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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. © 2017 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: conditioned place preference, endocannabinoid, reward, songbird, vocal communication.

INTRODUCTION

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).

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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.

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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,

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