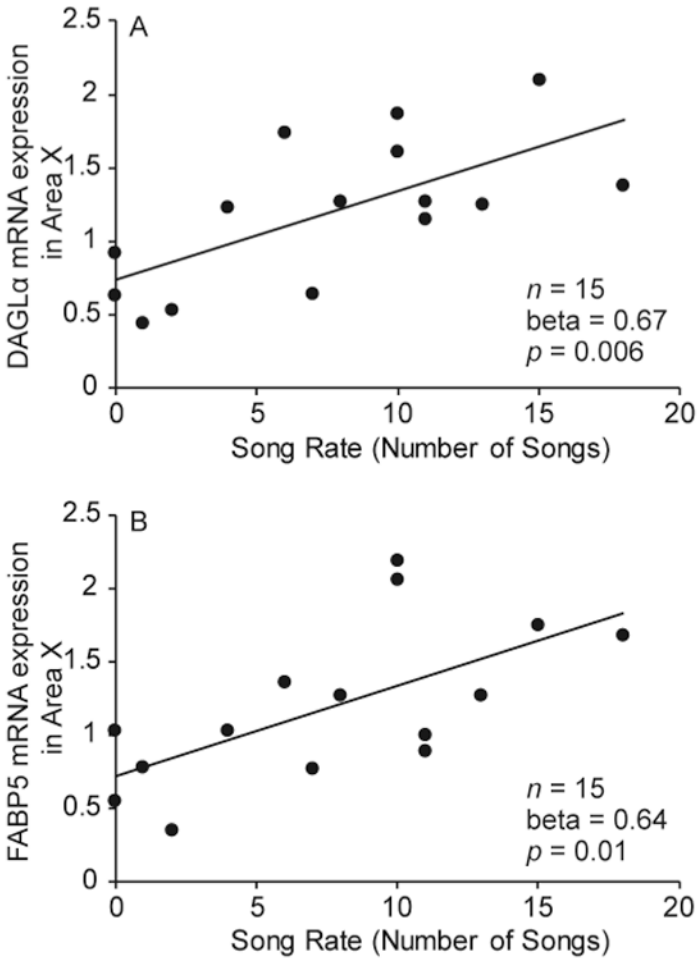

Figure 5.

Correlations between the number of songs and (A) DAGLα mRNA expression in Area X and (B) FABP5 mRNA expression in Area X ($n = 15$). Lines indicate a significant correlation ($p \leq 0.01$).

Table 1

Primers used for endocannabinoid-related genes (CB1, FABP7, FABP5, FAAH, DAGLα) and reference genes (GAPDH, HMBS).

Gene	Accession No.	Direction	Sequence	T_a (°C)	Product (bp)
CB_1	NM_001038652.1	Forward	GGTCTTCTGTGGACTTAGGG	58.5	113
		Reverse	CTCCTCTATTCCTTTGTTGCTC		
FABP7	XM_002188827.3	Forward	TGGTAGACAGCCACAACTTC	61.7	237
		Reverse	CTCCATCCAGGGTCACAAC		
FABP5	NM_001245600.1	Forward	TTTGGGTAAATGGTGCCTC	58.3	143
		Reverse	GACGGTGATTGTGTCCC		
FAAH	XM_014872201.1	Forward	AGGTCTGCTCTATGGAGTGC	59.5	109
		Reverse	GTCCTCTGCTGCAGGTTTAT		
DAGLα	XM_014878708.1	Forward	GTGATTGAGTTCGTGTACGCC	61.5	118
		Reverse	CCACCCAGTTACACACCACC		
GAPDH	NM_204305.1	Forward	AGCAATGCTTCCTGCACTAC	57.5	121
		Reverse	CTGTCTTCTGTGTGGCTGTG		
HMBS	XM_002187761.1	Forward	ACCTGCCAACTTCTCTTCCT	58	140
		Reverse	GGTTCCAATCACACTCTTTTCA		

Table 2

Sanger sequencing of the qPCR reaction product from primers for each gene

Gene	qPCR reaction product sequence
CB_1	TAATTTTTCCCCCTCCTTTTCTTTCACATGCATATGAGACTAACAGCAACAAAGG AATAGAGGAGA
FABP7	GGGGTCGCACACTGTAGNNNGGNATGTGACTAAANNCNCTGTGCATTATCANG NTNGCAGGGGGACANGNGTANTGATCNGAANCCNNNGCACTTTTCNAAAACAT AGAAATCNGCCTTTAAACTCGAGATAGGAATTTGANGAAACTACTCCCGATGAT AGAAANTGCANNTCANTTGTGACCCTGGATGGAG
FABP5	GGACTNGGTGTGGGTATGGCTATGAGAAAAATGGGAAGCATGGCCAAACCANC ACGTTTTACNTAATTAAGGATGGGGACACAATCACCGTTCA
FAAH	CATNGNCGCCAGGAGTNAGTACTTCCACGNTTGGGGCTTCATAAAACACCGTCC ATAAACCTGTCTGGCAGNCNGACA
DAGLα	CAGTACTCACCTCCTGCACGACATCACAGCCAAGAGTGTCACCCCTGGGAATGG TGGTGTGTAACTGGGGTGG
GAPDH	AGGGCTGGCAGGTATCCATGACACTTTGGCATCGTGGAGGGTCTCATGACCACT GGTCCATGCCATCACAGCCACACAGAAGACAGAT
HMBS	TCGCAAAGGGAAACCCACCTTGNATGCTGTTGTCTTTCATCCCAAAAACTGTGG AAAAACACTGAGCCTCCTTCCTGAAAAGAGTGTGATTGGAACNAA

Table 3

Results of regression models demonstrating behaviors and brain regions in which endocannabinoid-related expression explain variance in song-associated reward (measured via CPP).

Endocannabinoid-related gene	Regression model	Variables	Beta	*p*
CB_1 receptor $n = 14$	Adjusted $R^2 = 0.770$ $F_{5,8} = 9.69$ $p = 0.0030$	VTA	0.374	0.0466 *
		PAG	-0.468	0.0131 *
		POM	-0.028	0.8724
		Number of songs	0.398	0.0495 *
		Non-vocal behaviors	0.007	0.9653
FABP7 $n = 13$	Adjusted $R^2 = 0.687$ $F_{5,7} = 6.27$ $p = 0.0160$	VTA	-0.058	0.7932
		PAG	-0.460	0.0476 *
		POM	0.771	0.0133 *
		Number of songs	-0.020	0.9475
		Non-vocal behaviors	-0.091	0.6432
FABP5 $n = 13$	Adjusted $R^2 = 0.409$ $F_{5,7} = 2.66$ $p = 0.1172$	VTA	-0.047	0.8541
		PAG	-0.132	0.7388
		POM	0.751	0.1366
		Number of songs	0.298	0.3319
		Non-vocal behaviors	-0.078	0.7933
FAAH † $n = 12$	Adjusted $R^2 = 0.565$ $F_{4,7} = 4.57$ $p = 0.0394$	PAG	-0.186	0.3908
		POM	-0.281	0.2485
		Number of songs	0.369	0.1787
		Non-vocal behaviors	0.452	0.0867
DAGLα †† $n = 15$	Adjusted $R^2 = 0.385$ $F_{3,11} = 3.92$ $p = 0.0398$	POM	0.027	0.9033
		Number of songs	0.611	0.0340 *
		Non-vocal behaviors	0.177	0.4934

* Indicates a significant predictor variable ($p \leq 0.05$).

† Indicates VTA was not included in regression model (see text for details).

†† Indicates VTA and PAG were not included in regression model (see text for details).