Singing in the Brain: Decoding the Neurobiological Mechanisms Underlying Gregarious Song in European Starlings and Implications Across Vertebrates

By

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Dissertation Abstract

Birdsong is well-known for being performed during the breeding season to attract mates and defend territories. However, many birds, such as the European starling (*Sturnus vulgaris*), also sing in large flocks during the non-breeding season. This behavior, referred to here as gregarious song, is thought to be intrinsically motivated and important for social cohesion within the flock. Relatively little is known about the mechanisms that produce gregarious song. In this dissertation, I explore a conserved neural circuit that may regulate gregarious song and other non-sexual, social behaviors across vertebrates. In **Chapter 1**, I further characterize the avian nucleus accumbens (NAC) and its subregions by comparing them to mammals. I find that immediate early gene activity positively correlates with gregarious song produced in flocks, but not with songs produced while breeding. In **Chapter 2**, I discovered that pharmacologically stimulating mu-opioid receptors within the NAC and the medial preoptic area (mPOA) increases gregarious song and leads to the establishment of a conditioned place preference. In **Chapter 3**, I used RNA-sequencing of the starling's mPOA to uncover possibly novel modulators of gregarious song, including a cluster of co-expressing glutamatergic genes that correlated with song. In **Chapter 4**, I compared the RNA-sequencing profile of the mPOA in singing starlings with that of rats performing juvenile play, to uncover a shared neurotranscriptomic profile of intrinsically motivated social behaviors. This included multiple glutamatergic genes and multiple microglia-specific genes. The work presented here provides evidence for an interconnected, highly conserved circuit in the brain that works to regulate gregarious song and other non-sexual social behaviors across vertebrates.

General Introduction

As their moniker suggests, songbirds use song as a form of communication that serves a multitude of functions dependent on the natural context. Male songbirds are renowned for singing elaborate courtship songs to attract mates and defend their territories; however, many songbirds also sing outside of the breeding season. European starlings (*Sturnus vulgaris),* a passerine found across North America, are one such species. Starlings gather in enormous, overwintering flocks during the non-breeding season where both males and females sing at high rates. The inherently social nature of flocking provides continual opportunities to interact with other birds to develop and practice necessary social skills [1]. Starlings are open-ended learners that learn new songs throughout their entire lives, so it has been proposed that learning and practicing new song during the non-breeding season may allow males to produce songs that are attractive to mates in the breeding season [2]. Singing within the flock may also increase social tolerance and contribute to the maintenance of flock cohesion [1, 3, 4]. Due to the fundamental sociality of this singing in flocks, I will refer to it here as gregarious song.

Understanding the proximate motivators of performing gregarious song is difficult, as there is not a clear, pronounced reward for the behavior. In this context, starlings do not directly sing to each other, nor do they appear to gain any obvious, external reinforcement in response to song, like copulation or departure of a rival [5]. Because of this, it has been proposed that gregarious song is an intrinsically motivated and intrinsically rewarded behavior, similar to juvenile play observed in many young animals [1]. The performance of gregarious song has been shown to be linked to a positive affective state through the establishment of a conditioned place preference, a common way to measure the reward

value of a stimulus [6-8]. This explanation aligns with the proposal that gregarious song increases social cohesion in a flock [9]: performing gregarious song could lead to a place preference within the flock, thus providing an immediate, intrinsically generated reward response for staying in the group. This is then adaptive for the birds, as staying in a large group leads to increased awareness of predators and improved foraging efficiency [10-13]. Although the behavioral mechanisms are somewhat understood, more targeted research is required to understand the neurobiological pathways that underlie gregarious song, which is largely overshadowed by studies on sexually-motivated song in the breeding season.

The nucleus accumbens (NAC) is a highly conserved region implicated in many types of reward. It is part of the mesolimbic pathway and regulates reward associated with feeding, sexual behavior, and drug addiction [14-16]. The NAC also serves an important role in the rough-and-tumble play behavior of juvenile rats, which like gregarious song, is coupled with a positive affective state [17-19]. This suggests it may also play a role in gregarious song. In songbirds, correlational data implicate the NAC in prosocial behaviors such as allopreening (one bird preening another) and synchronized flight [20]. In starlings specifically, the stimulation of mu opioid receptors— known widely for their role in encoding the hedonic value of rewarding stimuli—within the rostral portions of the NAC have been demonstrated to increase the production of gregarious song [3].

One challenge to studying the role of the NAC in songbirds is that there is some disagreement within the field of avian neurobiology as to its exact location [21]. Additionally, a lack of detailed information of the songbird NAC [22-24] makes it difficult to study, as the NAC is well-known for its heterogeneous nature in mammals, with functionally distinct subregions (i.e. core, shell, and rostral pole) [25]. Therefore, it is

necessary to characterize the starling NAC in more detail to understanding its role in gregarious song.

In addition to the NAC, previous literature points to the medial preoptic area (mPOA) as being important for song in many contexts. The mPOA is a structure that is functionally conserved among vertebrates and is part of the neural social behavior network [26, 27]. In starlings, lesions of the mPOA tend to increase singing in flocks [28], and the positive affective state associated with gregarious song correlates positively with the expression of opioid-related genes in the mPOA [6]. Downregulation of mu opioid receptors in the mPOA reduces singing and also breaks the link between positive affect and singing behavior [29]. As reviewed above, multiple studies show that opioids are implicated in the production of gregarious song in the mPOA, and more recently, the NAC has been implicated as well [30]. The mPOA accesses the canonical mesolimbic reward pathway through projections to the ventral tegmental area, which in turn sends projections to the NAC [31]. Additionally, the mPOA receives afferents directly from the NAC [32, 33]. This suggests that the mPOA and NAC may work together to influence the production of gregarious song. However, our understanding of each region's relative contribution to both the production of and the reward associated with this behavior remains incomplete.

It is apparent from the above literature review that most research in understanding gregarious song has concentrated on opioid receptors in the mPOA. Systems such as the dopaminergic [9, 34], noradrenergic [35], and endocannabinoid systems [36] in the mPOA have received comparatively minimal attention. Given the complexity of the behavior, it is likely that many unexplored systems within the mPOA work to control the production of gregarious song. As such, it is necessary to take advantage of advances in high throughput

methodologies, such as RNA-sequencing, to uncover possible novel modulators of this behavior. A broad-scale, molecular systems approach also allows for transcriptome wide comparison across different taxa. Previous RNA-seq research has identified molecular "toolkits" that can modulate functionally similar behaviors across distantly related species [37, 38]. Given the likelihood that mechanisms that regulate gregarious song in the mPOA are conserved to regulate non-sexual, affiliative behaviors across other vertebrates, comparative brain transcriptomics will allow for the testing of cross-species hypotheses.

This dissertation examines the hypothesis that the NAC and mPOA are part of a conserved, interconnected circuit that regulates motivation and reward relevant to the production of gregarious, non-sexual song and other affiliative social behaviors. In **Chapter 1,** I adopted a comparative approach to substantiate the location of the songbird NAC by characterizing its subregions in starlings relative to mammals. I also began to investigate each subregion's association with gregarious song through immediate early gene activation. In **Chapter 2,** I pharmacologically manipulated mu opioid receptors within the starling NAC and mPOA to understand each of their opioid-specific roles in the production of gregarious song. In **Chapter 3**, I performed RNA-sequencing on the mPOA of the starling to uncover candidate genes that may play an important role in the regulation of gregarious song. Lastly, in **Chapter 4**, I resume the comparative approach by comparing the transcriptomic profile of the mPOA of gregarious singers to the profile of juvenile playing rats to identify possible analogous drivers of gregarious behaviors across vertebrates.

Chapter 1

Immunolabeling Provides Evidence for Subregions in the Songbird Nucleus Accumbens and Suggests a Context-Dependent Role in Song in Male European Starlings (*Sturnus vulgaris*)

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Abstract

Birdsong is well known for its role in mate attraction during the breeding season. However, many birds, including European starlings (*Sturnus vulgaris*), also sing outside the breeding season as part of large flocks. Song in a breeding context can be extrinsically rewarded by mate attraction; however, song in non-breeding flocks, referred to here as gregarious song, results in no obvious extrinsic reward and is proposed to be intrinsically rewarded. The nucleus accumbens (NAC) is a brain region well-known to mediate reward and motivation, which suggests it is an ideal candidate to regulate reward associated with gregarious song. The goal of this study was to provide new histochemical information on the songbird NAC and its subregions (rostral pole, core, and shell), and to begin to determine subregion-specific contributions to gregarious song in male starlings. We examined immunolabeling for tyrosine hydroxylase (TH), neurotensin, and enkephalin (ENK) in NAC. We then examined the extent to which gregarious and sexually-motivated song differentially correlated with immunolabeling for the immediate early genes FOS and ZENK in each subdivision of NAC. We found that TH and ENK labeling within subregions of the starling

NAC was generally similar to patterns seen in the core and shell of NAC in mammals and birds. Additionally, we found that gregarious song, but not sexually-motivated song, positively correlated with FOS in all NAC subregions. Our observations provide further evidence for distinct subregions within the songbird NAC and suggest the NAC may play an important role in regulating gregarious song in songbirds.

Introduction

Male birdsong is a well-studied behavior that is best known for its role in mate attraction and territory maintenance [39]. However, many gregarious species, such as European Starlings (*Sturnus vulgaris*), also sing at high rates outside the context of breeding. Song in a non-breeding context is common in flocks and is considered essential for song learning and maintenance [1] It is also thought to play an important role in the maintenance of social cohesion within a flock [40]. Although much is known about the neural regulation of song learning and the production of courtship song, relatively little is known about the neural mechanisms that facilitate and maintain song in gregarious contexts.

Unlike courtship or territorial song, gregarious song in flocks does not result in any immediate responses by conspecifics. Starlings do not orient themselves towards other birds, and their songs do not result in immediate mate attraction or the departure of a rival [5]. In the absence of an obvious extrinsic reinforcer, it has been proposed that gregarious song may be facilitated by an intrinsic reward state and that the act of producing song may itself be rewarding [1, 29, 41]. Although singing in this context may result in as yet unidentified extrinsic rewards, multiple studies show that songbirds develop strong

preferences for places in the environment that are associated with gregarious singing behavior, indicating that the act of producing gregarious song is tightly coupled to a positive affective state [6, 36, 42].

The nucleus accumbens (NAC) is a key brain region that is well-known in mammals to mediate reward associated with social behavior, sexual behavior, feeding, and the use of drugs of abuse [14-16, 43]. The NAC in mammals has three distinct subregions: the rostral pole (ACR), the accumbens shell (ACS), and accumbens core (ACC) [25]. The ACS and ACC have been well studied in mammals. The ACS underlies the reinforcing properties of rewarding stimuli such as food and drugs [14, 43-45] and social reward related to pair bonding [46, 47], while the ACC plays a significant role in approach to motivational stimuli and learning [25, 48]. The ACR appears to consist of both shell- and core-like projections with the lateral ACR sharing efferent projections with the core and the medial part consisting of shell-like projections [49]. Although core and shell receive more attention, ACR has also been shown to be sensitive to drug-induced reward [50].

The role of NAC in reward and motivation suggest it is an ideal candidate to regulate reward associated with gregarious singing behavior. In birds such as domestic chicks and pigeons, NAC subregions have been proposed based on hodology, the receipt of projections from the ventral tegmental area [51-53]. and the distribution of various neuromodulators [21, 51, 54, 55]. It appears in songbirds that the subregions of NAC, identified based on matching neuroanatomical landmarks in chicks, may be functionally distinct, but evidence varies across studies. For example, in zebra finches increased levels of dopamine within the ACR are found in newly paired birds [56] and dopamine transmission in the ACR was shown to increase in response to playback of male song [57]. In contrast, other studies

have found no relationship between dopamine levels or immediate early gene protein expression in ACR and playback of male song [58, 59]. Another study in which the precise location of NAC was not indicated also implicated NAC in responses to male song [60]. In starlings, studies suggest a role for the caudal NAC (both ACS and ACC combined) in female nesting behaviors [61] and a study in zebra finches differentially implicated each subregion in distinct affiliative interactions between pair-bonded males and females; the ACR was associated with synchronized flight and the ACC and ACS were associated with allopreening [20]. Together these past studies suggest a role for NAC in social behaviors and raise the possibility of functionally distinct subdivisions; however, few behavioral or other studies in songbirds consider subdivisions of NAC [22-24].

We had two goals for the following studies. The first was to provide new information on NAC and its subregions by studying the distribution of immunolabeling for markers primarily associated with dopamine and endogenous opioids, because of their known roles in motivation and reward within this region [18, 62]. The second goal was to begin to determine the extent to which the individual subregions of the NAC may contribute to gregarious song by correlating singing behavior with immunolabeling for the immediate early genes (IEG) FOS and ZENK. Relationships between ZENK and FOS expression in brain regions and behavior only partially overlap [63-65]. For example, ZENK is expressed in the preoptic area of rats during juvenile play, but FOS is not [66]. Therefore, the use of these two indirect indicators of neural activity could potentially provide more information on brain regions regulating song than either IEG could by itself. We also compared the degree to which relationships between song and IEG labeling patterns were

specific to gregarious song by comparing them to IEG labeling associated with sexually-motivated song in a reproductive context.

Materials and Methods

All studies were run under animal care and use protocols approved by the University of Wisconsin Animal Care and Use Committee and in accordance with guidelines of the National Institutes of Health.

Study 1- Immunolabeling for dopamine and opioid-related proteins in the NAC

We examined 40um thick coronal sections of male starling brains that contained NAC and had been immunolabeled for the dopamine-related proteins neurotensin (NT) and tyrosine hydroxylase (TH) and the opioid-related protein enkephalin (ENK) as part of past research (Table 1). Primary and secondary antibodies used for immunolabeling and references to published papers with detailed labeling protocols can be found in Table 1. All antibodies were validated using Western immunoblots and by leaving out the primary antibodies. We examined labeling in the ACR, ACC, and ACS. To provide readers with rostro-caudal references for the location of each subregion we provide below the y-coordinate (in μm) of the most closely matching area in the 3D starling atlas [67] relative to MRIcron's (v12.0.20201102) default origin voxel (Fig. 1). The ACR is the region previously referred to as the rostral ventromedial part of the lobus parolfactorius that is located in the ventral part of the striatum around the lateral ventricles at the level of the olfactory tubercle [21, 24, 68]. In our sections this area appeared when Area X became circular as we moved caudally. In the 3D starling atlas [67], ACR is located rostro-caudally at approximately 63 μm on the y-axis from the origin voxel. The locations for ACC and ACS

were identified using landmarks from studies on domestic chicks [51] that show these areas to be located just lateral to the bed nucleus of the stria terminalis located along the ventral tips of the lateral ventricles at the level of the tractus septomesencephalicus as it forms a slightly inward curving pyramid in coronal sections, with the ACC located just dorsal to the ACS. In the 3D starling atlas, the ACC and ACS are located rostro-caudally at approximately 46 μm on the y-axis from the origin voxel.

For each label, we selected sections from 7 birds that were all in a spring-like condition as part of prior studies (Table 1) and measured the optical density (OD) and percent area covered (PAC) by TH and ENK labeling within each subregion to have a quantitative comparison. Measurements were not taken from NT due to tissue folds and damage in the targeted areas, so labeling is described qualitatively. Images of brain sections were acquired using a digital camera (Lecia DFC310 FX) connecting a microscope to a PC computer. All images were taken using MetaMorph software (Molecular Devices, LLC.). The exposure times for each stain (TH = 20.66 ms, NT = 19.84 ms, ENK = 20.31 ms) were determined by averaging together the results of the auto-expose function on MetaMorph from seven random regions of a brain section of each stain. Shading correction and background subtraction were performed in MetaMorph for all stains. OD and PAC were measured using the Fiji Distribution of ImageJ [69]. The selection of ACR was within an area of 0.43 × 0.56 mm, ACC within 0.62 × .19 mm, and ACS within 0.62 × 0.38 mm (Fig. 1). Measurements were taken from both hemispheres and were averaged together for statistical analyses.

Study 2- Immunolabeling for immediate early genes in NAC and gregarious song

In this study we counted the number of IEG labeled cells in ACR, ACC, and ACS in tissue from previously published studies on male starlings that were singing either gregarious or sexually-motivated song [63-65]. In brief, the tissue used in this study was collected from male starlings that were trapped near Madison, WI and behaviorally tested in flocks in outdoor aviaries. Details related to trapping, housing, and starling care are provided in three published studies [63-65]. We present only details relevant to the present study here.

Photoperiod manipulations were used to place males into 1) non-breeding season (fall/winter-like) conditions under which they naturally form large flocks and spontaneously sing high rates of gregarious song and 2) breeding-season (spring-like) conditions under which males naturally acquire nest sites and sing high rates of sexually-motivated song to attract females. Males in the spring-like condition also received testosterone implants which help to facilitate sexually-motivated song in aviary housed males (further details are found in [64]). Males in the gregarious condition (n=18) were split between two flocks housed in two outdoor aviaries. Each flock was observed for 50 minutes a day, three days in a row. During each period, spontaneous song production was recorded using a point sampling method, in which every minute it was marked whether an individual male was singing or not. Males in the sexually-motivated condition (n=24) were also split between two flocks housed in two outdoor aviaries. Sexually-motivated song is triggered by the presence of a female, so to stimulate song for birds in this condition a breeding-season condition female was released into each aviary and each flock was observed for 50 minutes a day, three days in a row. During each period, song production after female introduction was recorded as described for gregarious song. The sum of the 3

observation days was used in analysis. We note here that when only the last test day was considered, as is typical for studies of immediate early gene expression, there were no significant correlations and only slight positive trends in the ACR and ACC. We interpret this finding in the discussion below.

Ten minutes after the final behavioral observations, all males in a social group were sacrificed via rapid decapitation and brains were removed and fixed in a 5% acrolein solution. Primary and secondary antibodies used for immunolabeling and references to published papers with detailed labeling protocols can also be found in Table 1. All sections were mounted onto gel-coated slides, dehydrated, and cover slipped. The tissue used in this study had been labeled for the IEGs FOS and ZENK as part of the past published papers, but NAC had not been examined. We selected only birds with sections that had high quality label in NAC, resulting in 14 birds for FOS labeling and 18 birds for ZENK labeling.

Quantification

A researcher blind to the reproductive condition and behavior of each bird performed counts of ZENK labeled cells and FOS labeled cells within the ACR, ACC, and ACS. Imaging hardware and software was the same as listed previously. The exposure times for each stain (ZENK=5.43 ms, FOS=4.79 ms) were calculated using the same method listed above, as was shading correction and background correction.

All cell counts were performed in the Fiji Distribution of ImageJ [Schindelin et al., 2012]. The numbers of FOS labeled cells and ZENK labeled cells were counted in the NAC subregions with the same area measurements listed previously. Due to the dark staining for ZENK, background was also subtracted on ImageJ with a rolling ball radius of 10. If dust or artefact was present, the threshold for cell counts was manually adjusted by an

experimenter blind to the bird's condition of behavior to maintain accuracy. The numbers of FOS labeled and ZENK labeled cells were counted bilaterally on three consecutive sections if tissue was intact. If tissue was damaged or sections were lost, a fourth section was counted. If the fourth section was also damaged or missing, counts were taken from the two intact sections. Values from these counts were averaged and the mean value for each region was used for analysis.

Statistical Analysis

Data were analyzed with R (Version 4.0.5) in RStudio (Version 1.4.1106) [70]. Separate repeated measures ANOVAs were run to compare OD and PAC across ACR, ACC and ACS for TH and ENK. When significant, these were followed by Holm-Bonferroni posthoc comparisons. For immediate early gene analysis, a simple linear regression analysis was run for each NAC region and seasonal condition, with maximally stimulated song (either gregarious or sexually-motivated) as the independent variable, and IEG count (either FOS or ZENK) as the dependent variable. Model assumptions were tested with residual plots, and assumptions of parametric statistics were met. Subjects were deemed model outliers if they were ± 2 standard deviations about the mean. However, the removal of outliers did not change whether the results were significant or non-significant, so all subjects were included in the analysis below.

Results

Study 1- Characterizing the avian nucleus accumbens using immunolabeling

Tyrosine Hydroxylase (TH)

TH label was present in the ACR (Fig. 2), with a large, dense, patch adjacent to the midline that was slightly ventral to the most ventral tip of the TSM. The densest label formed an oblong, oval that stood out from the rest of the ventral striatum. TH label also filled the entire ACC and ACS with label in ACC appearing less dense than the ACS. Results of an ANOVA revealed significant differences in PAC of TH across the regions ($F(2, 12) = 4.67$, $p = 0.032$; $n = 7$) with the ACS PAC significantly greater than ACR ($p = 0.033$, 95% CI[1.71, 8.80]) and nearly significantly greater than ACC ($p = 0.055$, 95% CI[1.27, 15.34]) (Fig. 3). There were no significant differences in OD of TH between any of the subregions ($F(2, 12) = 1.52$, $p = 0.26$; $n = 7$).

Neurotensin (NT)

NT-immunoreactive fibers were present in ACR (Fig. 4). There was a dense grouping of fibers in the same location as the densest TH label described above. NT labeling was also dense in ACS and ACC. The label gradually became less dense more lateral to the midline. Labeling appeared to be fairly uniform across ACS and ACC, but differences were difficult to differentiate because tissue folds were common in caudal sections of NAC (which was not the target region for the initial study).

Enkephalin (ENK)

For ENK the densest fiber cluster was located within a similar region to that described for TH (Fig. 5). ENK labeled fibers in ACR were noticeably denser than the rest of the ventral striatum. Dense labeling was found both in the ACC and ACS with no particularly noticeable distinction between the ACC and ACS subregions, which was confirmed quantitatively by the finding that there were no significant differences in either OD or PAC of ENK between any of the subregions for OD ($F(2, 12) = 3.33$, $p = 0.071$; $n = 7$)

or PAC ($F(2, 12) = 0.44$, $p = 0.65$; $n = 7$) (Fig. 6).The label gradually became less dense in fibers more lateral from the midline.

Study 2- Immediate early genes and song

Singing behavior and FOS immunolabeling

FOS immunolabeled cells appeared to be uniformly spread throughout each subregion, with no consistent clustering within a given area. There was a visibly higher number of FOS immunolabeled cells within all subregions of the NAC in high singing starlings than in low singing starlings (Fig. 7). We performed simple linear regressions, in which FOS cell counts within the ACR, ACC, and ACS were regressed on total song over three observation days in fall-like birds singing gregarious song and spring-like birds singing sexually-motivated song. There were significant, positive correlations between gregarious song and the number of FOS immunolabeled cells in ACR ($R^2 = 0.50$, $p = 0.022$; $n = 10$), ACC ($R^2 = 0.42$, $p = 0.041$; $n = 10$), and ACS ($R^2 = 0.46$, $p = 0.046$; $n=9$) (Fig. 7 & 8). There were no significant relationships between sexually-motivated song and label in any of the NAC subregions (R^2 and p-values shown in Fig. 8).

Singing behavior and ZENK immunolabeling

There were no noticeable, visible differences in the number of ZENK immunolabeled cells within all subregions of the NAC between high and low singing starlings. Again, we performed simple linear regressions, where ZENK cell counts within the ACR, ACC, and ACS were regressed on total song over the three observation days in fall-like birds singing gregarious song and spring-like birds singing sexually-motivated song. There was one significant, positive correlation between gregarious song and the number of ZENK immunolabeled cells in ACR ($R^2 = 0.23$, $p = 0.04998$; $n = 17$; Fig. 9). There were no other

significant relationships between ZENK labeled cells and sexually-motivated song or gregarious song within the NAC (R^2 and p-values shown in Fig. 9).

Discussion

The results of this study show that immunolabeling for dopamine- and opioid-related markers in starling NAC is generally similar to that reported in mammals and birds. We also saw that immediate early gene activity in all three NAC subregions correlated positively with gregarious but not sexually-motivated song. These findings provide further evidence for similar NAC subdivisions in birds and mammals and a potential role for the songbird NAC in gregarious singing behavior that is tightly coupled to a positive affective state.

Patterns of immunolabeling in the starling NAC are similar to those in other species

Few studies have examined NAC core-shell differences in birds, but in rodents distinct patterns have been reported in the distribution of dopamine- and opioid-related proteins with TH and NT tending to be denser in ACS than ACC [71-80] and ENK immunolabeling tending to be patchy and either denser in core or relatively uniform across the core and shell [81-84]. Consistent with what is reported in rodents, we found the area covered by TH labeling in starlings tended to be greater in ACS than ACC, although this differs somewhat from TH immunolabeling in pigeons which appears to be uniformly dense across both the ACC and ACS [85], although this was not quantified in the paper. ENK labeling in starlings also appeared to be uniform across ACS and ACC, which matches reports in rodents and developing chickens [86]. This is also supported by our finding that there were no differences in optical density or the area covered by labeled fibers across

subregions. In contrast to what is observed in rodents, in starlings NT labeling appeared to be uniform across the ACS and ACC; however, due to tissue folds in caudal sections of NAC it was difficult to conclude the degree to which similar labeling differences between the ACS and ACC are present in starlings.

Few studies in rodents have focused on immunolabeling in the rostral pole of NAC. In birds, dense TH immunolabeling has been observed in ACR in quail, zebra finches, and pigeons within a region that appears similar to the distinct oblong, rostral region that we observed in starlings [20, 85, 87]. A similar region in starlings was also densely innervated with NT and ENK fibers and distinct from surrounding striatum. NT immunolabel has also been identified within the ACR of chicks [88], and similarly dense met-ENK labeling is observed in this same region in budgerigars and chickens [86, 89]. Although further characterization of NT is needed, together results suggest neurochemical homology for subdivisions of the avian and rodent NAC. See Table 2 for summary.

IEG cell counts in the NAC correlate positively with gregarious but not sexually-motivated song

IEG protein products are typically measured within two hours after the production of behavior [66, 90, 91]. However, in this study positive correlations were found between IEG measurements and the sum of song measurements across all three test days but not song produced on the day of brain collection. One interpretation is that IEG expression in this study reflects an individual bird's general propensity or motivation to sing which may be better reflected in song sampled across several test periods. This would suggest that the correlations identified here reflect a close association between the motivation to sing and activity in NAC whether the bird sang during the observation period.

This measure of an individual's propensity to produce gregarious song was positively correlated with numbers of FOS labeled cells within all subregions of the NAC, and with ZENK cell counts in ACR. In contrast, there were no significant correlations between FOS or ZENK counts and the propensity to produce sexually-motivated song. As reviewed in the introduction, a growing number of studies suggest that in contrast to sexually-motivated song, which can be rewarded by mate attraction and copulation, gregarious song is associated with an intrinsic reward state [6, 36, 42]. Gregarious song has been proposed to be a form of play behavior that allows birds to develop important social skills for use in more serious reproductive contexts [1]. The NAC, and specifically stimulation of mu opioid receptors in NAC, is centrally involved in reward induced by social play in rodents [17-19], and NAC is considered vital for positive, reward-seeking behavior [92]. Recently, we found that pharmacological stimulation of mu opioid receptors in ACR in starlings stimulated gregarious song [3]. This demonstrates that the correlations reported here may reflect causal involvement of this region in gregarious song and supports the hypothesis that the NAC plays a role in social reward that is conserved across vertebrates. Studies are now needed to causally test roles for ACC and ACS in singing behavior.

We investigated two different IEGs to gain more complete insight into potential relationships between song and activity in NAC. As mentioned previously, the response of these IEGs can differ in the same brain regions in response to the same stimuli [66, 93]. Additionally, in immediate early gene studies, the absence of expression does not indicate the absence of neuronal activity, and not every immediate early gene is expressed in every brain area [64]. Thus, the lack of correlations between FOS or ZENK labeled cells and sexually-motivated song does not preclude a role for NAC in song in this context. Unlike

gregarious song, this type of song is not tightly coupled to a reward state [29, 42]. but reflects a highly motivated state of reward seeking (i.e., males sing to attract females which can be rewarded by copulation). NAC is known to be involved in sexually-motivated behaviors in mammals [15, 94] and motivated responses to other rewards [14, 43-45]. Thus, it is possible that NAC would be involved in song in this context; and we interpret our results to indicate that the role of NAC in gregarious and sexually-motivated song differs. A next step will be to explore distinct roles for the NAC in song in these two contexts.

It is also important in future research to explore functionally distinct roles for the ACR, ACS, and ACC in gregarious song. As reviewed in the introduction, in rodents the ACS is well-known for its role in hedonic reward [14, 43-45][Mcbride et al., 1999; Alderson et al., 2001; Pecina, 2005; Van Der Plasse et al., 2012] so it is possible that it is primarily responsible for the positive affective state that is associated with gregarious song in songbirds [6, 36, 42]. In contrast, the ACC in rodents is not closely associated with hedonic pleasure but implicated in motivated responses to rewards and learning [25, 48] so it is possible that its role in song relates primarily to motivation. The ACR has been implicated in drug-induced reward in rodents [50]; however, there are few functional studies concentrated on the rostral pole in mammals. It is possible that ACR may contribute to functions served by both ACC and ACS based on studies in rodents that show that the lateral ACR shares efferent projections with the core and the medial ACR shares projections with shell [49]. Future studies are needed to explore the specific functional contributions of subregions of NAC to gregarious song.

Conclusion

The results of this study 1) provide additional support for neurochemically distinct rostral pole, core, and shell subdivisions of the NAC in birds that are neurochemically homologous to the mammalian NAC, and 2) implicate the NAC in a form of non-sexual, affiliative, rewarding social communication. Results are also consistent with the hypothesis that NAC is part of a central, conserved circuitry that underlies rewarding social behaviors across vertebrates. Given the role of intra-NAC dopamine in motivation and opioids in reward, studies are now needed to examine the roles of these modulators in ACR, ACC, and ACS in gregarious as well as sexually-motivated song. Finally, here we focused solely on immunolabeling for proteins in NAC related to dopamine and opioids, given our interest in motivation and reward; however, in future studies the use of calcium binding proteins [53] and tract-tracing studies are needed to further uncover potential subdivisions and homologies with mammals.

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Statement of Ethics

All subjects were tested and collected for prior studies. All study protocols were reviewed and approved by the University of Wisconsin- Madison Institutional Animal Care and Use

Committee, approval numbers L00350 (TH), L005163 (NT), L00379 (ENK), and L00344 (FOS and ZENK).

Conflict of Interest Statement

Authors report no conflict of interest.

Funding Sources

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Author Contributions

B.J.P was responsible for conceptualization, data curation, statistical analysis, and writing the original draft. S.A.H was responsible for data curation. L.V.R was responsible for conceptualization, funding acquisition, and writing review and editing.

Data Availability Statement

Data will be made freely available upon request.

Table 1. Antibody information for each immunolabel from prior publications using the same tissue

Immunolabel	Primary antibody	Secondary antibody	Reference
Tyrosine hydroxylase	Mouse anti-tyrosine hydroxylase at 1:10,000, Immunostar Inc., Hudson, WI, USA; CAT#22941	Goat anti-mouse at 1:500, Vector Laboratories, Burlingame, CA, USA	Heimovics and Riters, 2008
Neurotensin	Rabbit anti-neurotensin at 1:5,000, Immunostar Inc., Hudson, WI, USA; CAT#20072	Goat anti-rabbit at 1:1,000, Vector Laboratories, Burlingame, CA, USA	Merullo et al., 2015
Enkephalin	Rabbit anti-enkephalin at 1:2,500, Immunostar Inc., Hudson, WI, USA; CAT#20065	Goat anti-rabbit at 1:2,500, Vector Laboratories, Burlingame, CA, USA	Kelm-Nelson et al., 2013
FOS	Rabbit anti-FOS at 1:18,000, Santa Cruz Biotechnologies, Santa Cruz, CA, USA; sc253	Goat anti-rabbit at 1:250, Jackson ImmunoResearch Laboratories, West Grove, PA, USA	Heimovics and Riters, 2005, 2006
ZENK	Rabbit anti-ZENK at 1:5,000, Santa Cruz Biotechnologies, Santa Cruz, CA, USA; c19	Goat anti-rabbit at 1:500, Jackson ImmunoResearch Laboratories, West Grove, PA, USA	Heimovics and Riters, 2007

Table 2. Description of immunolabeling within each subdivision of the NAC in starlings and in prior studies in mammals.

Immunolabel	Starling NAC summary	Mammalian NAC summary
Tyrosine hydroxylase	Label present in ACR, ACS appeared denser than ACC	Label present in ACR, ACS appeared denser than ACC
Neurotensin	Label present in ACR, ACS appeared uniform with ACC although tissue folds made conclusions difficult	Label present in ACR, ACS appeared denser than ACC
Enkephalin	Label present in ACR, ACS appeared uniform with ACC	Label present in ACR, ACS appeared uniform with ACC

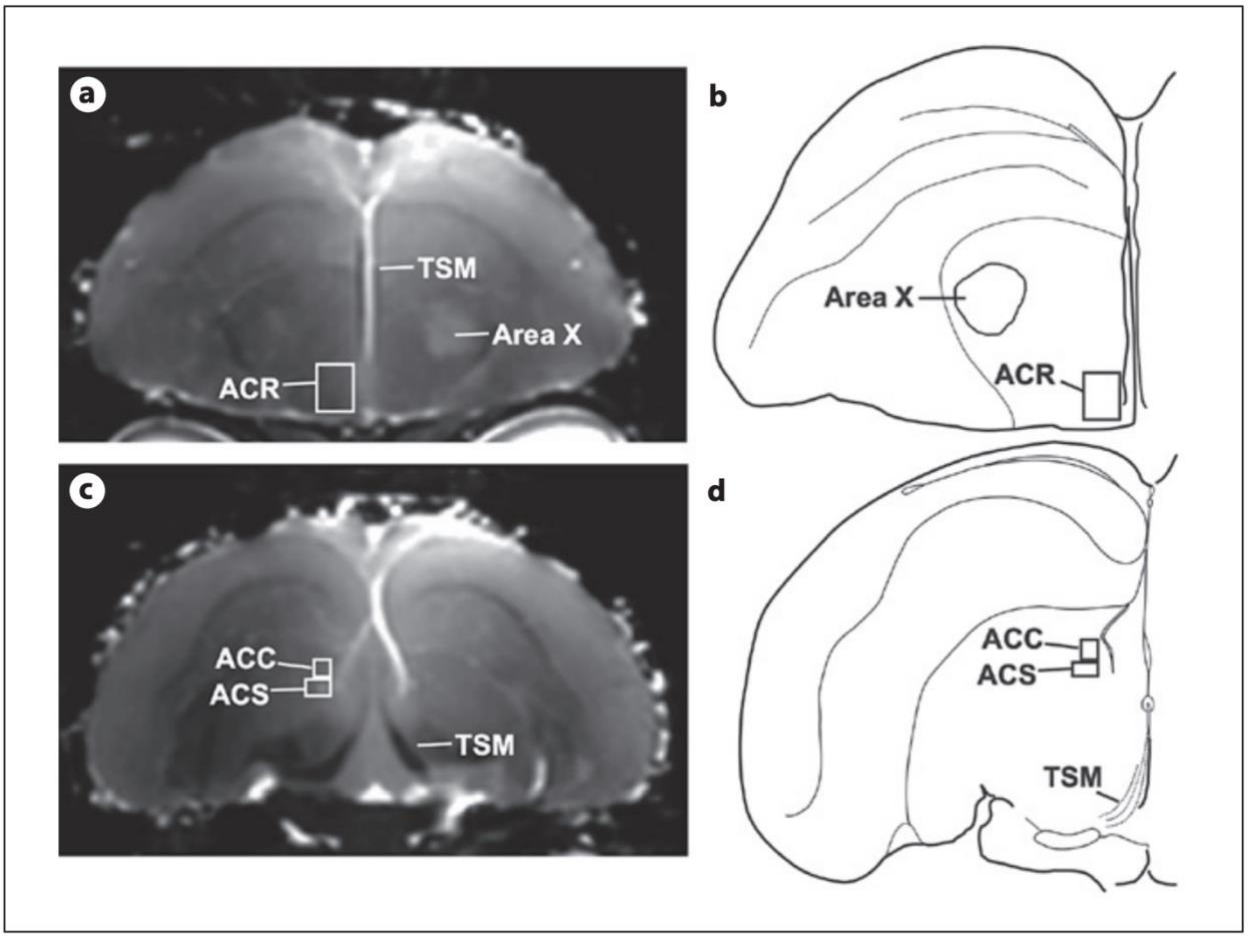

Figure 1. Images adapted from the 3D starling atlas (A and C) and illustrations (B and D) showing locations of the rostral pole of the nucleus accumbens (ACR) (panels A and B), and the accumbens core (ACC) and accumbens shell (ACS) (panels C and D) in the left hemisphere of coronal sections. TSM = tractus septomesencephalicus.

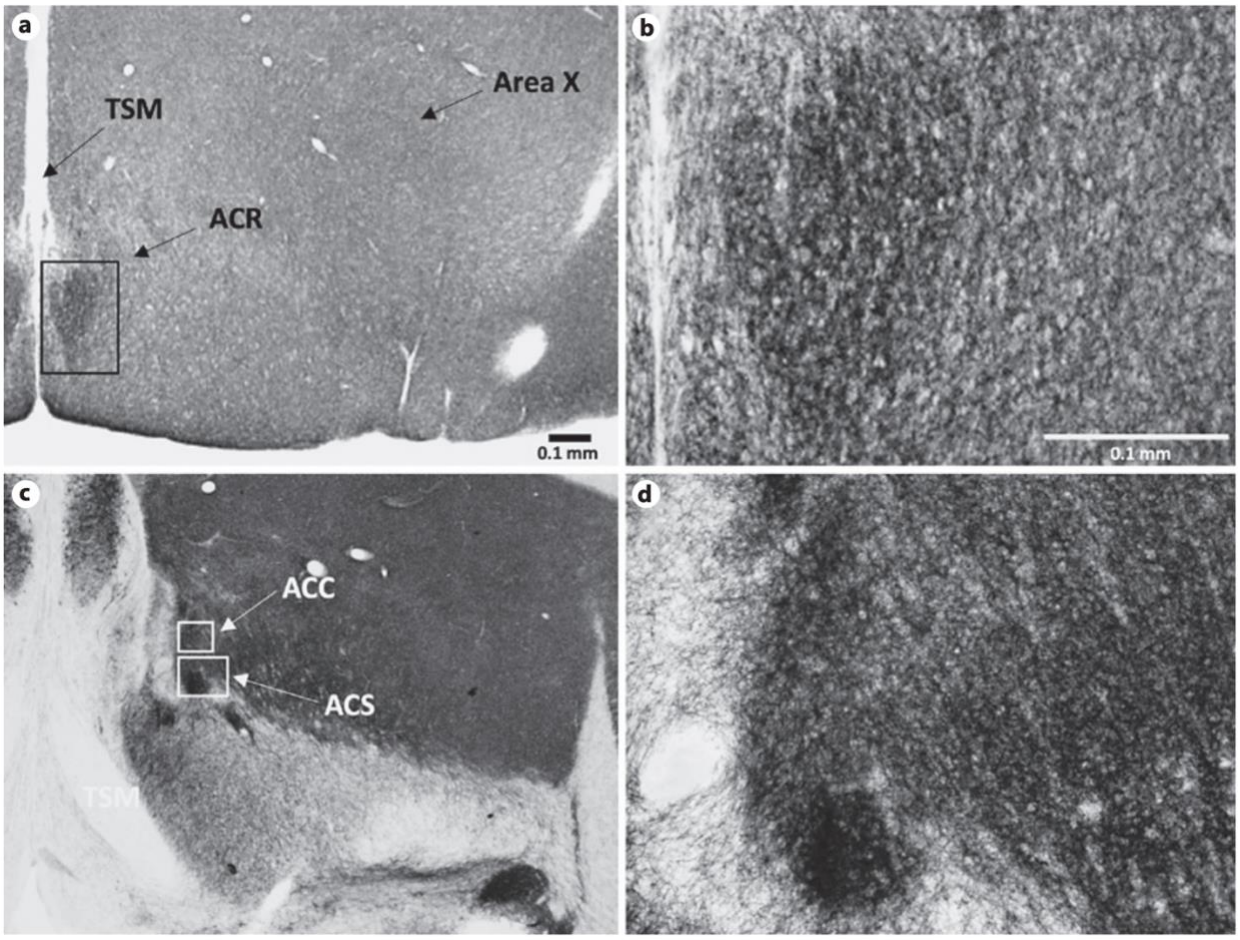

Figure 2. Photomicrographs of tyrosine hydroxylase (TH) labeling in the rostral pole of the nucleus accumbens (ACR), nucleus accumbens shell (ACS) and nucleus accumbens core (ACC). **A.** TH labeling in the ACR at 2x magnification. **B.** 10x magnification of the ACR. **C.** TH labeling in the ACC and ACS at 2x magnification. **D.** 10x magnification of the ACS and ACC. Arrows highlight the tractus septomesencephalicus (TSM) and location of Area X for orientation.

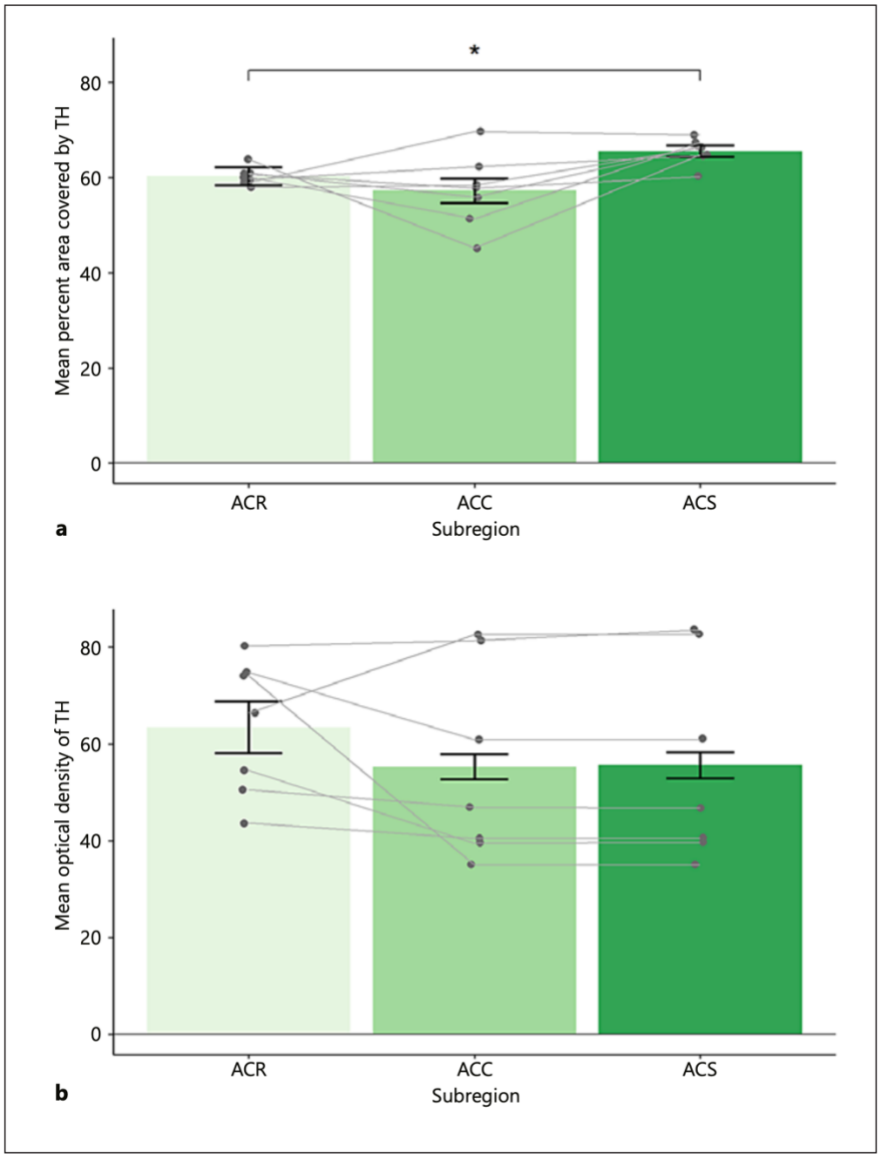

Figure 3. Percent Area Covered (A) and Optical Density (B) of tyrosine hydroxylase labeling in the rostral pole of the nucleus accumbens (ACR), nucleus accumbens shell (ACS) and nucleus accumbens core (ACC). Error bars represent one within-subject standard error about the mean. *P<0.05

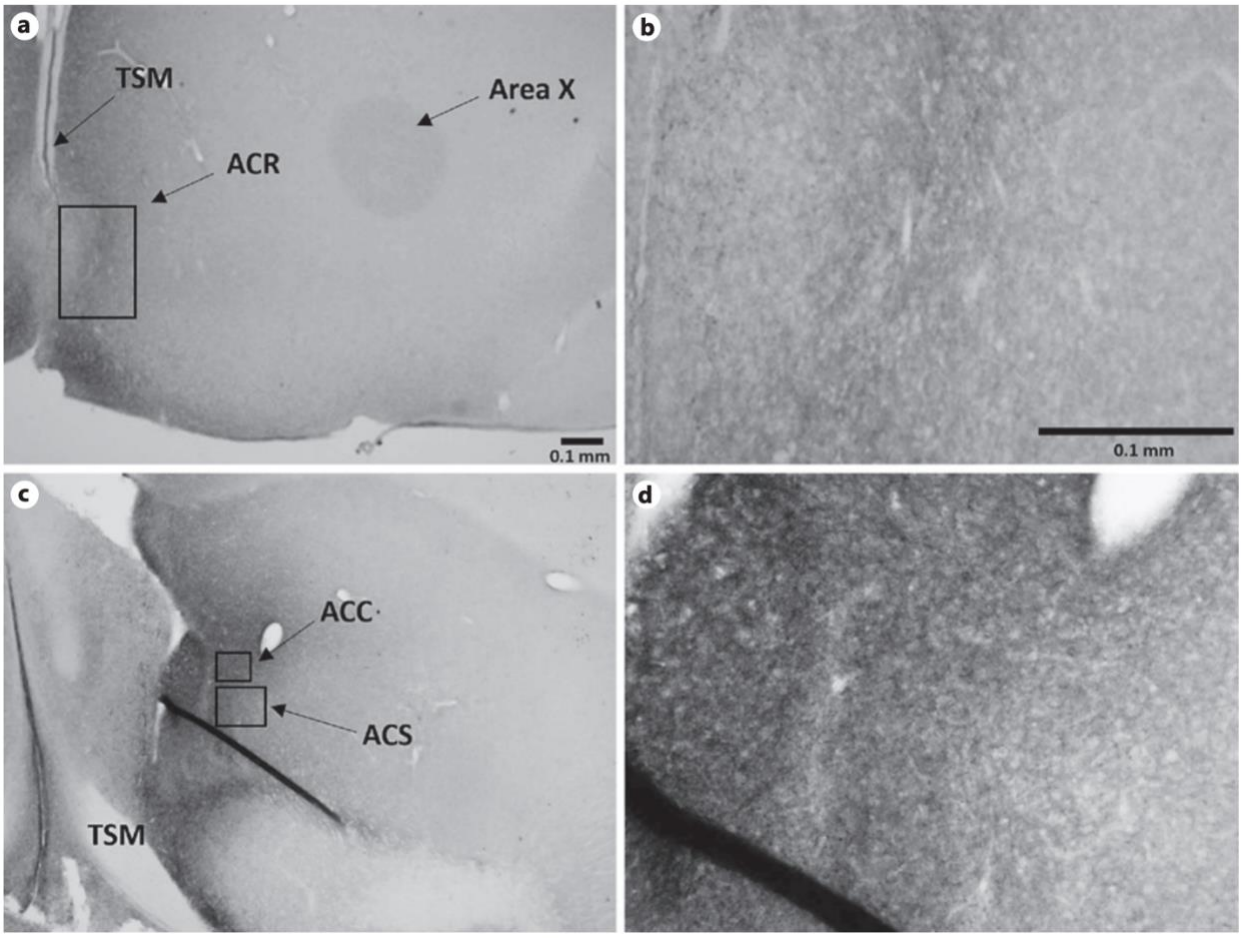

Figure 4. Photomicrographs of neurotensin (NT) in the NAC. See Figure 2 for additional details.

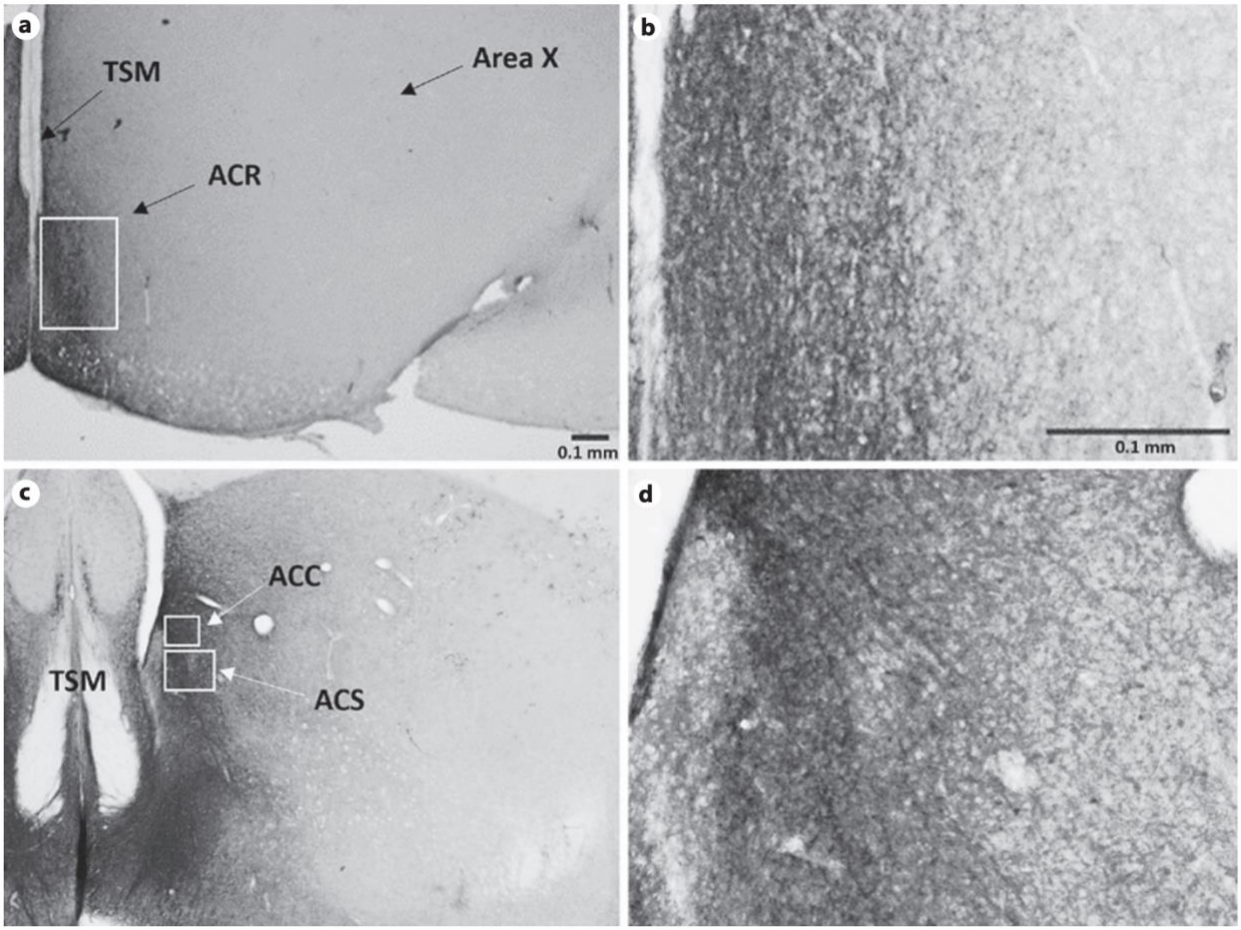

Figure 5. Photomicrographs of enkephalin (ENK) in the NAC. See Figure 2 for additional details.

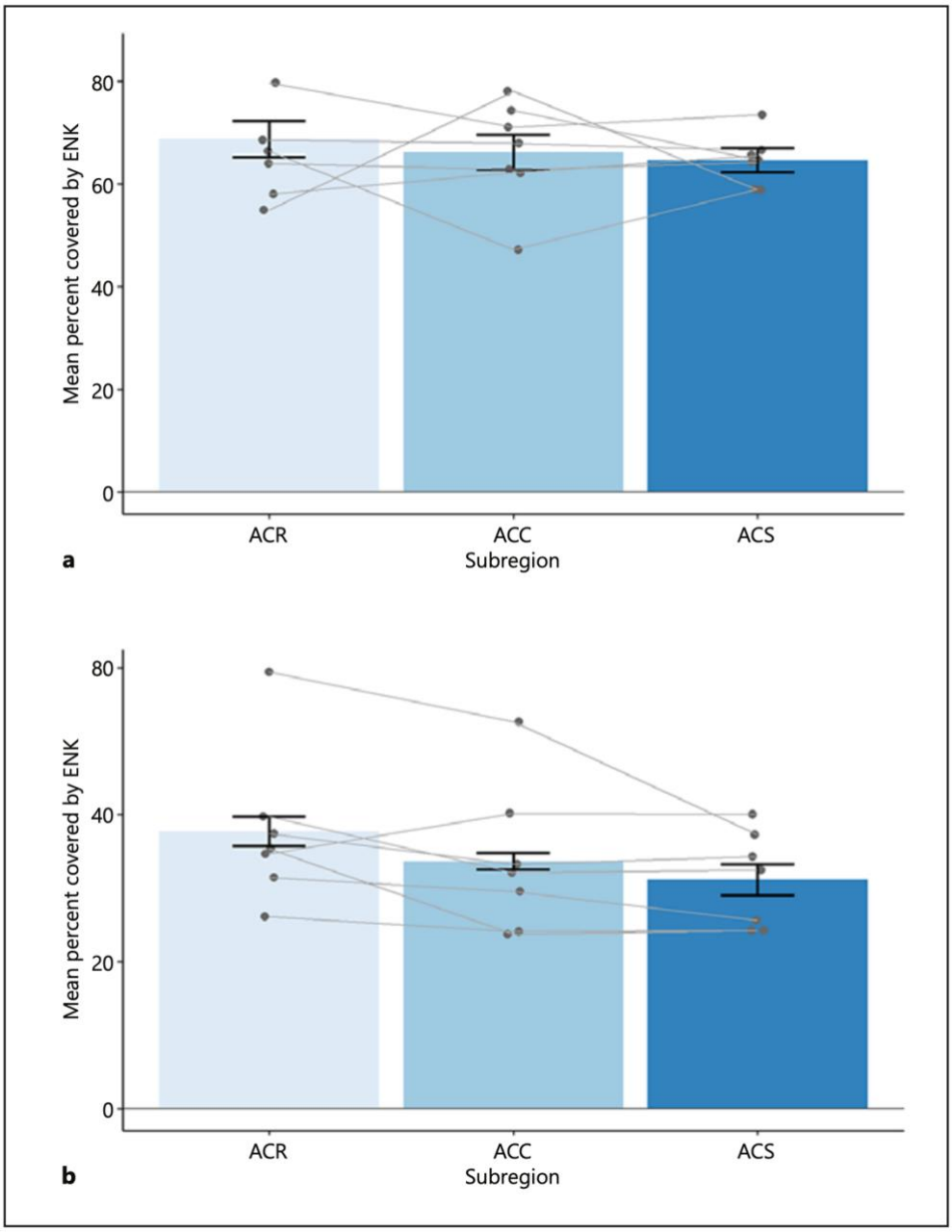

Figure 6. Percent Area Covered (A) and Optical Density (B) of enkephalin labeling in the rostral pole of the nucleus accumbens (ACR), nucleus accumbens shell (ACS) and nucleus accumbens core (ACC). Error bars represent one within-subject standard error about the mean. No significant differences were found between any of the subregions for either variable.

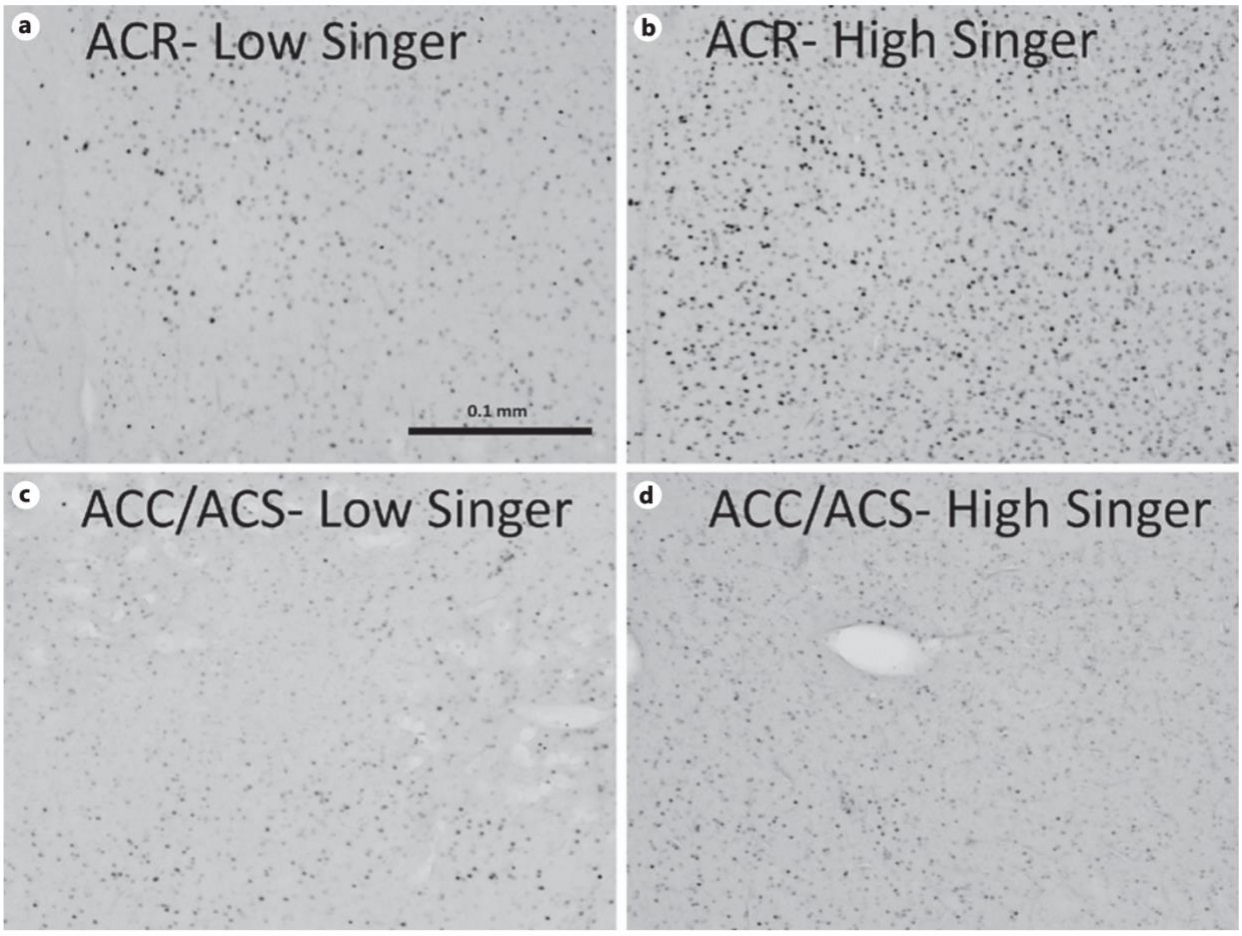

Figure 7. Photomicrographs showing the comparison of FOS in the NAC of low singing (song = 5) (left) and high singing (song = 32) (right) European starlings singing in a gregarious context. Images are centered within the same locations as shown in Figures 2, 4, & 5.

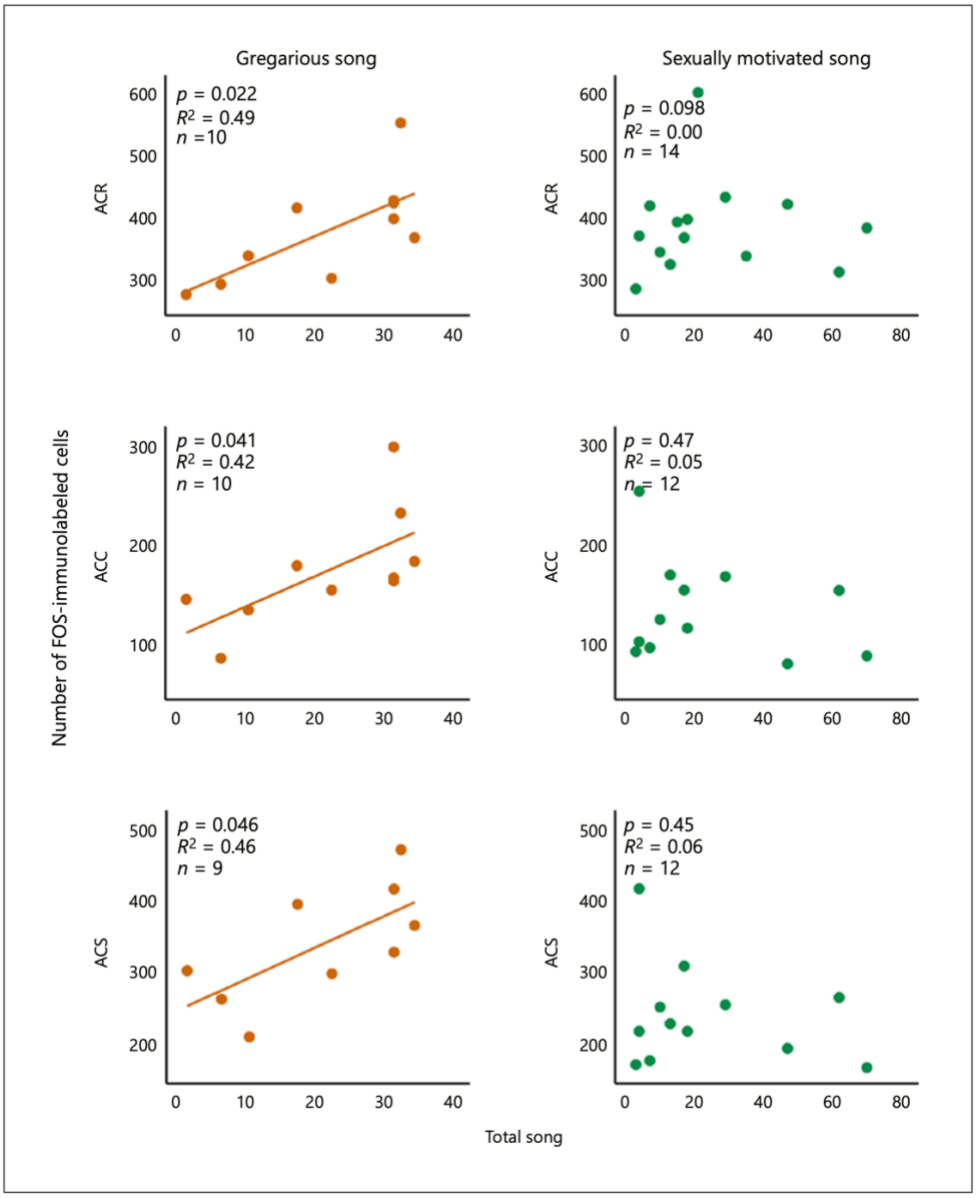

Figure 8. Numbers of FOS labeled cells within the rostral pole of the nucleus accumbens (ACR), accumbens core (ACC), and accumbens shell (ACS) correlate positively with gregarious song production. Data shown are the number of FOS-labeled cells within the ACR, ACC, and ACS correlated with total song (point sampling every minute for an hour) for each individual. Each point represents one starling. Individuals singing gregarious song are in the left column and individuals singing sexually-motivated song are in the right column. The inclusion of a regression line indicates a significant correlation ($p<0.05$).

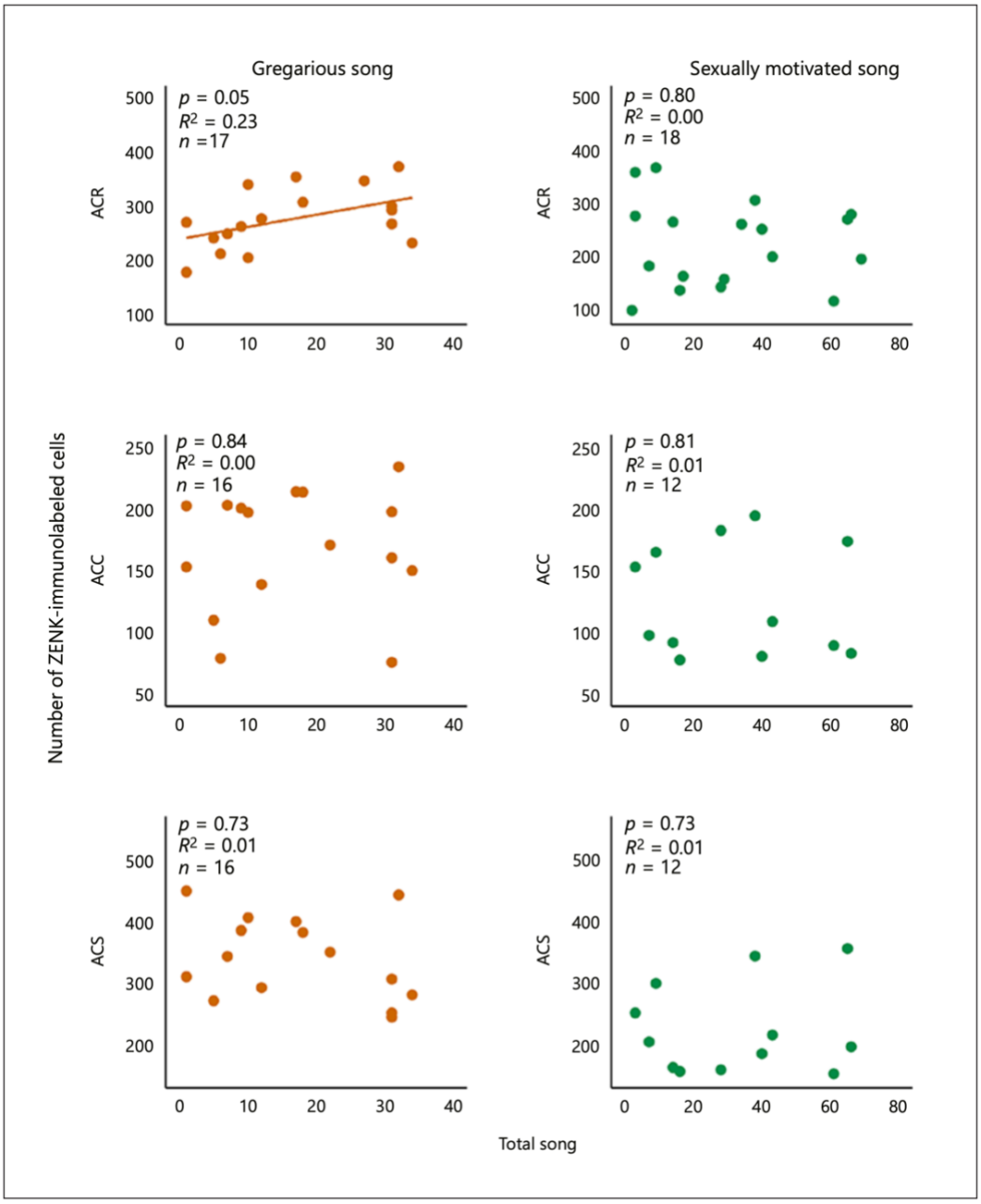

Fig. 9. Numbers of ZENK labeled cells within the rostral pole of the nucleus accumbens (ACR) correlates with gregarious song production. Data shown are the number of FOS-expressing cells within the ACR, ACC, and ACS correlated with total song (point sampling every minute for an hour) for each individual. Each point represents one starling. Individuals singing gregarious song are in the left column and individuals singing sexually-motivated song are in the right column. The inclusion of a regression line indicates a significant correlation ($p<0.05$).

Chapter 2

Mu opioid receptor stimulation in the medial preoptic area or nucleus accumbens facilitates song and reward in flock European starlings

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Abstract

It has been proposed that social cohesion in gregarious animals is reinforced both by a positive affective state induced by social interactions and by the prevention of a negative state that would be caused by social separation. Opioids that bind to mu opioid receptors (MORs) act in numerous brain regions to induce positive and to reduce negative affective states. Here we explored a potential role for MORs in affective states that may impact flocking behavior in mixed-sex flocks of nonbreeding European starlings, *Sturnus vulgaris.* Singing behavior, which is considered central to flock cohesion, and other social behaviors were quantified after infusions of the MOR agonist D-Ala2, N-Me-Phe4, glycinol5-ENK (DAMGO) into either the medial preoptic area (POM) or the nucleus accumbens (NAC), regions previously implicated in affective state and flock cohesion. We focused on beak wiping, a potential sign of stress or redirected aggression in this species, to provide insight into a presumed negative state. We also used conditioned place preference (CPP) tests to provide insight into the extent to which infusions of DAMGO into POM or NAC that

stimulated song might be rewarding. We found that MOR stimulation in either POM or NAC dose-dependently promoted singing behavior, reduced beak wiping, and induced a CPP. Subtle differences in responses to MOR stimulation between NAC and POM also suggest potential functional differences in the roles of these two regions. Finally, because the location of NAC has only recently been identified in songbirds, we additionally performed a tract tracing study that confirmed the presence of dopaminergic projections from the ventral tegmental area to NAC, suggesting homology with mammalian NAC. These findings support the possibility that MORs in POM and NAC play a dual role in reinforcing social cohesion in flocks by facilitating positive and reducing negative affective states.

Keywords: positive reinforcement, negative reinforcement, social behavior, affiliation songbirds, birdsong, affective state, communication

Contribution to the field

The formation of non-sexual, affiliative social groups provides safety and opportunities to develop important social skills. It has been proposed that group cohesion may be reinforced by both a positive affective state induced by social interactions and by the reduction of a negative state that would be caused by social isolation, yet little is known about the underlying neural mechanisms. Mu opioid receptors (MORs) in the medial preoptic area (POM) and nucleus accumbens (NAC) are implicated in the regulation of affective states and social behaviors. Here we provide evidence in affiliative flocks of European starlings that MOR stimulation in POM or NAC induces reward and facilitates singing behavior that is important for flock cohesion. These findings are consistent with the possibility that MORs in POM and NAC play a dual role in reinforcing flocking behavior by reducing negative and promoting positive affective states. Because the POM and NAC

appear to be conserved and similar across vertebrates, studies on social cohesion in songbirds have the potential to reveal core, evolutionarily conserved circuits that reinforce affiliative non-sexual social behaviors across vertebrates.

Introduction

Numerous studies on the neural control of social behavior focus on behaviors in reproductive contexts, including mating, bonding, and maternal behaviors. Although not as well studied, many animals also engage in important affiliative, non-sexual social behaviors outside a breeding context. This includes the remarkable flocking behaviors observed in many birds outside the breeding season [1, 95-99]. The formation of these flocks enhances safety and foraging efficiency [10, 100-102] and contributes to reproductive success through the development of social skills [1, 2, 103, 104]. It has been proposed that social cohesion in these flocks may be reinforced by both a positive (i.e., pleasurable) affective state induced by social interactions within the flock and by the removal of a negative (i.e., aversive) state that would be caused by social separation [98, 105]. Here we define a rewarding a stimulus as one that elicits an approach response either because it induces pleasure or because it reduces an aversive state [106].

Opioids that bind to mu receptors both induce positive and reduce negative affective states [107-110], suggesting a potential role for opioids in reward associated with flocking behavior. This idea is supported by a growing number of studies that implicate mu opioid receptors (MORs) in behaviors considered critical for flock cohesion. For instance, peripheral injections of the opioid receptor antagonist naloxone in male zebra finches, *Taeniopygia guttata*, reduce the production of undirected song, a type of song proposed to promote flock cohesion [111]. Likewise, in flocks of male and female European starlings,

Sturnus vulgaris, peripheral administration of the MOR agonist fentanyl both facilitates singing behavior and reduces beak wiping [29], which in some species, including starlings, is considered a potential sign of stress or redirected aggression under stressful conditions (i.e., "displacement" behavior performed because an alternative response is prevented, [112-116]). Multiple studies using conditioned place preference (CPP) tests indicate that production of singing behavior in flocks of starlings and zebra finches is tightly coupled to an intrinsically rewarded state [6, 29, 36, 42]. Studies in starlings also demonstrate that this form of gregarious song is associated with opioid-mediated analgesia (i.e., the reduction of a negative state) [41]. Together these studies suggest that opioids may act at MORs to promote flock cohesion by both inducing a positive affective state and reducing a negative affective state [105].

The medial preoptic area (commonly abbreviated POM in birds) is a critical site in which opioids induce both CPP and analgesia in rodents [117-120]. In starlings, correlational studies demonstrate positive associations between MOR mRNA in POM and song-associated reward, as measured using CPP tests [6]. More recently causal studies demonstrate that experimental siRNA downregulation of MORs in POM suppresses gregarious singing behavior and disrupts song-associated CPP [29]. These CPP test results suggest that the reward state that accompanies singing behavior in flocks may function to reinforce flocking behavior [105]. Although shy of statistical significance, there was also a clear trend for the siRNA downregulation of MORs to increase beak wiping [29]. These studies raise the possibility that MOR in POM may both enhance positive and reduce negative states to facilitate and reward song and flock cohesion.

The POM directly accesses the canonical mesolimbic reward pathway via direct projections to the ventral tegmental area (VTA), which then projects to the nucleus accumbens (NAC) [53, 121, 122]. The NAC is well known for its role in reward, but this region is also involved in responses to negative stimuli, learning, and motor activity [25, 48]. A more contemporary view is that NAC plays a critical role in promoting responses to positive and the avoidance of negative stimuli [123]. In rats, stimulation of MORs in NAC can induce reward (as measured using self-administration and CPP tests) [124, 125] and reduce stress-related behaviors [126]. It is thus possible that the MORs in NAC also play a dual role in rewarding, and thus reinforcing, flocking behavior by inducing positive and reducing negative affective states. Studies on starlings have begun for the first time to explore a role for the NAC in the regulation of gregarious song. Positive correlations were found in male starlings between gregarious song and the numbers of cells immunolabeled for FOS and ZENK (aka egr-1) in NAC [4]. A second study suggested these relationships to be causal, with a few (3 of 6) birds beginning to sing after infusion of the highly selective MOR agonist DAMGO into the NAC at the highest dose used in the study [30]. At this dose no effects were observed on beak wiping; however, this has not been tested thoroughly.

Unlike the POM, only recently has the NAC in songbirds begun to be studied. For many years the location of NAC in birds was unclear [8]. Studies in pigeons, chicks, and now in songbirds delineate rostral, shell, and core subdivisions for NAC [4, 21, 24, 68, 127-130]. In the present study, we focused on the rostral portion of NAC identified by Reiner [21], which in mammals contains shell- and core-like anatomical connections [49, 131]. The pharmacology study reviewed above demonstrated that MOR stimulation in this rostral portion of NAC induced locomotion and feeding, which is similar to what is observed in

mammals [30]. Yet studies have not confirmed that this location in songbirds receives dopaminergic input from the ventral tegmental area, which is a key characteristic of NAC in mammals.

The goal of the present study was to provide insight into the hypothesis that MORs in the POM and NAC may promote gregarious behaviors and social cohesion by promoting positive and reducing negative affective states. We were also interested in the possibility that the two regions may play distinct roles in social behavior and affective states. To do this, we compared the effects of infusion of the MOR agonist DAMGO into POM or NAC on song and other social behaviors. We focused on beak wiping to provide insight into a presumed negative state and then used CPP tests to provide insight into the extent to which infusions of DAMGO into POM and NAC that stimulated song are rewarding. Finally, we performed a tract-tracing study to confirm the presence of dopaminergic projections to NAC from the ventral tegmental area to provide further insight into homology.

Methods

All experiments were performed under animal care and use protocols approved by the University of Wisconsin Animal Care and Use Committee and according to guidelines of the National Institutes of Health.

Effects of MOR stimulation in the POM and NAC on gregarious song and establishment of CPP

Twenty-two adult European starlings, *Sturnus vulgaris*, were used as experimental animals (16 males, 6 females). Fewer females than males were tested because fewer females sang during prescreening, which was required for inclusion in the experiment (as detailed below). All birds were captured in fall and winter months from a local farm on the

west side of Madison, Wisconsin and housed in same-sex cages on 18L:6D for more than 6 weeks so birds would become “photorefractory”. Photorefractoriness is a characteristic of early fall wherein starlings begin to sing in large, mixed-sex flocks [132]. For this study, starlings were housed in indoor aviaries (2.13 × 2.4 × 1.98 m) with flock mates, so each aviary had a total of 8 birds. Following testing and subsequent removal of a bird from an aviary, a replacement bird was added to maintain 8 birds in each flock. The aviaries contained natural and artificial perches and the birds had *ad libitum* access to food, drinking water, and bathing water. During the 18 hrs of light, talk radio was played to acclimate birds to voices and extraneous noise.

This study was conducted from May 2021 to November 2021. Three researchers observed birds in flocks for 20 min a day, for 5 consecutive days to identify singing birds to be used as focal animals. Each focal bird was observed by only one observer from the beginning to the end of its testing to control for inter-observer variability within each experimental bird. At the beginning of each observation, an audio recording of starling song was played to facilitate singing behavior (Marius Travell, YouTube). Focal birds were not selected until at least 3 birds in the aviary sang for more than 3 days in a row. Once focal birds were selected, a cannula guide was surgically implanted to target either the POM or NAC (see below). Two birds from each aviary were selected to be tested on alternate days, and observers were intentional about leaving at least one singing starling in each aviary unmanipulated to help facilitate song from other birds. Observers were blind to the cannula location of the focal birds during their observations.

Cannula Surgery

For all surgeries, birds were anesthetized with isoflurane and placed into a stereotaxic apparatus (Kopf, Tujunga, CA, USA) with the beak approximately 45° below the horizontal plane of the ear bars, and a 26-gauge stainless steel cannula guide (C315G-5UP/SP; Plastics One) was implanted in either the POM or NAC. Specific details of surgical procedures can be found in [133]. For the POM, the rostrocaudal coordinate was placed 1 mm posterior to the stereotaxis zero. The lateral target was placed 0.5 mm from skull zero, either to the right or left hemisphere (the side of the hemisphere was counterbalanced across birds). The vertical target was placed 6.5 mm below the skull zero. For the NAC, the cannula targeted the rostral pole of the NAC [4, 21, 30]. The cannula guide was angled at 4.5° in the stereotaxis, and the rostrocaudal coordinate was placed 1.5 mm anterior to the stereotaxis zero. The lateral target was placed 0.5 mm to the left or right hemisphere and was similarly counterbalanced. The vertical target was placed 6.4 mm below the skull zero. The cannula was secured to the skull using screws and dental cement (clear Ortho-Jet powder combined with acrylic liquid; Lang Dental Manufacturing Company, Inc.). After surgery, birds were moved to a cage outside of the aviary for 2 days to recover.

Pharmacological Manipulations

Starlings were moved back to their original aviaries following recovery. Experimenters observed the birds until both focal birds sang for at least 2 days in a row. Focal birds were then treated and tested for 4 days, with each treatment day separated by at least 1 "treatment washout" day. Focal birds in the same aviaries were tested on alternate days so that only one bird per aviary was tested on a single day. First, a habituation "injection" was performed for each bird by placing an empty cannula in the cannula holder for the same amount of time as for treatment injections (~5 min). This was performed to habituate the

bird to being captured, anesthetized, and put in a temporary cage to recover. After habituation, each experimental sequence consisted of 4 treatments: vehicle (sterile saline, 0.85%; 0.50 µl), 2 doses of the MOR agonist D-Ala2, N-Me-Phe4, glycinol5-ENK (DAMGO; Sigma-Aldrich, catalog #100929–53-1; low dose, 2.5 µg; high dose, 25 µg (all dissolved in 0.50 µl sterile saline), and treatment with the MOR antagonist Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH(2) (CTAP; 3 µg dissolved in 0.50 µl sterile saline) immediately followed by the low dose of DAMGO. The latter manipulation was used to test for MOR specificity by determining the degree to which CTAP prevented DAMGO effects on behavior. For this manipulation the low dose of DAMGO was selected a priori based on a recent study in starlings that showed that this dose in NAC facilitated song [30]. Here we also added a higher dose of DAMGO (25 µg) because in the same study doses lower than 2.5 did not stimulate singing behavior [30]. Each starling received each treatment once in a counterbalanced order. Treatments were color-coded so the observer was blind to treatment conditions with the exception of the CTAP treatment due to the nature of the paired injection.

On each test day, injections began between 09:00 and 15:30; however, each focal bird was injected and observed within approximately the same time period each day to control for potential circadian variation in singing within an individual bird. Experimental birds were caught rapidly with a net and then anesthetized using isoflurane and oxygen with the aid of a nose cone. The dummy cannula was carefully removed, and a 33-gauge cannula was connected to PE50 tubing (catalog #C232CT, Plastics One) with the treatment solution drawn into it. A vacuum syringe (Hamilton) connected to a Nanomite Syringe Pump (Harvard Apparatus) injected 0.50 µl of the treatment solution over a 2 min period.

Following the injection, the cannula was left in place for 3 min to allow for diffusion and equalization of pressure from the cannula tip. Infusion volume was confirmed by examining the movement of an air bubble in the tubing. After cannula removal, the cannula dummy was replaced, and the bird was placed in a draped cage for 15 min of recovery before being placed back into its home aviary.

Behavioral Observations

A researcher observed each focal bird for 20 min. Starlings produce long, complex songs that commonly begin with introductory whistles, followed by variable phrase types, clicks and trills [96, 134-136]. In this study we counted continuously: introductory whistles, song fragments [1-2 sec variable songs], full songs [song longer than 2 sec, past studies show this song on average to be approx. 25 sec [135, 137]]). We additionally recorded bouts of beak wiping (separated by 1 sec); displacements (a focal bird approaches another bird and that bird leaves within 1 sec); and flights to new perches.

Conditioned Place Preference (CPP)

Our goal was to run CPP tests on the same birds that we observed singing. Cannula remain affixed to the skull firmly for 3-4 weeks, before they lose adhesion, presumably because birds are flying, exploring, and interacting freely in aviaries with branches and flock mates. In an effort to test the same birds after singing and for CPP we pilot tested and then ran what we found to be the most rapid CPP approach supported by past research [138] (discussed further in the discussion section). We were unable to test 4 of the NAC birds because cannulae did not remain securely affixed to the skull, so only 6 NAC birds were tested.

CPP testing began at least 1 day after the final behavioral observation. The CPP apparatus consisted of a cage (118 cm × 59 cm × 59 cm) divided into 2 visually unique compartments with one compartment decorated with a red background with a white circle in the center and the other with a blue background with a white cross in the center (Figure 1). There were 3 phases each occurring on consecutive days: conditioning day 1, conditioning day 2, and test day. Observers were blind to the treatment conditions. On conditioning day 1, a focal bird was injected with either 0.5 μl of the low dose of 2.5 μg DAMGO or vehicle solution and allowed to recover using the above protocol (dose selected a priori based on [30]). After recovery, the bird was placed singly into one side of the CPP apparatus and restricted to one of the distinct chambers for 45 minutes. Following conditioning, the bird was returned to its home aviary. The next day, conditioning day 2, the bird received whichever treatment it did not receive on conditioning day 1, recovered and was placed in the opposite, distinct compartment of the apparatus for 45 minutes and returned to its aviary afterward. The treatment order, side of the cage (left or right), and compartment (i.e., blue w/ cross or red w/dot) paired with each treatment were all counterbalanced across birds.

On test day, the bird was caught and immediately placed in the center of the CPP apparatus with the divider of the two compartments removed, which allowed the starling to freely move between compartments. Lights were off in the room when the bird was placed in the center of the CPP apparatus, and lights were switched on to start the observation period. Observers then sat behind a one-way mirror and recorded how much time was spent on each side of the apparatus.

Cannula Tip Verification

Immediately following the CPP test day observation, birds were anesthetized and injected with 1.0 µl of Chicago Blue 6B dye (Thermo Fisher Scientific) to identify the tip of the cannula. After infusion, the birds were sacrificed via rapid decapitation, brains were extracted and flash frozen with dry ice, and stored at -80°C. Brains were sectioned in 50 µm sections using a cryostat (catalog #CM1850, Leica Biosystems). The sections were mounted on slides and analyzed using a microscope to determine whether the blue dye (i.e., the injection point) was accurately in the NAC or POM. There were 10 "hits" for the NAC (3 female, 7 male) and 5 "hits" for the POM (1 female, 4 males) (Figure 2). Seven of the cannula tips were located outside of either NAC or POM (2 females, 5 males), which is common for stereotaxic surgery in wild birds. These "misses" were in multiple locations (Figure 2 and detailed in results).

Identifying TH+ projections to rostral NAC from the VTA

Retrograde Tracing

We selected the rostral NAC identified by Reiner as the site of infusion for this study [21]. To explore further the degree to which this brain region is homologous to mammalian NAC, a tract-tracing study was performed in four birds (3 males, 1 female) that were not behaviorally tested to reveal the extent to which our NAC target received dopaminergic input (indicated by tyrosine hydroxylase [TH] labeling) from the ventral tegmental area (VTA), which characterizes NAC in mammals [139-141]. Birds were anesthetized with isoflurane, and 0.5 µl of the retrograde tract tracer Fluoro-Gold (FG, Fluorochrome Inc., Denver, CO) was injected into NAC using identical methods as described above for cannula surgery. After infusion, starlings were returned to a cage for 10 days to allow for retrograde transport.

Double Fluorescence immunohistochemistry for FG and TH with Tyramide Signal Amplification (TSA)

Birds were anesthetized with isoflurane and transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4). Brains were post-fixed overnight in the same fixative and then cryoprotected with 0.1 M PB containing 30% sucrose at 4°C for two days. Brains were snap-frozen and 40-µm coronal sections were sliced on a cryostat and stored in cryoprotectant solution at −20°C until processing. Sections from just rostral to Area X through the VTA and periaqueductal gray were collected.

Brain sections were washed 5×5 min with 0.02 M PBS to remove cryoprotectant, and then incubated in 1.5% H_2O_2/50% methanol for 30 min to inhibit endogenous peroxidase activity and enhance the penetration of antibodies into tissue sections. They were rinsed 3×10 min in 0.3% Triton X-100/0.05% normal goat serum (NGS)/ 0.02 M PBS, incubated for 60 min in blocking solution (10% NGS/0.3% Triton X-100/0.02 M PBS), and then incubated overnight with a mixture of two primary antibodies: rabbit anti-FG (Fluorochrome, LLC, Denver; diluted 1:1000) and mouse anti-TH (MAB318, Millipore, Bilerica, MA, USA; diluted 1:1000) at 4°C in primary antibody incubation solution (PAIS, 0.3% Triton X-100/1% NGS/1% blocking reagent/0.02 M PBS). After primary antibody incubation, sections were washed and incubated for 1 h with HRP-conjugated goat anti-rabbit antiserum (Cell Signaling; diluted 1:100 in TBST), washed 3x10 min with wash buffer, then incubated for 10 min in Cy3-conjugated tyramide (TSA™ Plus Cyanine 3 kit, PerkinElmer, Waltham, MA; red for FG labeling) by diluting TSA stock solution 1:50 in 1x Amplification Diluent. After washing 3x10 min with wash buffer, sections were again incubated for 30 min with 3% H_2O_2 in TBS to quench peroxidase activity from the initial

TSA reaction, incubated for 1 h with HRP-conjugated horse anti-mouse antiserum (Cell Signaling; diluted 1:100 in TBST). They were washed 3x10 min with wash buffer and then incubated for 30 min in Alexa Fluor 488-conjugated tyramide (Molecular Probes, Eugene, OR; green for TH labeling) by diluting TSA stock solution 1:100 in 1x Amplification reagent. Following washing 3x10 min with wash buffer, sections were mounted onto slides using DePeX mounting medium (Serva, Heidelberg, Germany), air-dried, and stored in the dark at 4°C. Cells double labeled for FG and TH in VTA, which demonstrates dopaminergic projections from VTA to TH, were detected on images taken using an inverted Zeiss LSM 710 Meta laser scanning confocal microscope (Zeiss; Oberkochen, Germany).

Statistical analysis

Although both females and males were included in this study, during pre-tests fewer females sang in the aviaries, resulting in too few females in the study for statistical comparisons between sexes. However, we indicate the sexes in the figures. Sphericity was tested using Mauchly's W. Assumptions of homogeneity of variance and normality were tested using Levene's tests and Q-Q plots. When data violated assumptions, they were transformed using Log (x+0.05). In cases for which assumptions were not violated or for which transformation corrected violations parametric repeated measures ANOVAs were run with the behavioral variable entered as a dependent measure, treatment (saline, low dose DAMGO, high dose DAMGO, and CTAP + low dose DAMGO) entered as a repeated measures variable and region (POM or NAC) entered as a between-subjects variable. Significant ANOVAs were followed by pairwise post-hoc Tukey tests. When transformation did not correct violations, non-parametric Friedman ANOVAs were run. When significant,

these were followed by pairwise Durbin-Conover post-hoc comparisons. Analyses were run using jamovi 2.3.5. Figures were made using the ggplot2 package in R v4.1.2 [70, 142].

Results

Cannulae successfully targeted POM in 5 birds (4 males, 1 female) and NAC in 10 birds (7 males, 3 females). Cannulae missed the targets in 7 birds (5 males, 2 females) We briefly describe our results of cannula that missed the POM or NAC "misses" separately below.

Singing behaviors

Two birds in the NAC group (1 male and 1 female) never sang again during experimental observations after surgery. These two birds were removed from analysis resulting in n=8 birds in the NAC group for song analyses.

Introductory whistles

Introductory whistle components of song were observed in 0/8 NAC saline treated birds, 4/8 NAC low dose DAMGO treated birds, 6/8 NAC high dose DAMGO treated birds, and 3/8 CTAP + DAMGO treated birds. For POM the results were 0/5, 4/5, 1/5, and 3/5, respectively. Results of Friedman ANOVAs (performed because data violated assumptions as described in methods above) revealed significant effects of DAMGO treatment in NAC ($\chi^2_3 = 11.9$, $p = 0.008$) and POM ($\chi^2_3 = 9.88$, $p = 0.020$) (Figure 3). For NAC, post hoc Durbin-Conover tests indicate that compared to saline, birds whistled more after treatment with the low ($p = 0.022$) and high ($p < 0.001$) doses of DAMGO, but the low and high doses did not differ significantly ($p = 0.077$). Whistling was also significantly lower in the CTAP + DAMGO treatment compared to the high dose of DAMGO ($p = 0.005$) but was not significantly different from either saline ($p = 0.228$) or the low dose of DAMGO ($p = 0.228$) (Figure 3). For POM, post hoc Durbin-Conover tests indicate that compared to saline, birds

whistled more after treatment with the low ($p < 0.001$) but not high ($p < 0.381$) doses of DAMGO and that the low and high doses differed significantly ($p = 0.003$). Whistling after the CTAP + DAMGO treatment was significantly lower relative to the low dose DAMGO treatment ($p = 0.018$) but did not differ from saline ($p = 0.094$) or the high dose of DAMGO ($p = 0.381$) (Figure 3).

Fragments

Fragments of song were observed in 1/8 NAC saline treated birds, 5/8 NAC low dose DAMGO treated birds, 6/8 NAC high dose DAMGO treated birds, and 4/8 CTAP + DAMGO treated birds. For POM the results were 1/5, 3/5, 1/5, and 1/5, respectively. Results of Friedman ANOVAs revealed significant effects of DAMGO treatment in NAC ($\chi^2_3 = 11.73$, $p = 0.008$) but not for POM ($\chi^2_3 = 2.25$, $p = 0.522$) (Figure 3). For NAC, post hoc Durbin-Conover tests indicate that compared to saline, birds produced fragments more after treatment with the high ($p < 0.001$) but not low ($p = 0.094$) doses of DAMGO, and the low and high doses differed significantly ($p = 0.016$). Production of fragments was also significantly lower in the CTAP + low dose DAMGO treatment compared to the high dose of DAMGO ($p = 0.008$) but was not significantly different from either saline ($p = 0.159$) or the low dose of DAMGO ($p = 0.773$) (Figure 3).

Full songs

Full songs were observed in 0/8 NAC saline treated birds, 2/8 NAC low dose DAMGO treated birds, 4/8 NAC high dose DAMGO treated birds, and 1/8 CTAP + DAMGO treated birds. For POM the results were 0/5, 1/5, 0/5, and 0/5, respectively. Results of Friedman ANOVAs revealed significant effects of DAMGO treatment in NAC ($\chi^2_3 = 9.55$, $p = 0.023$) but for POM only one bird sang full songs (i.e., a male sang 3 times after the low dose of

DAMGO) so no analysis was run on POM (Figure 3). For NAC post hoc Durbin-Conover tests indicate that compared to saline, birds sang full songs more after treatment with the high ($p = 0.002$) but not low ($p = 0.288$) doses of DAMGO, and the low and high doses differed significantly ($p = 0.028$). Production of full song was also significantly lower in the CTAP + low dose DAMGO treatment compared to the high dose of DAMGO ($p = 0.008$) but was not significantly different from either saline ($p = 0.591$) or the low dose of DAMGO ($p = 0.591$) (Figure 3).

Beak wiping

The female that never sang again during experimental observations after surgery also failed to beak wipe and was removed from analysis, resulting in n=9 birds in the NAC group for beak wipe analysis. For beak wiping, analysis of untransformed data revealed a significant main effect for treatment ($F_{3,36} = 10.75$, $p < 0.001$). No significant main effect was revealed for region ($F_{1,12} = 0.01$, $p = 0.922$) or the treatment x region interaction ($F_{3,36} = 0.37$, $p = 0.776$) (Figure 4). Post hoc Tukey tests indicate that compared to saline, birds beak wiped less after treatment with the high ($p = 0.001$) but not low ($p = 0.539$) doses of DAMGO. Beak wiping did not differ between low or high dose DAMGO treatments ($p = 0.261$). CTAP + low dose DAMGO treatment did significantly decrease beak wiping compared to saline ($p < 0.001$) and the low ($p = 0.039$) but not high ($p = 0.467$) doses of DAMGO (Figure 4).

Displacements

For displacements, analysis of untransformed data revealed a significant main effect for treatment ($F_{3,39} = 3.05$, $p = 0.040$). No significant main effect was revealed for region ($F_{1,13} = 0.019$, $p = 0.893$) or the treatment x region interaction ($F_{3,39} = 0.115$, $p = 0.951$) (Figure 4).

Post hoc Tukey tests indicate that compared to saline, birds displaced other birds more after treatment with the high ($p = 0.025$) but not low ($p = 0.102$) doses of DAMGO, but displacements did not differ between low or high dose DAMGO treatments ($p = 0.858$). CTAP + low dose DAMGO treatment did not significantly alter displacements compared to saline ($p = 0.065$), low ($p = 0.926$) or high ($p = 0.991$) doses of DAMGO (Figure 4).

Flying

For flying, analysis of untransformed data revealed a significant main effect for treatment ($F_{3,39} = 10.42$, $p < 0.001$). No significant main effect was revealed for region ($F_{1,13} = 0.825$, $p = 0.380$) or the treatment x region interaction ($F_{3,39} = 0.483$, $p = 0.696$) (Figure 4). Post hoc Tukey tests indicate that compared to saline, birds flew to new perches more after treatment with the low ($p = 0.002$) and high ($p = 0.001$) doses of DAMGO, and flying did not differ between low or high dose DAMGO treatments ($p = 0.677$). CTAP + low dose DAMGO treatment increased flying compared to saline ($p < 0.001$) but did not alter flying relative to birds treated with the low ($p = 0.777$) or high ($p = 0.987$) doses of DAMGO (Figure 4).

Conditioned place preference for DAMGO

In the two compartment CPP test used in this experiment the time on the previously saline paired side of the apparatus is reciprocally related to the time on the previously DAMGO paired side of the apparatus. Thus, the two variables were not sampled independently as required to run paired t-tests. To address this issue, we subtracted the time spent on the saline side from the time spent on the DAMGO paired side for each bird. If birds do not demonstrate a preference the difference would be zero while more positive scores reflect DAMGO-induced place preferences. We then ran single sample t-tests to

compare the difference scores statistically to zero. A single sample t-test revealed that the difference scores for birds receiving DAMGO into NAC and into POM were significantly above zero for NAC (t_5 = 0.3.19, p = 0.024) and for POM (t_4 = 6.97, p = 0.002) (Figure 5), indicating DAMGO-induced place preferences.

Results for infusions that missed NAC or POM

The "misses" were in multiple locations. One was in the 3rd ventricle, 1 in the lateral ventricle, 1 in the tractus septomesencephalicus (TSM), 2 were ventral to intermediate portions of POM labeled as ventrolateral thalamus (VLT) in the Stokes et al canary brain atlas [143], 1 targeted the lateral hypothalamus (LH), and 1 targeted the ventromedial portion of the hypothalamus (VMH); Figure 2)). Statistical analyses were not run on the "misses" due to low sample sizes (n=1 for most regions; Figure 2). We report behavioral results in Table 1 but do not discuss these results further.

Tract tracing and tyrosine hydroxylase labeling

After infusions of the retrograde tract tracer Fluro-Gold (FG) into NAC, many FG-positive cells were observed, with several cells double labeled for FG + TH being found within the ventral tegmental area in all four birds (Figure 6).

Discussion

The results of this study demonstrate a causal role for MORs in both the NAC and POM in the facilitation of gregarious song, the reduction of a potentially negative state reflected in beak wiping, and reward demonstrated by the CPP tests. Collectively, these findings are consistent with the possibility that MORs in NAC and POM may play a dual role in flock cohesion by both reducing negative and inducing positive affective states. During pre-tests, few females sang frequently enough to be included in the study, similar to past studies on

MOR in POM and MOR in NAC [29, 30], thus females could not be compared statistically to males. However, when results of all the studies to date that include males and females are considered together there do not appear to be any obvious sex differences in the effects of DAMGO in either region on behavior. This is somewhat expected given that the song produced by males and females in this context likely plays a shared function related to group cohesion.

Mu opioid receptor stimulation in NAC or POM increases singing

Infusion of the selective MOR agonist DAMGO into NAC or POM stimulated singing behaviors differentially across doses, with subtle differences observed for distinct components of song. Specifically, the low dose of DAMGO in POM and both low and high doses of DAMGO in NAC stimulated low levels of introductory whistles, while song fragments and full songs were stimulated more robustly by the high dose of DAMGO in the NAC only. These effects were reversed by treatment with the MOR antagonist CTAP, indicating that effects on behavior were specific to MORs in both the NAC and POM.

Although birds produced few full songs after any of the treatments, song in this context is highly sensitive to disruption, and birds stop singing when caught and injected [29, 30]; therefore, restoring full song production with injections of DAMGO into NAC is striking. When specific components of song were considered, DAMGO treatment in either NAC or POM was found to robustly increase the production of song fragments. This component of starling song consists of the production of short 1-2 second fractions of song referred to as warbling or variable song types [96, 134]. The function of fragments is not known; however, it is possible that the ongoing production of these short songs functions to

maintain social cohesion or contact, similar to simple contact calls produced by numerous species [144].

Although DAMGO only increased the number of introductory whistles in some birds from zero to one or two, we find it striking that none of the birds whistled after saline treatment while for the NAC infused group 4 of 8 whistled after the low and 6 of 8 whistled after the high dose of DAMGO. For POM, 4 of 5 birds also whistled after a low dose of DAMGO. Introductory whistles are components of song that are proposed to convey information about species and individual identity [145]. They are produced spontaneously or in response to another individual [97, 146, 147]. Whistles increase seasonally in fall after the breeding season as starlings form and maintain overwintering flocks, and this component of song may promote flock cohesion by allowing birds to recognize other starlings and individuals within flocks [146, 148]. Collectively, these results establish a role for MOR in NAC in song production that was hinted at in a prior study [30] and demonstrate effects to be dose dependent.

It was somewhat surprising to find that DAMGO in POM did not significantly facilitate the production of fragments or full songs given that MOR downregulation in POM suppresses singing behavior [29]. This finding certainly does not preclude a role for MOR in POM in these components of song but suggests that NAC may be a more sensitive target for opioid stimulation of song in this context.

The POM is found to access directly regions underlying vocal production in passerines (i.e., “the song control system”) through a projection to the dorsomedial part of the nucleus intercollicularis and to indirectly access this system through projections to the ventral tegmental area, the periaqueductal gray, and the locus coeruleus [121]. The pathway by

which the NAC accesses the song system has not been studied; however, in mammals NAC is commonly considered a central limbic-motor interface (e.g., [149]). NAC is reciprocally connected to both the POM and the VTA [25, 32, 33, 71, 131, 150], offering indirect routes by which NAC can impact singing behavior. These and other pathways can be identified in future studies.

Mu opioid receptor stimulation in NAC or POM reduces beak wiping

The increases in singing behavior induced by DAMGO in NAC and POM were accompanied by stepwise, dose-dependent decreases in beak wiping, which is a proposed reflection of a negative state of stress or redirected aggression in starlings [112, 113], and no differences in effects were observed between the two regions. One interpretation is that the presence of flock mates naturally leads to the release of opioids that bind to MOR in NAC and POM to reduce a negative state that facilitates singing and flock cohesion. However, unexpectedly, the DAMGO-induced suppression of beak wiping was not reversed, but in fact appeared to be enhanced, by pre-treatment with the MOR antagonist CTAP, suggesting effects may not be MOR specific. There is some precedent in the literature for this with administration of low doses of opioid receptor antagonists found to enhance effects of the MOR agonist morphine on analgesia [151]. These effects appear to be caused by low doses of the antagonists preferentially inhibiting excitatory opioid receptors, which then enhances effects of activation of inhibitory opioid receptors by [152]. If this is the case, higher doses of CTAP may be needed to observe the expected reversal of DAMGO effects. The degree to which effects are specific to MOR needs to be determined in future studies.

Mu opioid receptor stimulation at a dose that facilitates song also induces a CPP

In addition to influencing song and beak wiping, strong conditioned place preferences were induced by infusions of the low dose of DAMGO (dose selected prior to the experiment based on a past study suggesting that this was a dose in NAC capable of triggering song [30]). CPP tests are classic tests used to evaluate the rewarding properties of drugs of abuse [153], and the present findings are consistent with CPP studies in rodents that demonstrate that MOR stimulation in NAC and enkephalin infusion in POM induce strong place preferences [119, 125]. Positive results in rodents have been taken as evidence that MOR stimulation in POM or NAC is rewarding [125, 138, 154], although there are studies that do not show this effect for NAC, which may reflect discrepancies in injection sites [154, 155]. This reward may be induced by a pleasurable outcome or by the reduction of an aversive state, and CPP tests do not distinguish between the two [156, 157]. With respect to drugs that stimulate MORs, experiences in humans suggest that likely both states are induced.

Past studies in songbirds show clear linear, positive correlations between gregarious song and an individual's intrinsic reward state, as measured using CPP tests [6, 29, 36, 42], and it has been proposed that either a reward state induces singing behavior or that the act of singing itself induces a reward state. Although the present findings do not rule out the latter interpretation, the finding that MORs in the POM or NAC induce CPPs in starlings supports the interpretation that the induction of a reward state by MOR activation facilitates gregarious singing behavior. Together the DAMGO-induced increase in singing, CPP, and reduction in beak wiping reported here lend support to the proposal that social cohesion in these flocks may be rewarded both the induction of a positive state resulting from behaviors that occur within the flock and by the reduction of a negative state.

The CPP design we selected was based in part on a meta-analysis of CPP tests for opiates [138], which suggested that 2 rather than 3 compartments for the apparatus is most effective, that a 45-minute pairing is better than 30 min, and the omission of a preconditioning phase results in larger effects. The rationale for this relatively short test is that we have only 3-4 weeks after cannula surgery to complete the studies. Birds take time post-surgery to resume singing, therefore we had a very limited time in which to perform the CPP test. Our pilot tests and the results reported here indicate that DAMGO infusion can induce a CPP following this design; however, there is a large literature on CPP testing, best practices, and caveats, some of which recommend inclusion of a neutral chamber and pretesting for initial side preferences [153, 156, 158]. It is thus possible that results do not reflect rewarding effects of DAMGO; however, given that the present findings are consistent with opioid CPP as well as self-administration studies in rodents [119, 125, 158] we consider reward to be a parsimonious interpretation.

Mu opioid receptor stimulation in NAC or POM increases displacements

For both POM and NAC, high doses of DAMGO significantly increased displacements, and no differences were observed between the two regions. These effects were prevented by pre-treatment with CTAP, suggesting results are MOR selective. The results for NAC are consistent with a past paper in which the same low dose of DAMGO used here increased displacements in starlings [30]. Here we also report for the first time that MOR stimulation in POM also increases displacements. It is important to note that displacements in starling flocks are mildly agonistic and relatively non-threatening. Starlings do not maintain strong dominance hierarchies and are observed sharing food outside the breeding context. They tend to maintain approximately one body width of distance between themselves

presumably to avoid being pecked by other birds. Therefore, the function of displacements has been proposed to relate to the optimization of social spacing [30, 159]. The present findings suggest that activation of MORs in POM and NAC may play a role in optimizing social spacing to maintain flock cohesion.

Mu opioid receptor stimulation in NAC or POM stimulates motor behaviors

Stimulation of MORs in NAC or POM increased motor behavior as reflected in numbers of flights to new perches, with no differences observed between the two regions. Similar to beak wiping, CTAP did not reverse motor behavior. The results for NAC are consistent with past studies in birds and mammals [30, 160]. Although we found no reports on the impact of MOR in POM on motor activity, prior studies have revealed a role for the POM in voluntary wheel running in mice [161-163].

Behavioral and anatomical data suggest findings in birds may generalize to other vertebrates

Unlike the POM, only recently has the NAC in songbirds begun to be studied. The results of the tract-tracing study showed that after infusion of the retrograde tract tracer FG into NAC, FG+TH positive cells were identified in VTA. This confirms that our NAC target site receives dopaminergic input from the VTA, an essential characteristic of the NAC in mammals. The behavioral results also confirm that manipulations of MOR in songbirds induce similar effects to those observed in mammals, including a reduction in proposed stress-related behavior and increased motor behaviors, reviewed above. Moreover, stimulation of MOR in the starling NAC induced a CPP which is consistent with findings from several rodent studies [124, 125]. Together the present findings add to prior studies that support homology between the avian and mammalian NAC [164-167]. Along with past

studies supporting homology between the avian and mammalian POM (e.g.,[164]), this suggests that findings in birds related to social reward and cohesion will generalize to mammals. We thus propose that the findings reported here for NAC and POM are revealing new information about critical mechanisms and circuitry by which opioids reinforce essential, positive, stress-reducing non-sexual social behaviors.

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Table 1. Effects on behavior of DAMGO infusions outside NAC or POM.

Song (whistle, fragments, full)			—		
Sex	Region	Saline	Low DAMGO	High DAMGO	CTAP + Low DAMGO
Male	TSM	0	3,3,0	2,16,1	0
Female	3rd vent	7,0,5	3,0,0	9,0,0	0,0,0
Male	lat. vent	0	0	0	0
Male	VLT	0	0	0	0
Female	VLT	0	0	0	0
Male	LH	0	0	1,0,0	3,0,0
Male	VMH	1,0,2	0	0	0
Beak wiping					
Male	TSM	6	3	0	0
Female	3rd vent	10	1	0	0
Male	lat. vent	4	8	0	0
Male	VLT	30	22	0	0
Female	VLT	51	0	0	0
Male	LH	3	17	1	0
Male	VMH	1	0	0	0
Displacement					
Male	TSM	0	9	11	21
Female	3rd vent	2	10	18	29
Male	lat. vent	4	0	6	7
Male	VLT	8	9	133	62
Female	VLT	1	0	0	0
Male	LH	2	4	10	3
Male	VMH	2	14	57	94
Flying					
Male	TSM	6	72	152	202
Female	3rd vent	26	105	174	152
Male	lat. vent	36	22	113	40
Male	VLT	43	19	229	96
Female	VLT	43	0	0	55
Male	LH	62	100	175	133
Male	VMH	37	100	120	162
CPP					
Sex	Region	Secs saline side	Secs DAMGO side	Difference score	—
Male	TSM	307	593	286	—
Female	3rd vent	900	0	−900	—
Male	lat. vent	470	430	−40	—
Male	VLT	326	574	248	—
Female	VLT	0	900	900	—
Male	LH	0	900	900	—
Male	VMH	752	148	−604	—

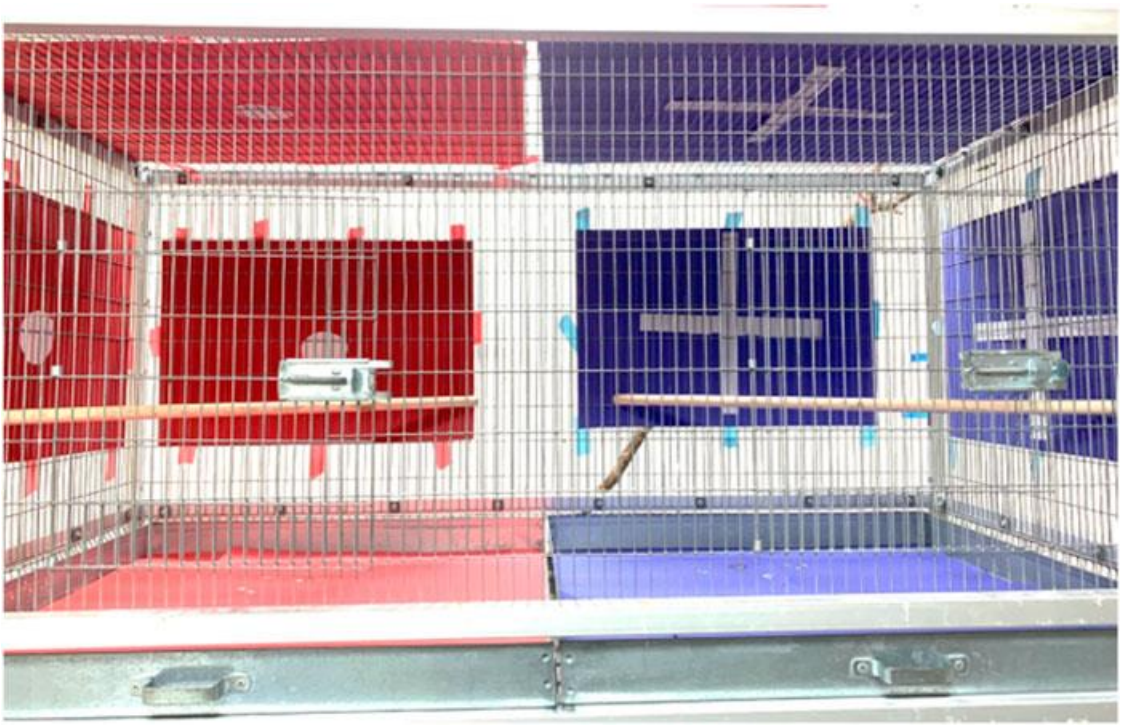

Figure 1. Photograph of the CPP apparatus to illustrate the 2 distinctly decorated conditioning compartments. On two separate days, birds were restricted to one side after saline treatment and to one side after DAMGO treatment (sides and treatments counterbalanced across birds). The next day a partition separating the two sides was removed (as in the photo) and the amount of time each bird spent on each side was recorded. See text for additional details.

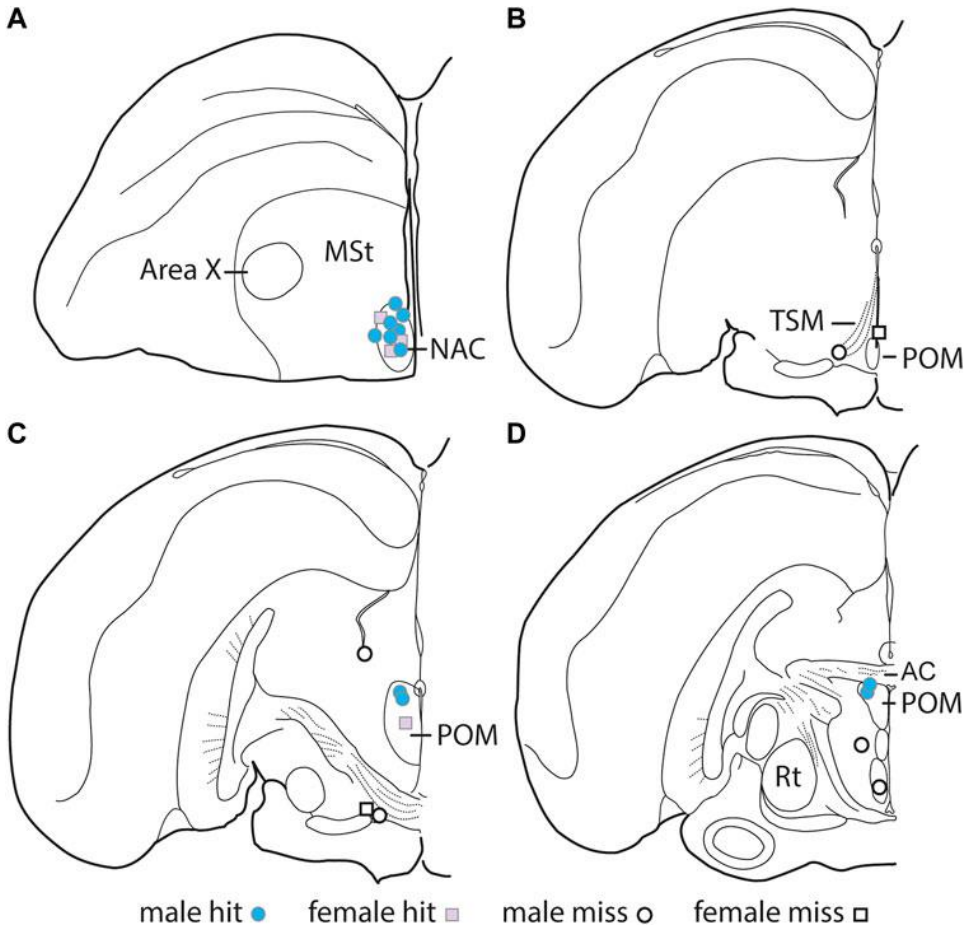

Figure 2. Location of DAMGO infusion sites. Illustration of one hemisphere of starling brain, with hits represented by filled-in shapes and misses represented by open shapes. Males are represented by circles, and females are represented by squares. AC, Anterior commissure; MSt, medial striatum; Rt, nucleus rotundus; TSM, tractus septomesencephalicus.

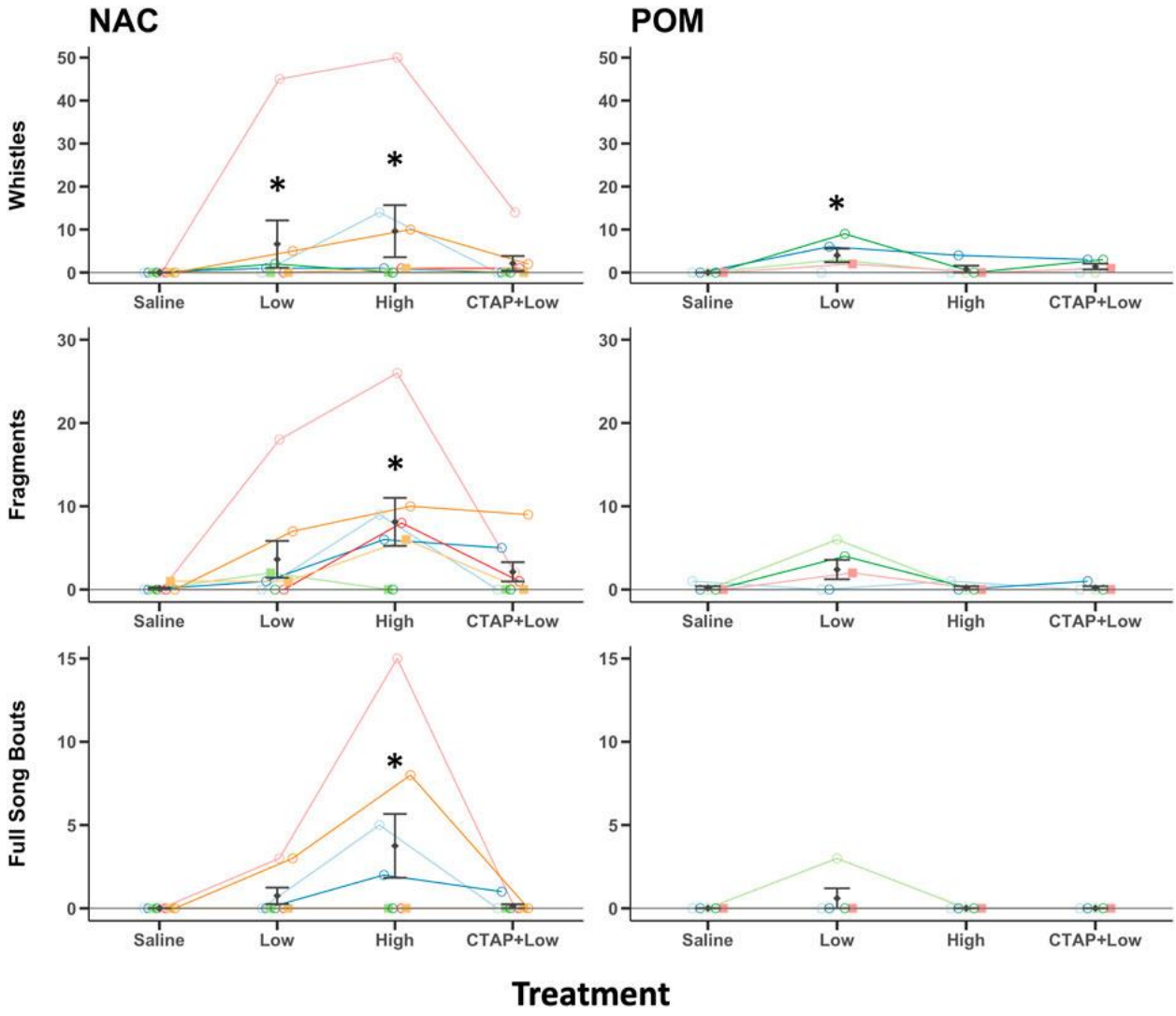

Figure 3. MOR stimulation in NAC or POM facilitates singing behaviors. Effects of intra NAC and intra POM treatments of Saline, low dose DAMGO (2.5 µg; Low), high dose DAMGO (25 µg; High) and the MOR antagonist CTAP followed by low dose DAMGO (CTAP + Low) on introductory whistles, song fragments, and full song bouts. Note: because whistle rates were low in some birds, they are just visible above the 0 line; however, for NAC 0/8 saline treated birds, 4/8 low dose DAMGO treated birds, 6/8 high dose DAMGO treated birds, and 3/8 CTAP + DAMGO treated birds whistled. For POM the results were 0/5, 4/5, 1/5, and 3/5, respectively. Mean ± sem is indicated for each group. Individual repeated data points are shown for males (circles) and females (squares). * indicates $p < 0.05$ compared to saline. See results for additional statistical details.

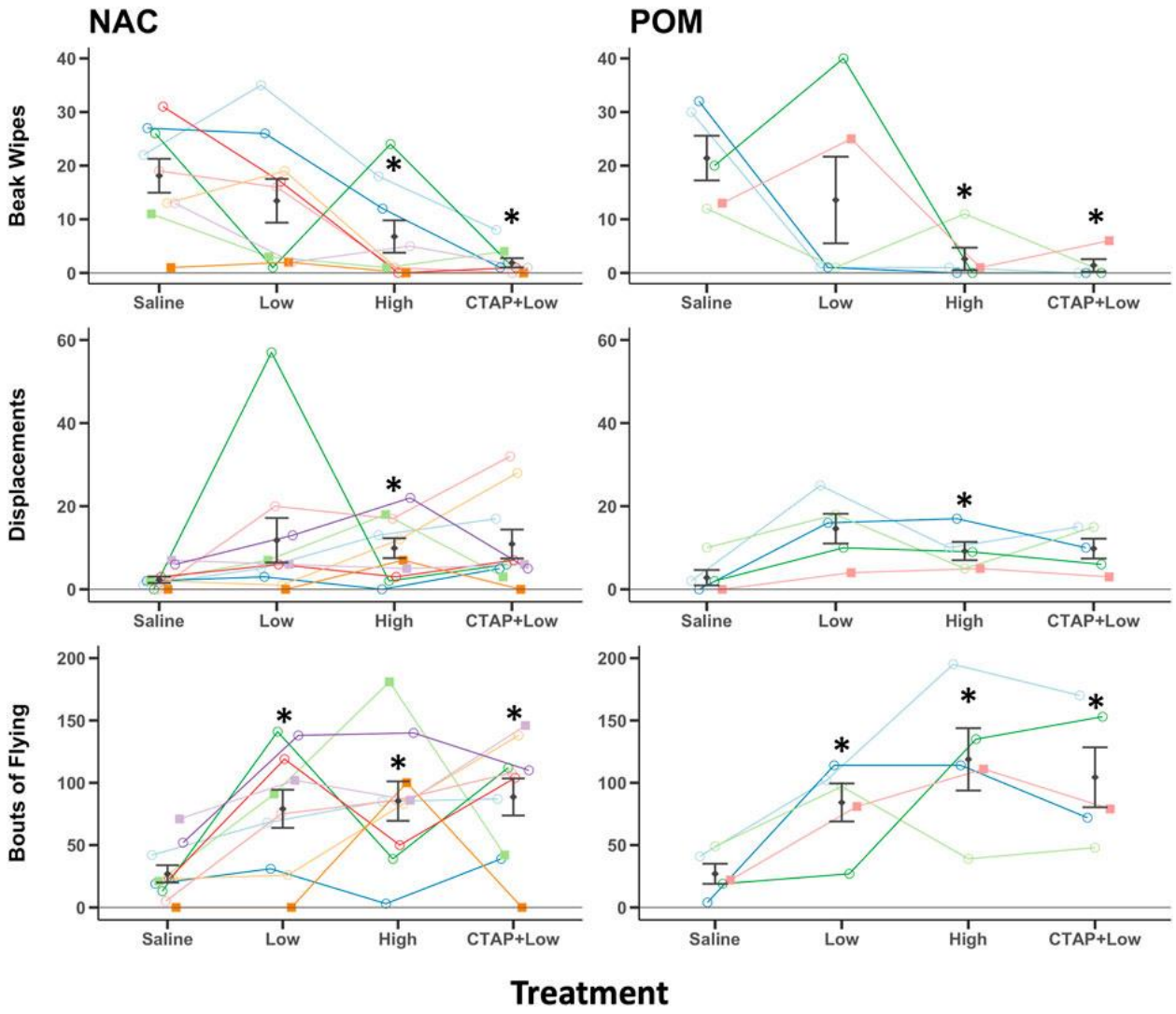

Figure 4. MOR stimulation in NAC or POM suppresses beak wiping but facilitates displacements and locomotion. Effects of intra NAC and intra POM treatments of Saline, low dose DAMGO (2.5 µg; Low), high dose DAMGO (25 µg; High) and the MOR antagonist CTAP followed by low dose DAMGO (CTAP + Low) on beak wiping, displacements, and flights. Mean ± sem is indicated for each group. Individual repeated data points are shown for males (circles) and for females (squares). Site of injection was not a significant factor, but we show results for NAC and POM separately here so points for each region can be seen clearly. * indicates $p < 0.05$ compared to saline for NAC and POM infusions combined. See results for additional statistical details.

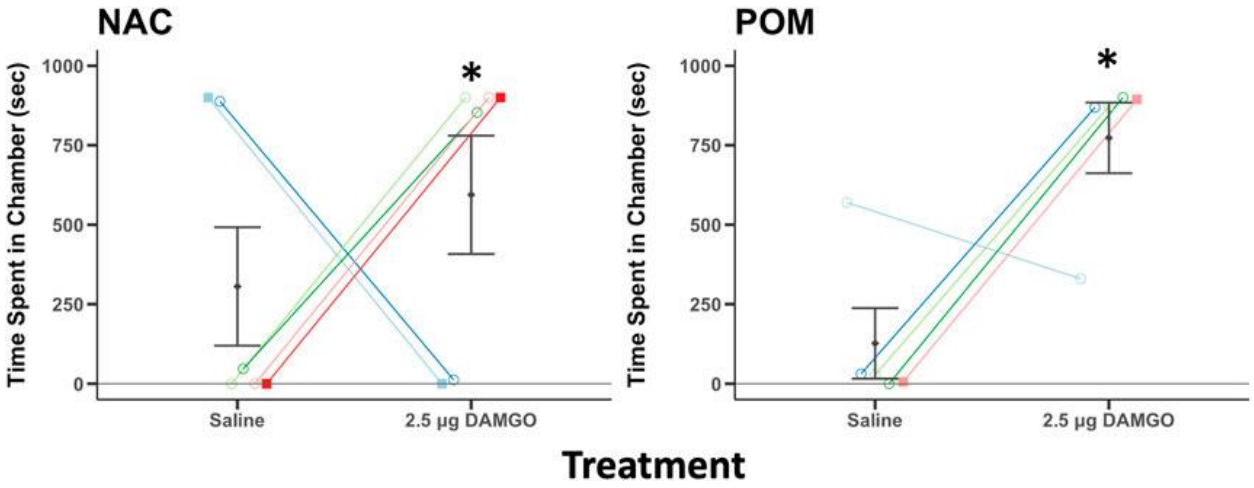

Figure 5. MOR stimulation in NAC and POM induces a conditioned place preference. Time spent in a chamber that had been paired previously with intra NAC or intra POM infusions of saline and 2.5 µg DAMGO. Mean ± sem is indicated for each group. Individual repeated data points are shown for males (circles) and for females (squares). * indicates that the difference score between the time spent by each individual on the side that had been paired with 2.5 DAMGO minus the side that had been paired with saline differed significantly from zero ($p < 0.05$). Note: for analysis we subtracted the time spent on the saline side from the time spent on the DAMGO paired side and compared this statistically to zero (which would reflect no preference). We present here the individual data points to provide readers with information on each individual. See results for additional statistical details.

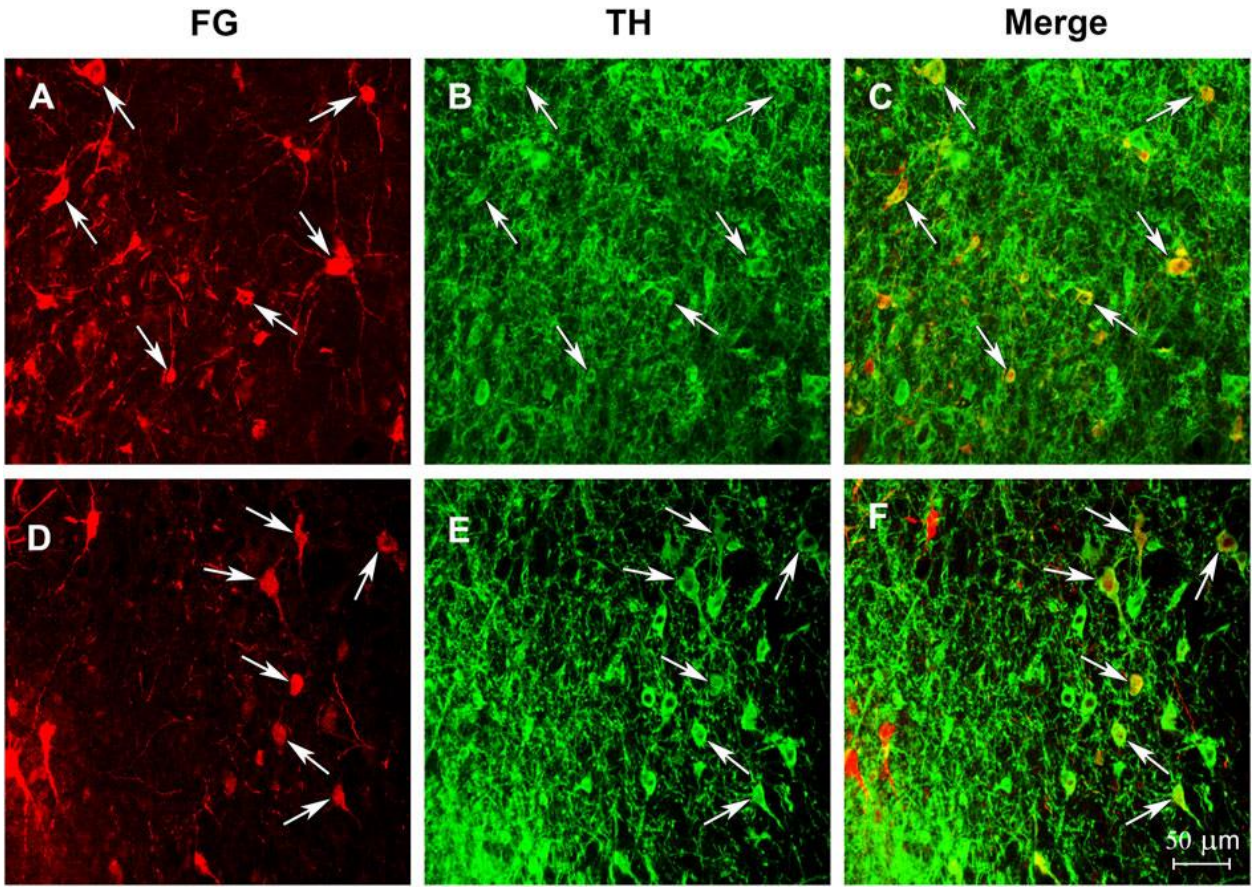

Figure 6. Photomicrographs illustrate that the starling NAC receives dopaminergic input from VTA. Confocal images showing immunolabeling in the VTA from two birds (one bird per row) that received injections of the retrograde tract tracer Fluro-Gold into the NAC. A and D illustrate neurons labeled with tracer. B and E illustrate neurons labeled for TH. C and F illustrate neurons double labeled for both the tracer and TH. Arrows highlight the same double labeled cells in each image. Left is lateral for each image.

Chapter 3

Distinct patterns of gene expression in the medial preoptic area are related to gregarious singing behavior in European starlings (*Sturnus vulgaris*)

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Abstract

Background

Song performed in flocks by European starlings (*Sturnus vulgaris*), referred to here as gregarious song, is a non-sexual, social behavior performed by adult birds. Gregarious song is thought to be an intrinsically reinforced behavior facilitated by a low-stress, positive affective state that increases social cohesion within a flock. The medial preoptic area (mPOA) is a region known to have a role in the production of gregarious song. However, the neurochemical systems that potentially act within this region to regulate song remain largely unexplored. In this study, we used RNA sequencing to characterize patterns of gene expression in the mPOA of male and female starlings singing gregarious song to identify possibly novel neurotransmitter, neuromodulator, and hormonal pathways that may be involved in the production of gregarious song.

Results

Differential gene expression analysis and rank rank hypergeometric analysis indicated that dopaminergic, cholinergic, and GABAergic systems were associated with the production of gregarious song, with multiple receptor genes (e.g., DRD2, DRD5, CHRM4, GABRD) upregulated in the mPOA of starlings who sang at high rates. Additionally, co-expression network analyses identified co-expressing gene clusters of glutamate signaling-related genes associated with song. One of these clusters contained five glutamate receptor genes and two glutamate scaffolding genes and was significantly enriched for genetic pathways involved in neurodevelopmental disorders associated with social deficits in humans. Two of these genes, GRIN1 and SHANK2, were positively correlated with performance of gregarious song.

Conclusions

This work provides new insights into the role of the mPOA in non-sexual, gregarious song in starlings and highlights candidate genes that may play a role in gregarious social interactions across vertebrates. The provided data will also allow other researchers to compare across species to identify conserved systems that regulate social behavior.

Background

Birdsong has been well studied in males for its role in mate attraction and territory defense [168]. However, some species, such as European Starlings (*Sturnus vulgaris*), also sing at high rates outside of the breeding context. In starlings, song in a non-breeding context is produced at high rates by both males and females in flocks and is considered essential for birds to develop and practice songs that are later used in breeding contexts [2, 169]. Song in a non-breeding context is also considered important for maintaining flock

cohesion [40]. Although much is known about the neural regulation of song learning and the production of male courtship songs, relatively little is known about the neural mechanisms that facilitate and maintain song in gregarious contexts, which will be referred to here as gregarious song.

Most of the existing literature has centered around the medial preoptic area, commonly abbreviated POM in birds and the mPOA in mammals. Here we refer to the structure as the mPOA to indicate that findings may generalize beyond birds, as highlighted below. The mPOA is a highly conserved structure that is well known for its role in sexually-motivated behaviors but more recently has also been shown to be important in the control of non-sexual social behaviors, including gregarious song [8]. Lesions of the mPOA tend to increase gregarious song, and the presence and activation of mu opioid receptors (inhibitory receptors) in the mPOA stimulates song, suggesting an inhibitory role for the mPOA in gregarious song [29, 170, 171]. Studies show that a positive affective state associated with gregarious song correlates positively with the expression of opioid-related genes in the mPOA [6], and downregulation of mu opioid receptors in the mPOA reduces singing and breaks the link between positive affect and singing behavior [29]. Results of a recent study also show that mu opioid receptor stimulation in the mPOA stimulates components of song (i.e., introductory whistles), reduces a potential sign of stress (i.e., beak wiping), and induces reward (i.e., a conditioned place preference), suggesting that opioid activity in the mPOA may both stimulate and reward gregarious singing behavior [34]. Although opioids have been best studied, studies focused on other candidate modulators of motivation and reward also implicate dopamine D1 receptors [34], endocannabinoids [36], and possibly alpha 2 norepinephrine receptors [35] in the mPOA in gregarious singing

behavior; however, these studies are limited by a focus on previously-known candidate genes. There are likely many more neurochemicals and signaling cascades within the mPOA driving this behavior that remain to be uncovered.

The mPOA is part of a social behavior network that is highly conserved across vertebrates [26]. We have already found that the role of mu opioid receptors in the mPOA identified by studies of gregarious song [3, 6, 29, 171] extends to other non-sexual rewarding social behaviors in rodents (i.e., rat social play) [66]. Thus, identifying novel neuromodulators within the mPOA associated with gregarious song is expected to reveal critical, conserved mechanisms that regulate other important non-sexual social behaviors across vertebrates.

This study aimed to uncover potentially novel neurotransmitters, neuromodulators, and hormone pathways within the mPOA associated with gregarious song. To do this, we observed male and female European starlings singing in flocks, then performed RNA-sequencing (RNA-seq) in punches from the mPOA to compare relative gene expression between birds that sang at low and high rates. We applied multiple bioinformatics approaches to gain insight into new candidate systems associated with gregarious song. These ranged from hypergeometric analysis to compare expression profiles across sexes, to using multiple methods of co-expression analyses to find likely "hub" genes associated with differences in gregarious song production that likely regulate other prosocial behaviors in vertebrates.

Materials and Methods

Animals

Twenty-two adult European starlings (11 males [with testes], 11 females [with ovaries]) were used in this study. All starlings were trapped from the wild on a farm on the west side of Madison, Wisconsin in October of 2019. No specific permissions were necessary for capturing starlings from the wild. They are considered invasive and are not endangered, protected, or covered under the Migratory Bird Treaty Act in the United States. After capture, all birds were housed in indoor stainless-steel cages at the University of Wisconsin-Madison. Birds were put on an 18L:6D cycle (lights on at 6:00am) for at least 6 weeks to induce a state of "photorefractoriness". This is a physiological state in early fall in which starlings begin to sing in large, mixed-sex flocks [172]. All studies were approved by the University of Wisconsin Institutional Animal Care and Use Committee and in accordance with the Guidelines of the National Institutes of Health.

Behavioral observations

Focal birds were moved to indoor aviaries (2.12 × 2.4 × 1.98 m) with natural tree branches and given *ad libitum* access to food, drinking water, and bathing water. Birds were in mixed-sex flocks of 8 birds (4 males, 4 females). Talk radio was played during daylight hours to acclimate birds to voices and extraneous noise. All observations were conducted from September 2020-November 2020 by the same observer behind a one-way mirror.

Observations began once 4 or more birds were singing within an aviary. For each observation period, a male or female focal bird was selected based on the rate of song observed during earlier observation periods. This strategy was employed to ensure a broad representation of song rate production in the birds included in our study. Focal observations were performed on the targeted bird in the aviary for 20 minutes a day, 5

days in a row. All observations took place between 0900 and 1200h each day. Immediately preceding each observation, a recording of starling song was played for 5 minutes to instigate singing from the aviary. Then song was measured by a point sampling method. Every 20 seconds during the observation period, the observer was cued by a quiet vibration of a smartwatch and recorded a tally if the bird was singing. The quantitative measure of the song was the number of tallies that were recorded over 5 days (maximum of 300 possible). The observer also continuously recorded locomotion, feeding, drinking, and agonistic interactions. All behavioral data are available in File S1.

Thirty minutes after the final observation period, the bird was caught by net in the aviary and sacrificed by rapid decapitation. We waited 30 min as mRNA for immediate early gene expression tends to peak 30 min after stimulation [173]; however, for this study we were interested in constitutive gene expression associated with the propensity to sing. Thus, we examined relationships between patterns of gene expression and song measurements summed across the 5 test days. The brain was flash-frozen using dry ice and stored in -80 °C freezer until observations of the last birds were completed in November. A replacement bird was then moved from the cages in the animal facilities to the aviary to maintain a total of 8 birds in an observation aviary. Once 4 or more birds were singing again, the above protocol was repeated until 11 male brains and 11 female brains were collected.

Tissue collection

Whole brains were cut in 200 μm sections in the coronal plane using a cryostat at -15 °C and mounted onto glass slides. Two mm punches (Fine Science Tools Sample Corer, 2mm, Item no. 18035-02) for the mPOA were taken from a single slice in each bird caudal

to the end of the visible tractus septomesencephalicus and rostral to the anterior commissure, in the center of the brain surrounding the 3rd ventricle (Fig. 1) then stored at -80 °C. An example photo of an mPOA punch can be found in Fig 1.

RNA processing and RNA-seq

All sequencing was performed by the University of Wisconsin-Madison Biotechnology Center's Next Generation Sequencing Facility. The comprehensive methods used for total RNA verification and processing for RNA sequencing, which align with our prior work [66], are as follows. Total RNA was extracted with the Aurum Total RNA Fatty and Fibrous Tissue Kit (Bio-Rad, Hercules, California). A NanoDrop One Spectrophotometer was used to verify RNA purity and an Aglient 2100 Bioanalyzer was used to verify RNA integrity. The purified RNA was stored at -80°C until it was sent for sequencing. The samples met Illumina input guidelines and were then prepared using the Illumina® TruSeq® Stranded mRNA Sample Preparation kit (Illumina Inc., San Diego, CA, USA). The mRNA was purified from 150ng total RNA using poly-T oligo-attached magnetic beads for each library preparation. Afterwards, each poly-A enriched sample was fragmented using divalent cations. SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) was then used to synthesize fragmented RNA into double-stranded cDNA. Random primers were used for first strand cDNA synthesis and then second strand cDNA synthesis using DNA Polymerase I. RNase H was used to treat the double-stranded cDNA to remove mRNA and was then purified by paramagnetic beads (Agencourt AMPure XP beads, Beckman Coulter). The cDNA products were exposed to Klenow DNA Polymerase, which attached an 'A' base (Adenine) to the 3' end of the blunt DNA fragments. DNA fragments were ligated to Illumina unique dual adapters with a single Thymine overhang on the 3' end. This as

amplified in a Linker Mediated PCR reaction for 12 cycles using Phusion™ DNA Polymerase and then purified using paramagnetic beads. The finished libraries were assessed for quality using an Agilent HS DNA chip (Agilent Technologies, Santa Clara, CA, USA) and assessed for quantity using a Qubit® dsDNA HS Assay Kit (Invitrogen, Carlsbad, CA, USA). Libraries were standardized on 2 nM and paired-end 2x150 base pair sequencing was performed using an Illumina NovaSeq6000 sequencer.

All samples (for 11 males and 11 females) had sufficient raw reads, so all were included in downstream analyses. For all analyses we used the 2021 zebra finch (*Taeniopygia guttata*) National Center for Biotechnology Information (NCBI) Release (2021-ZF) (GCA_003957565.4) for gene annotation. We also examined the slightly older 2019 zebra finch (*Taeniopygia guttata*) NCBI Release (GCA_003957565.2), and the European starling (*Sturnus vulgaris*) 2015 NCBI Release (GCA_001447265.1), but found results to be highly similar, so the 2021 annotation was used for all analyses because it is the most complete in terms of annotated genes. RNA expression was normalized using RSEM [174], a method allowing for improved RNA-seq assembly and quantification without a fully sequenced genome. The RSEM values for 2021 zebra finch and 2015 starling annotations can be found in File S1.

Differential gene expression analysis and rank-rank hypergeometric analysis

All analyses below were performed in R-Studio (v.1.3) with R (v.4.1.1).

Samples were separated by sex, then further separated by performing a median split based on song, creating a “low-singing” and “high-singing” group of birds within each sex (low-singing males; n = 6, high-singing males; n=5, low-singing females; n = 6, high-singing females; n = 5) (Figure 2). We then performed a differential gene expression (DGE) analysis

between the high and low singing groups for each sex (totaling 2 analyses) using the EdgeR Bioconductor Package, v. 3.1.2 [175]. Although this work concentrates on song-associated gene-expression changes, we also performed DGE analysis comparing the mPOA of both males and females to provide insight on sex differences in the mPOA in a non-breeding context.

Following this, rank-rank hypergeometric overlap (RRHO) analysis [176] was performed to compare patterns of gene expression direction across sexes. RRHO generated heatmaps that allowed us to see both congruent (same direction) and discordant (opposite direction) relationships across each sex's expression profile (e.g., which genes were consistently upregulated in both high-singing males and high-singing females, and vice versa).

WGCNA

We used the common systems biology approach of weighted gene co-expression network analysis (WGCNA) to create single scale gene co-expression networks and gene modules from gene expression data sets [177]. RRHO showed there was statistically significantly high overlap between the top 800 most differentially expressed genes between males and females, so the sexes were combined for network analyses. Before network analysis began, genes with low expression across samples within each dataset were removed, transitioning from 21,721 original genes to 9,648 filtered genes.

For the first analysis, a weighted network of genes (nodes) and their expression correlations (edges) were generated, with correlations raised to a soft-thresholding power β of 7. In this study, the β value for all WGCNA networks were chosen following the guidelines put forth by the authors of WGCNA [178]. We identified the β value as the point

in network topology analysis at which any increase in β would lead to only marginal improvements in the fit of the scale-free topology model. Initial parameters were as follows: unsupervised hierarchical clustering was used; signed mode; minimum module size = 30; deepSplit = 2; MergeCutHeight = 0.3. Due to our primary interest in neurotranscriptomic differences between the two groups of low- and high- singing birds, we also performed WGCNA's module preservation analysis to compare network connectivity between the two groups. Two separate networks were created, one with only low-singers and one with only high-singers. Additionally, genes not expressed in both low- and high- singing datasets were removed to ensure networks were comparable for module preservation analysis. WGCNA was performed on each dataset of 8,967 genes.

Two weighted networks of genes (nodes) and their expression correlations (edges) were generated, with correlations raised to a soft thresholding power β of 9 and all other parameters the same as above. We then used the modulePreservation WGCNA function to identify which modules of co-expressing genes showed no evidence of being preserved between the two analyses, implying possible functional differences between those modules [26]. Analysis was performed with parallelization disabled and nPermutations = 200.

Statistics for module preservation were used to determine if a specific module defined in the low-singing dataset (reference dataset) could also be found in the high-singing dataset (test dataset). Results are reported as a summary of Z-statistics (Zsummary). In short, the Zsummary is a composite measure of the density-based and connectivity-based preservation statistics generated by WGCNA's permutation test. For each module, four connectivity-based and three density-based preservation statistics are generated, each with its corresponding Z-statistic. These Z-statistics follow a normal

distribution if the module is not preserved; a higher Z-statistic value indicates a stronger likelihood that the preservation statistic surpasses what would be expected by chance. The Zsummary is calculated by taking the median of all the connectivity and density-based Z-statistics, summing them, and dividing by two. For a detailed mathematical explanation of this methodology, please refer to the original publication [179]. A Zsummary of >10 is indicative of high module preservation between the two networks (e.g., blue module in low-singers is almost indistinguishable from blue module in high-singers), Zsummary between 2 and 10 represents moderate module preservation, and Zsummary <2 indicates that there is almost no preservation between modules (e.g., genes in blue module in high-singers are almost entirely different from those in the blue module of low-singers). The hub genes in each module were chosen using the chooseTopHubInEachModule WGCNA function.

MEGENA & CTA

Multiscale embedded gene co-expression network analysis (MEGENA) is a hierarchical clustering framework that allows for the construction of gene co-expression networks and gene modules using planar-filtered networks and multiscale clusters [180]. This method identifies nested groups of highly correlated genes from a given tissue sample. MEGENA, unlike WGCNA, creates a multi-scale network, allowing one single network to include multiple variations of gene interactions (i.e., one gene can be found in multiple modules).

Our dataset included all animals, and genes were filtered out for low expression. 11,447 genes were processed by the MEGENA package in R. Parallelization (nCores = 8) was turned on to increase speed of analysis. The FDR cutoff, module significance p-value,

and connectivity significance p-value were all set at 0.05. The number of permutations for calculating FDR was set to 10 and the number of permutations for calculating the connectivity significance p-value was set to 100.

To link MEGENA gene modules to song, cluster-trait association (CTA) analysis was performed. CTA allows us to correlate expression patterns for groups of genes with performed behaviors. A principal components analysis was run on each of the modules using the ModulePrinComps function in the MEGENA package [180], and the first principal component of the expression levels of all genes within the module was used as a new variable, known as an eigengene (see File S4). This gives each gene module a representation as a continuous variable for each animal and allows for behaviors to be correlated with co-expressing groups of genes that are likely functionally connected. Pearson correlations were performed to compare module eigengenes with singing condition.

Enrichment analysis

Enrichment analysis was performed on gene sets produced by DGE and RRHO and on modules created by WGCNA and MEGENA. This method allowed us to input a custom list of genes and determine if those genes are over-represented in large gene sets with previously known functions. If a custom set of genes is overrepresented in a functionally known gene set, it is notated as “significantly enriched”. These previously identified gene sets include molecular pathways, drug actions, diseases, and many others. Enrichment of all MEGENA modules was performed using the moduleGO function in the DGCA package in R for general exploration [28]. Follow up enrichment tools for investigating our lists of interests included ToppCluster [181], GeneOntology [182, 183], and STRING [184]. All

basic gene functions listed were found using GeneCards [185]. STRING included the most widespread enrichment analysis, so all significant STRING enrichments for gene groups of interest are reported in the additional information.

Results

Males and females overlap in their patterns of differential gene expression

A full list of genes from most to least differentially expressed with directionality information of expression change (upregulated in high-singers = positive sign, downregulated in high-singers = negative sign) is provided in File S2. In males, 51 genes had an adjusted p-value (Benjamini-Hochberg False Discovery Rate (FDR)) of less than 0.05, with 1,389 genes having a raw p-value of less than 0.05. In females, only 4 genes had an adjusted p-value of less than 0.05 and had 888 genes with a raw p-value less than 0.05.

Previous work has shown that top differentially expressed genes that did not meet threshold for FDR were successfully validated with qPCR [186], so we expect many genes with a raw p-value of 0.05 to still be biologically meaningful. Due to the large amount of differentially expressed genes with a p-value of less than 0.05, we performed rank-rank hypergeometric overlap analysis to uncover genes concordant in males and females. Males and females showed remarkable similarity to one another, with high-singing and low-singing groups showing significant overlap across sexes (low-singers: $p < .0001$, high-singers: $p < .0001$) (Fig. 3). Out of the top 800 most differentially expressed genes (~top 7% of annotated genome), we found 305 genes consistently upregulated and 226 genes consistently down-regulated in male and female high-singing birds. Enrichment analysis in STRING for concordant expressing genes across sexes showed significant enrichment for neuromodulators associated with motivated behaviors such as dopamine and acetylcholine

as well as enrichment for GABA and glutamate (Table 1). Additionally, ToppCluster-enrichment showed many genes associated with autism, wherein affiliative social communication is heavily impacted.

Our DGE analysis comparing males and females found 1,194 genes with a raw p-value of less than 0.05, with 685 of these genes significantly upregulated in males relative to females, and 509 upregulated in females relative to males. Thirty-eight of the genes significantly upregulated in females were linked to the W chromosome (ChrW) of the 2021 Zebra finch NCBI genome annotation. These genes did not contain any notable enrichment. In this annotation, there were 165 ChrW-linked genes, but 123 of these genes were excluded due to low counts across groups. This left 4 ChrW genes not significantly upregulated in females relative to males. 383 of the genes upregulated in males were linked to the Z chromosome (ChrZ). These genes were enriched for multiple metabolic processes. There were 1,084 ChrZ-linked genes for this annotation, but 508 did not have differential expression due to low counts. This left 193 ChrZ genes not significantly upregulated in males relative to females. Full EdgeR results of DGE analysis comparing males and females (upregulated in females = positive sign, upregulated in males = negative sign) can also be found in Supplementary Material S2.

Birds show differences in gene connectivity across singing condition

For the initial WGCNA analysis, 15 modules were identified (see File S3), however, no module eigengenes significantly correlated with song. Although it did not reach significance ($p = .086$), the greenyellow module was most closely associated with song and consisted of 93 genes. It contained three glutamatergic signaling genes (GRM2, GRIN2A, SLC17A6), a cholinergic receptor gene (CHRNA5), and a GABA receptor subunit (GABRD).

For the module preservation analysis, we found multiple modules that were not preserved between low- and high- singers. For the high-singer dataset, WGCNA identified 16 modules of genes, while 12 modules were identified in the low-singer dataset. Module preservation analysis identified 3 gene modules with a Zsummary<2 and were therefore not preserved between the two analyses (Fig. 4). All module preservation analysis results and genes in the modules of interest can be found in File S3. One of these 3 was the grey module, which consists of genes that are "unassigned" to any module produced, so this module was not investigated further. The non-preserved pink module consisted of 241 genes and was significantly enriched (in STRING) for glutamate-related genes (Fig. 5). This includes CACNG3 and CACNG5, both AMPA receptor regulatory proteins, and this module's hub gene (the most connected gene) was SHISA6, which is thought to enable ionotropic glutamate receptor binding activity. The pink module also showed enrichment for GABAergic synapse, containing the GABA transporter SLC6A1 as well as the GABA receptor subunit GABRD, which was one of the previously identified differentially expressed genes. The pink module contained the immediate early gene EGR1, growth-hormone inhibiting hormone SST, in addition to two estrogen-related genes. This included GEPR1, an estrogen receptor, and CITED4, which enhances estrogen-dependent transactivation. The magenta module of 147 genes was also not preserved across singing condition, however, was not significantly enriched for any notable neural pathways.

A co-expressing module of glutamatergic genes significantly correlated with song

MEGENA identified 542 nested modules. CTA identified 145 of these modules' eigengenes (1st principal component) that significantly correlated with singing condition. Due to the numerous modules related to behavior, we performed an overlap analysis using

the GeneOverlap package [187] to determine which of these modules were significantly enriched for the 305 differentially-expressed genes in high singers. In our efforts to minimize interpretational noise due to extremely large module size, we further filtered those modules by removing those that contained more than 150 genes and had less than 5 differentially expressed genes, leaving 30 modules of interest (see File S4).

Seven of these modules were significantly enriched in STRING for glutamate pathways. Module #237 contained multiple glutamate receptor genes (GRM5, GRIA1, GRIA2, GRIN1, GRIN2B) and glutamate synapse scaffolding genes (SHANK2, HOMER2). This module contained genes that were significantly enriched for genetic pathways previously identified in human neurodevelopmental disorders linked to social impairments. Hub genes for module #237 were GRIA1, ADGRB3, and SIPA1L1 (Fig. 6). Two other modules were significantly enriched for thyroid hormone receptor binding including nuclear receptor co-repressor and co-activator (NCOR1, NCOA6). See Table 2 for information regarding the parent module (the larger module that a module of interest is nested in), hub genes, and eigengene-song p-values for standout MEGENA modules of interest. We plotted the relationships between the genes of interest identified by MEGENA and found significant positive Pearson correlations between song and GRIN1 and SHANK2 expression (Fig. 7) based on the raw p-value, however, we did not correct for multiple testing. The other 21 modules showed no notable significant enrichment and were not investigated further.

Discussion

Using multiple bioinformatics approaches we have identified several candidate genes associated with the production of gregarious song in European starlings. Male and female birds show consistent patterns of song-associated gene expression, with elevated

dopamine-, acetylcholine-, and GABA-related expression in the mPOA associated with high singing rates. We also identified a co-expressing complex of glutamate-related genes closely associated with song, most of which have been previously linked to phenotypes wherein social interactions are heavily dysregulated [188, 189].

Male and female singing groups show remarkably similar gene expression profiles

Our comparison of DGE results through RRHO revealed that low- and high-singing birds had similar genes upregulated and downregulated across sexes. We found this particularly interesting as low- and high-singing groups were created by median split of singing rate within each sex, not a median split across singing rates for all birds combined. In terms of raw singing rate, high-singing females (mean song = 17.2) were less similar to high-singing males (mean = 64.8) than low-singing males (mean = 8.667). Despite this, our RRHO comparison showed that the high-singing females' expression profile was more similar to that of high-singing males, with over 500 genes in concordant expression out of the top 750 most differentially expressed genes. This is noteworthy as it suggests that these genes are not simply representative of motor activity.

We did not find any obvious sex differences in song-related directionality as our most differentially expressed genes across singing condition remained largely the same across males and females. However, in our DGE analysis comparing males and females independent of singing condition, we found over 200 genes significantly differentially expressed between sexes. Although many of these genes were enriched for various metabolic processes, we did not find significant differences in sex steroid specific metabolism genes, such as CYP19A1 or the SRD5A variants (see additional files), which encode for aromatase and 5α-reductase respectively. This may be surprising given that the

mPOA is a steroid sensitive, sexually differentiated brain region [190, 191]. However, sex differences in protein or gene expression in the mPOA are commonly linked to steroid hormone concentrations [192], which are extremely low in non-breeding condition starlings [193]. It thus may be in the absence of high circulating testosterone and estradiol that sexually differentiated expression related to sex steroids is absent. We do see that androgen receptor (AR) and estrogen receptor (ESR1 and ESR2) expression may be marginally higher in female high-singers, but not in males. The biological relevance of these differences must be explored in future research [171, 194].

We also found that 38 genes linked to the avian female-specific ChrW were upregulated in females relative to males. This result is consistent with the expectations set by the sex-specific nature of the ChrW [195, 196]. Only 193 ChrW genes analyzed by DGE did not show significant differential expression across the sexes and 123 had insufficient counts to perform DGE analysis. Considering there is limited female-specific positive selection of genes on avian the ChrW, this outcome is not unexpected [197]. It is plausible that many of these genes may have redundant paralogs on the Z chromosome and others, reducing detectable expression differences across sexes. These findings contribute to a growing body of knowledge regarding the female songbirds' genome that has been historically understudied.

Neurotransmitter systems previously implicated in social behavior are differentially expressed across singing condition

We found that multiple dopamine-related genes were differentially expressed across singing condition. These included D5 (a D1-like receptor) and D2 receptors, a dopamine transporter (SLC18A2), a protein phosphatase (PPP3CC), and neurotensin

receptor (NTSR1). Dopamine in the mPOA in birds and rodents stimulates sexual motivation [198-200], including sexually-motivated birdsong [201]; and the mPOA directly accesses the mesolimbic dopaminergic system through projections to the ventral tegmental area (VTA), which is well-known to be involved in motivation in numerous sexual and non-sexual contexts [202-204]. It is thus possible that differential patterns of dopamine-related gene expression in the mPOA regulates motivational aspects of singing behavior.

Prior research on starlings demonstrates that optimal levels of D1 dopamine receptor stimulation in the mPOA facilitate sexually-motivated male song [205, 206]; however, its role in gregarious song has not been extensively studied. Previous research has found strong, positive linear correlations between D1- but not D2-like receptor density within the mPOA and gregarious song in male starlings [34]. This is inconsistent with recent work studying undirected song in zebra finches, which is similar to gregarious song in that it is performed in flocks in a non-sexual context [5, 8]. It was found that peripheral administration of the D2 receptor antagonist haloperidol reduced the rate of undirected song in male zebra finches, while the D1 receptor antagonist SCH23390 had no effect [207]. One likely reason of these conflicting findings is simply because antagonists were not injected directly within the mPOA; however, the effects of dopamine receptor manipulations in mPOA on gregarious song have not been tested. Our findings that D5 and D2 are upregulated in birds singing high rates of gregarious song suggest that dopamine receptors in mPOA may regulate song across contexts but this must be experimentally tested.

We also found multiple acetylcholine genes upregulated in high-singing starlings, including choline O-acetyltransferase (CHAT), a choline transporter (SLC5A7), and a

muscarinic cholinergic receptor (CHRM4). Multiple immunolabeling studies have shown high concentrations of cholinergic proteins in the song control system in the zebra finch brain [208-210], and cholinergic stimulation of the HVC via carbachol modulates zebra finch song [211]. However, the literature regarding its role in brain regions involved in social behavior is limited. A study in rats found that a direct injection of a cholinergic receptor agonist into the anterior hypothalamic-preoptic area induced 22kHz ultrasonic vocalizations, which are associated with an anxiety-like, negative emotional state [212, 213]. Although gregarious song is a vocalization associated with positive emotional state [8], it may be that acetylcholine acts within the mPOA to induce emotionally-salient vocal behavior, however, this needs to be investigated further.

Genes for GABA Type A ($GABA_A$) receptor subunit beta3 (GABRB3), alpha5 (GABRA5), and delta (GABRD) were upregulated in high-singing birds. GABRD was also a member of the module most associated with song in our first WGCNA analysis. Individual gene deficiencies in both GABRA5 and GABRB3 are strongly associated with reduced prosocial behavior in mice, including reductions in social exploration, contact, and vocalizations [214-216]. Similarly, dysregulated GABRD expression throughout pregnancy causes mouse dams to keep an increased distance from their pups postpartum [217]. These studies involved global manipulation of these genes and therefore did not identify specific regions of action. Our findings suggest that the mPOA is one region wherein $GABA_A$ receptor binding may influence social interactions, making it a promising region to perform viral manipulations of $GABA_A$ subunit receptor genes to understand their specific contributions to the regulation of behavior across social contexts.

Given that a body of research, reviewed in the introduction, demonstrates a role for mu opioid receptors in the mPOA in gregarious song and the reward that accompanies singing behavior, it was somewhat surprising that opioid-specific genes did not turn up in our analyses. This was unexpected, as downregulation of mu opioid receptors in the mPOA reduced song [29]. However, a previous study in the nucleus accumbens revealed the effect of opioid receptor stimulation on gregarious song to be small, only occurring when the highest receptor agonist dose was given [3]. Given this, it is possible that the expression signal of opioid related genes in the mPOA may also not have been a high enough magnitude to be detected by RNA-seq. Additionally, past studies reveal curvilinear, inverted U-shaped relationships between mu opioid receptors and gregarious song [133], which may be missed by the current analysis and is something to examine in future studies.

Multiple approaches implicate glutamatergic signaling genes in the mPOA in the production of gregarious song

DGE, RRHO, and both co-expression network analyses we performed uncovered numerous glutamate-related genes related to singing condition. This is particularly of note, as WGCNA and MEGENA use single-scale and multi-scale topologies, respectively, to cluster functionally related genes together, and both analyses highlighted glutamatergic systems as being related to singing condition. Many of these glutamatergic-related genes coincided with enriched pathways for major depressive disorder and neurodevelopmental disorders, which are both characterized by social dysregulation, and depression is specifically associated with anhedonia [218-220]. Given that gregarious song is highly social and tightly associated with a reward (i.e., hedonic) state [42, 171], these findings suggest that

the mPOA is a key, yet understudied, site in which glutamate regulation acts to influence rewarding, gregarious social interactions [8].

One of the modules related to song that we identified using MEGENA contained seven glutamate receptor and synapse scaffolding genes, all of which have been genes of interest in studies of neurodevelopmental disorders with socio-communicative deficits [188, 189, 221, 222]. One gene functionally implicated in the regulation of social interactions we found that significantly correlated with song was NMDA receptor subunit, GRIN1. GRIN1 knockdown mice have been identified and used as an autism model [189, 223, 224], and loss of GRIN1 activation in corticotropin-releasing factor neurons in male naïve mouse increases social stress [225]. Another functionally similar gene we found correlated with gregarious song was SHANK2, a glutamatergic synapse scaffolding gene [226, 227]. SHANK2 knockdown mice have also been proposed as an autism model [188, 228, 229], and its knockdown has been shown to induce deficits in sociability in naïve mice [230] and reduce parental bond in dams [231]. Interestingly, it was found that activation of the mPOA via DREADDS reestablishes social bonding in these mothers, highlighting the region-specific importance of SHANK2 in affiliative interactions. Our identification of GRIN1 and SHANK2 as possible candidate genes that act within the mPOA to regulate gregarious behaviors is consistent with this literature.

One hub gene identified in WGCNA associated with singing condition was SHISA6, an auxiliary AMPA receptor subunit [232], while another hub gene identified in MEGENA was GRIA1, another AMPA receptor subunit. Although there is no research on the functional role of SHISA6 in the mPOA, recent work in mice has shown that increased SHISA6 expression in the nucleus accumbens (NAc) is linked to decreased social

interaction in mice [233]. Additionally, antagonism of AMPA receptors in the NAc increases social interaction in social avoidant rats [234]. The NAc is indirectly connected to the mPOA, as it is part of a motivation and reward circuit that receives input from the VTA, which as previously mentioned, receives input from the mPOA [202-204]. The NAc also contains direct, reciprocal connections with both the VTA and mPOA [25, 131, 235]. The NAc has also been shown to regulate gregarious song in starlings [3, 4, 171], so our mPOA results suggest the possibility that AMPA receptors act across this reward circuit to modulate the production of song. Due to their identification as hub genes associated with the glutamatergic AMPA receptor complex, SHISA6 and GRIA1 standout as excellent candidates for direct manipulation in the mPOA to uncover their specific role in the motivation and production of gregarious song.

Conclusions

Using multiple bioinformatics approaches, we have identified multiple candidate genes within the mPOA that may play a role in producing gregarious song in starlings further enhancing our understanding of the genetic underpinnings of a complex social behavior. Standout genes include DRD2, GRIN1, and SHANK2, due to their previously identified in role in prosocial, affiliative behaviors. Although RNA-seq allows for a thorough analysis of the neuromodulators within a given region, it is important to note that this research is exploratory in nature and targeted manipulations of these candidate genes are required before drawing definitive conclusions of their role in gregarious song. Additionally, this study used bulk RNA-seq, which does not consider the various cell types in the mPOA. In an analysis of the mouse POA, over 70 different neuron and glial cell types were identified [236]. The starling mPOA likely contains similar cellular diversity, so it is

possible our analysis missed song-related genes that are limited to specific cell-types. Lastly, we did not perform individual hub gene validation using common methods such as quantitative polymerase chain reaction (qPCR). We note that the effectiveness of qPCR to validate RNA-seq is not clear due to differences in probe bias between the methods [237-240], so we do not believe this reduces the validity of our findings.

This study provides a detailed characterization of the genetic profile of an integral part of the brain's social behavior network, and the included data will allow other researchers to examine and compare genes across other social behaviors and species. Furthermore, because of the highly conserved nature of the mPOA across vertebrates, our candidate genes of interest may regulate prosocial, affiliative behaviors across taxa.

Declarations

Ethics approval and consent to participate

Institutional Animal Care and Use Committee at the University of Wisconsin-Madison reviewed and approved the animal study.

Consent for publication

Not applicable.

Availability of data and materials

All datasets generated and/or analyzed during the current study are included in this published article [and its supplementary information files].

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

BJP, SCG, SAS, and LVR contributed to concept and design of the study. BJP collected all behavioral data. SAS collected tissue and extracted RNA. BJP and SCG performed all bioinformatics analyses. BJP wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted manuscript.

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Additional File Information

S1. RSEM expected count values and behavioral data.

S2. All differential gene expression results.

S3. All WGCNA results.

S4. All MEGENA results.

Table 1. Genes of interest that differ in the mPOA of low- and high-singing European starlings.

Gene Symbol	Gene	Function	Group difference	p-value
Acetylcholine-related				
CHAT	choline O-acetyltransferase	Catalyzes acetylcholine production	High > Low	female = 0.009, male = 0.001
SLC5A7	solute carrier family 5 member 7	High-affinity choline transporter	High > Low	female = 0.002, male = 0.012
CHRM4	cholinergic receptor muscarinic 4	Acetylcholine receptor	High > Low	female = 0.026, male < 0.001*
Dopamine-related				
SLC18A2	Solute Carrier Family 18 Member A2	Monoamine transporter	Low > High	female = 0.004, male = 0.003
DRD2	Dopamine Receptor D2	Dopamine receptor	High > Low	female = 0.087, male = 0.036
DRD5	Dopamine Receptor D5	Dopamine receptor	High > Low	female = 0.03, male < 0.001
NTSR1	Neurotensin Receptor 1	Neurotensin receptor	High > Low	female = 0.07, male = 0.017
PPP3CC	Protein Phosphatase 3 Catalytic Subunit Gamma	Downstream regulation of dopaminergic signaling	High > Low	female = 0.045 male = 0.029
GABA-related				
GABRB3	Gamma-Aminobutyric Acid Type A Receptor Subunit Beta3	GABA receptor subunit	High > Low	female = 0.012, male = 0.004
GABRA5	Gamma-Aminobutyric Acid Type A Receptor Subunit Alpha5	GABA receptor subunit	High > Low	female = 0.066, male < 0.001*
GABRD	Gamma-Aminobutyric Acid Type A Receptor Subunit Delta	GABA receptor subunit	High > Low	female = 0.014, male = 0.004
LRRTM1	Leucine Rich Repeat Transmembrane Neuronal 1	Regulates presynapse assembly in GABA synapses	High > Low	female = 0.03, male = 0.029
Glutamate-related				
GRIN1	Glutamate Ionotropic Receptor NMDA Type Subunit 1	Glutamate receptor subunit	High > Low	female = 0.03, male = 0.002
GRIK3	Glutamate Ionotropic Receptor Kainate Type Subunit 3	Glutamate receptor subunit	High > Low	female = 0.047, male = 0.007
GRIA2	Glutamate Ionotropic Receptor AMPA Type Subunit 2	Glutamate receptor subunit	High > Low	female = 0.011, male < 0.001
GRM5	Glutamate Metabotropic Receptor 5	Glutamate receptor	High > Low	female = 0.051, male = 0.003
GRIA1	Glutamate Ionotropic Receptor AMPA Type Subunit 1	Glutamate receptor subunit	High > Low	female = 0.011, male < 0.001
SHANK2	SH3 And Multiple Ankyrin Repeat Domains 2	Encodes scaffolding for excitatory synapse	High > Low	female = 0.011, male = 0.004

*Indicates significant false discovery rate

Table 2. MEGENA modules of interest.

Module	Parent Module	Number of Genes in Module	Hub Genes (number of connections in module)	Eigen-gene-Song p-value
31	8	59	KCNS2(17) HIVEP2(14) FLRT2(14)	0.027
203	31	54	KCNS2(17)	0.026
237	48	114	ADGRB3(22) SIPA1L1(21) GRIA2(17)	0.025
602	237	16	GRIA1(12)	0.021
603	237	17	DOCK3(11)	0.01
300	71	17	EPHA7(10)	0.011
345	84	115	NCOA6(21) CELSR3(20) RBM5(18) RAP2A(17) KMT2A(16)	0.043
783	345	99	NCOA6(21) CELSR3(20) RBM5(18) RAP2A(17) KMT2A(16)	0.027
572	203	46	KCNS2(17) HIVEP2(14) FLRT2(14)	0.028

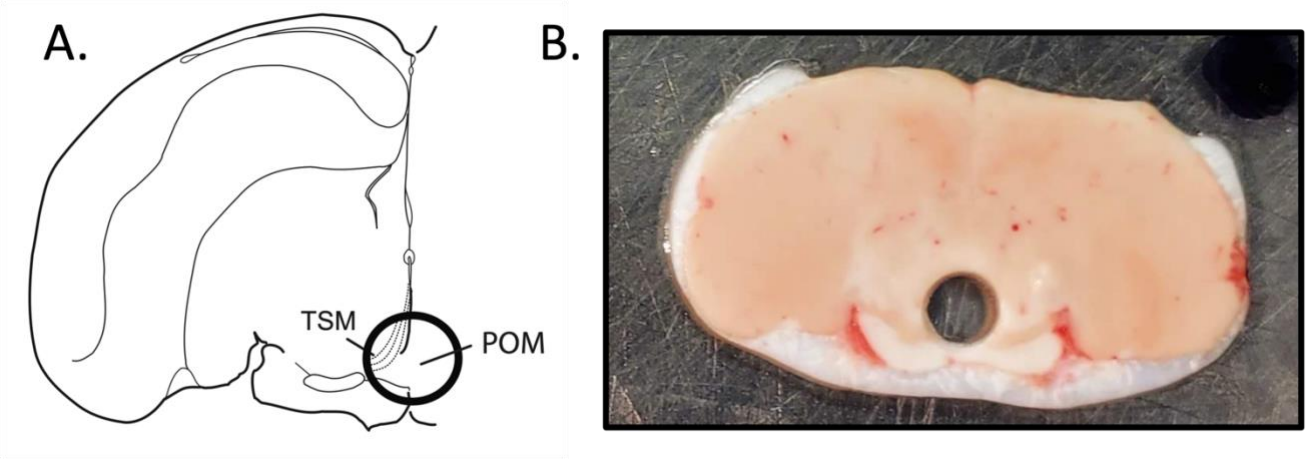

Figure 1. A) Illustration created in-house of one hemisphere of the starling brain showing the location of the mPOA where punches for RNA-sequencing were taken. Punches (2mm diameter) were taken slightly caudal to the end of the visible tractus septomesencephalicus (TSM) and rostral to the anterior commissure, in the center of the brain surrounding the 3rd ventricle. B) Example image of one of the mPOA punch areas.

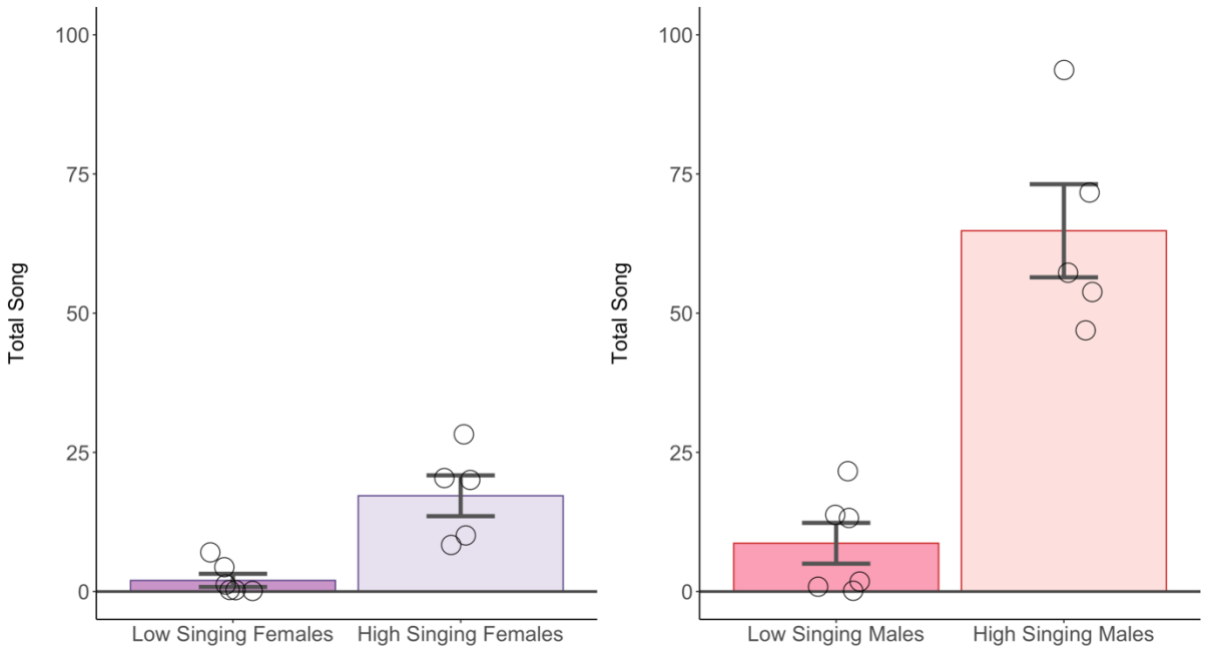

Figure 2. Shows total song of individual birds in the 4 groups used in differential gene expression and rank-rank hypergeometric overlap analysis. Birds were separated by sex, and a median split based on the total point-sampled song over 5 days was performed among males and females. Both low-singing and high-singing males sang more than their respective singing conditions in females.

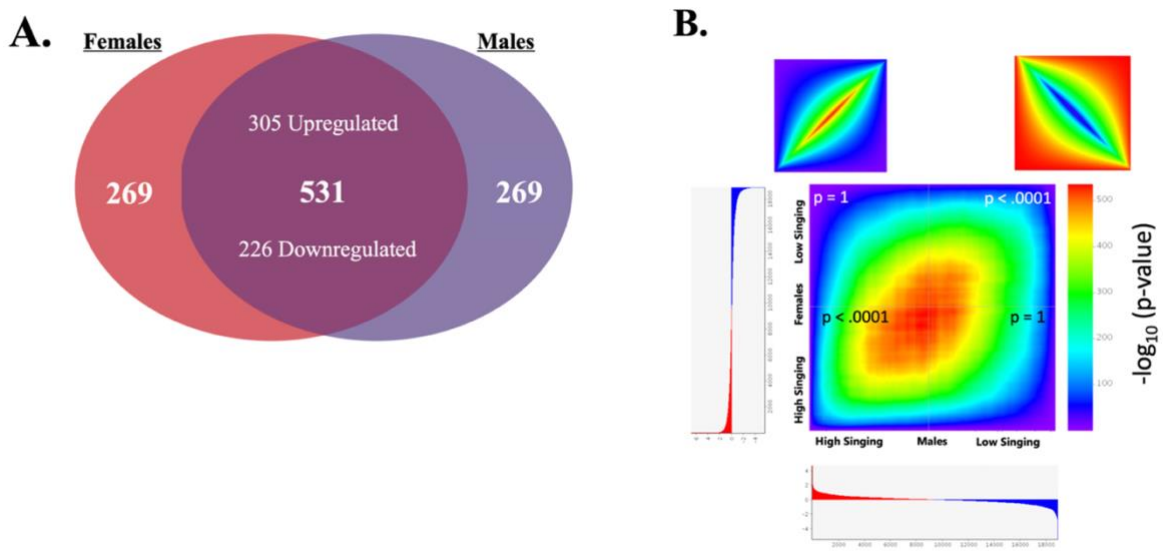

Figure 3. A) Venn diagram showing the overlap in expression of the 800 most differentially expressed genes relative to high singers when comparing males and females. B) Heatmap generated by rank-rank hypergeometric overlap analysis comparing high-singing and low-singing males and females in the medial preoptic area (mPOA) with p-values indicating the significance of the overlap between the top 800 most differentially expressed genes within each quadrant. Warmer colors represent higher proportions of overlap, while cooler colors represent lower portions of overlap. Examples of perfect correlations and perfect anti-correlations are included above for reference. The mPOA shows very similar expression profiles in males and females, with significant overlap in concordant gene expression, but no significant overlap in discordant gene expression.

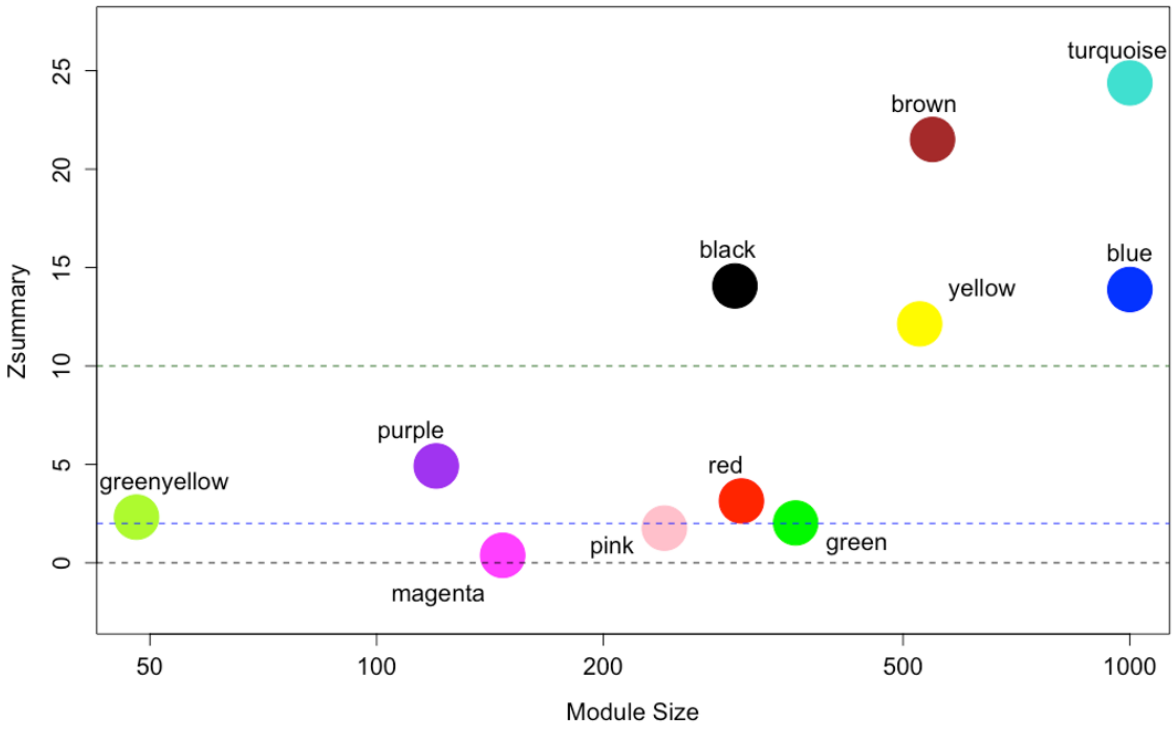

Figure 4. WGCNA module preservation analysis results between low-singing and high-singing starlings depicted as a Zsummary, a composite value of Z-statistics representing module connectivity and density, mapped against module size. The grey module is excluded. If a module had a Zsummary <2, it was considered not preserved across conditions. Pink and magenta were the only modules not preserved across condition, while all other modules maintained some form of preservation in both singing groups.

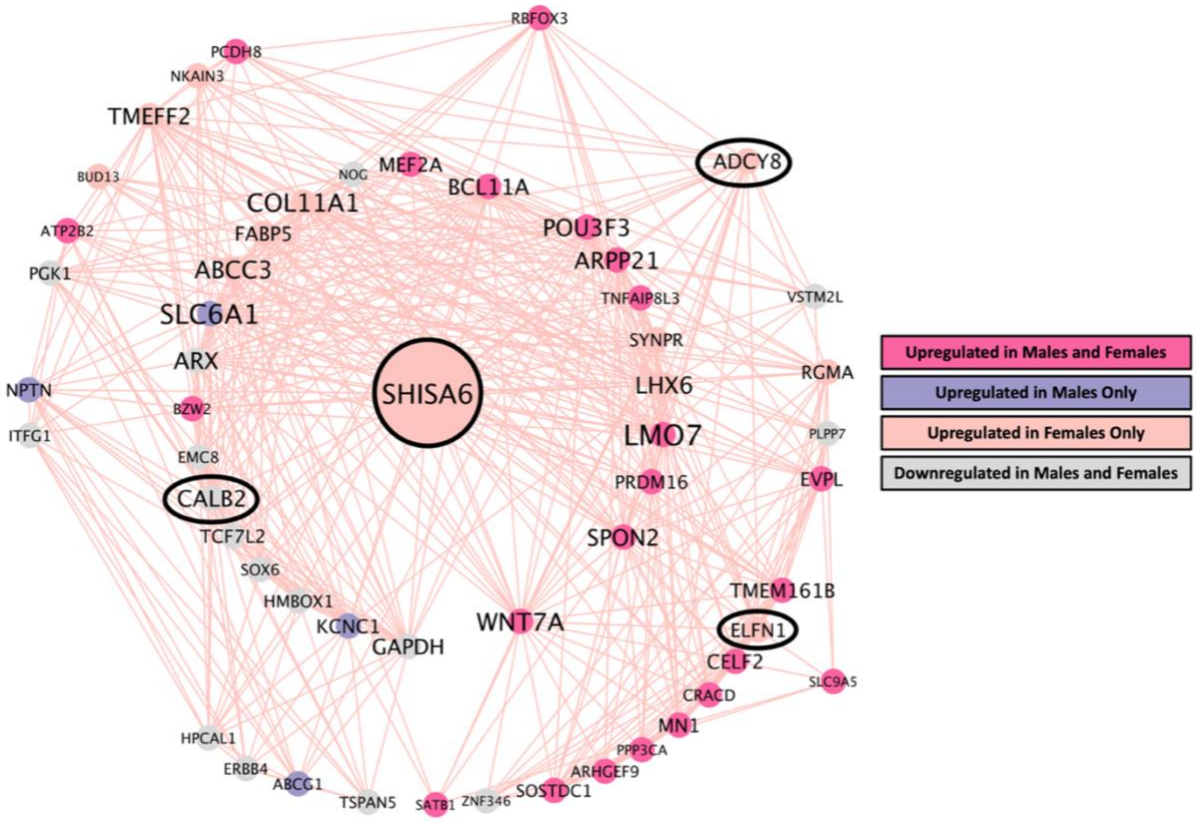

Figure 5. Top connected genes in the WGCNA pink module, a module that was not preserved across singing condition in the mPOA. Genes with less than 15 connections were excluded from visualization. Increased font size for the gene symbols indicates a greater number of connections between genes. Genes previously identified for a role in glutamatergic signaling are indicated by black circles. The large gene in the center (SHISA6) is the hub gene of this module identified by WGCNA. Protocols for identifying modules of interest are provided in the "Methods" section.

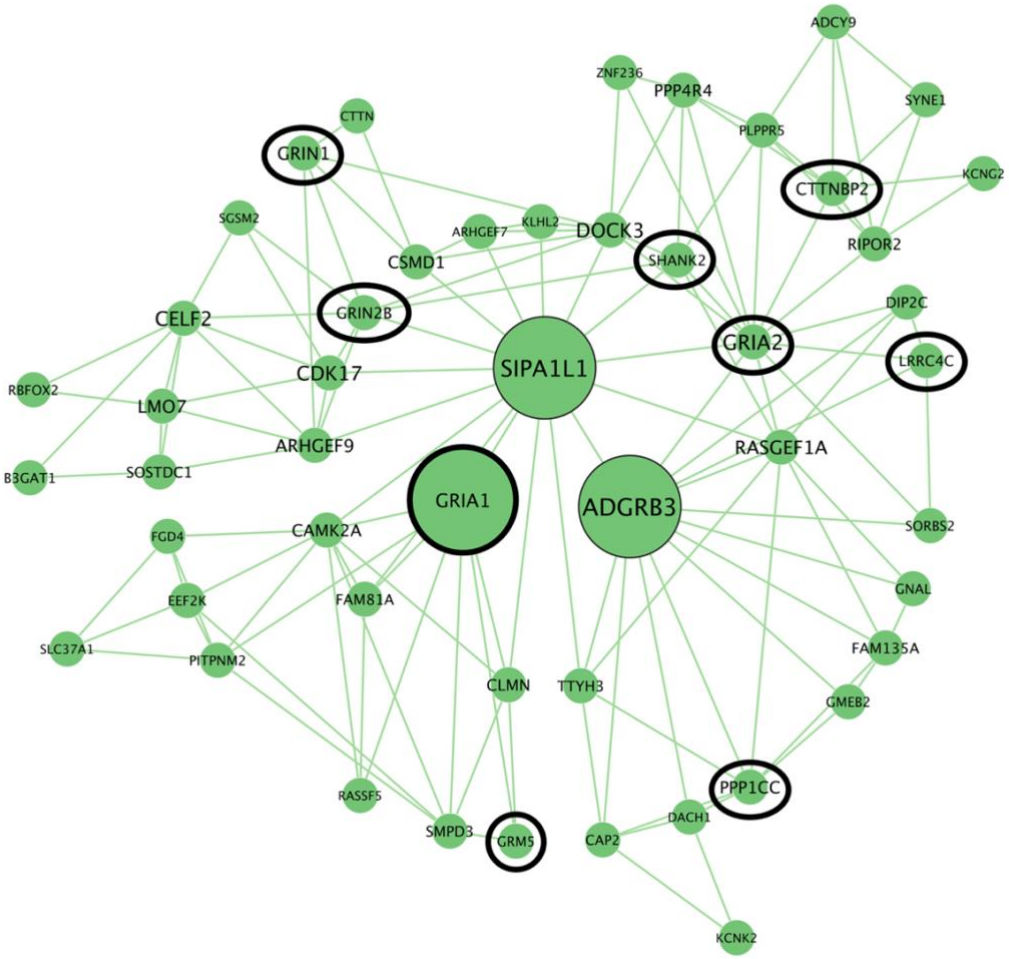

Figure 6. Top connected genes in MEGENA module #237, whose eigengene correlated with singing condition in the mPOA. All genes were upregulated in both males and females. Increased font size for the gene symbols indicates a greater number of connections between genes. Genes with fewer than 5 connections were excluded from visualization. Genes previously identified for a role in glutamatergic signaling are indicated by black circles. The large genes in the center (GRIA1, SIPA1L1, ADGRB3) are the hub genes of this module identified by MEGENA. Protocols for identifying modules of interest are provided in the "Methods" section.

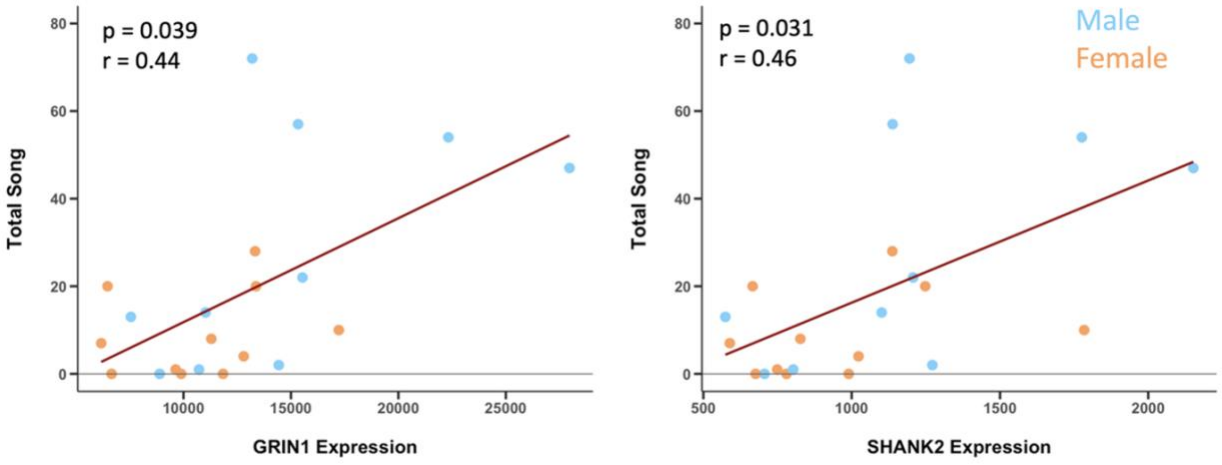

Figure 7. Pearson correlations between the expression (RSEM expected counts) of two glutamate related genes (GRIN1, SHANK2) and total song. Both genes were part of MEGENA modules whose eigengene significantly correlated with singing condition in cluster trait analysis. Total song is total number of point samples recorded over 5 days of observation. Dark red line indicates significant correlation based on raw p-value. Please note that these do not include corrections for multiple testing.

Chapter 4

Playful Song: A Shared Neurotranscriptomic Profile of Gregarious Song in European Starlings and Social Play in Juvenile Rats

Brandon J. Polzin, Changjiu Zhao, Stephen C. Gammie, and Lauren V. Riters

Abstract

Rough-and-tumble play in rats and gregarious song in European starlings (*Sturnus vulgaris*) are two distinct forms of non-sexual social behavior. Both are believed to play a role in the development of sociomotor skills such as aggression and reproduction, providing opportunities for low-stakes practice. Additionally, both behaviors are thought to be intrinsically rewarded and are associated with a positive affective state. Given the functional similarities of these behaviors, this study used RNA-sequencing to identify commonalities in their underlying neurochemical systems within the medial preoptic area (mPOA). This brain region is implicated in multiple social behaviors, including song and play, and is highly conserved across vertebrates. Through the use of DESeq2 and rank rank hypergeometric overlap analysis, I identified a shared neurotranscriptomic profile in highly gregarious (HG) animals (high-singing starlings and high-playing rats). Several glutamatergic receptor genes, such as GRIN1, GRIN2A, and GRIA1, were upregulated in HG animals. This aligns with previously identified candidates for social behavior within the mPOA of both male and female starlings. Moreover, multiple microglia-specific genes were

upregulated in HG animals, suggesting a potential role for microglial synaptic pruning in the mPOA to regulate gregarious song. This study is the first to directly investigate shared neuromodulators of gregarious behaviors across songbirds and mammals. It provides insight into a conserved neural circuit that may regulate non-sexual social behaviors across vertebrates.

Introduction

Given the wide diversity of vertebrates, it is notable that functionally similar, adaptive social behaviors have repeatedly evolved independently of each other. Examples of this include monogamy and the formation of large social groups, seen across multiple species of birds and mammals [241, 242]. The recurring evolution of these behaviors suggests they are beneficial for survival in a naturally competitive environment. In the aforementioned examples, the manifestations of these behaviors look similar to one another across taxa. However, sometimes functionally similar behaviors are "beneath the surface". That is to say, behaviors may serve a similar function despite being expressed differently across species with varying natural life histories.

One such behavior is play. Play behavior is largely influenced by natural history, as it has been proposed to allow animals to practice for social interactions and motor sequences they will use at a later timepoint, such as in reproductive behavior or agonistic interactions [243, 244]. Given this function, the manifestation of play is highly dependent on the recurring social behaviors present within that species.

The neural regulation of play has been particularly well-studied in rats, as juveniles perform rough-and-tumble play behavior at high rates throughout their postnatal development [103, 245]. This has been proposed to prepare rats for the locomotory skills

necessary for fighting with rivals, or even the motor skills associated with courtship [246]. Although fundamentally different in its presentation, it has been proposed that gregarious song performed in non-breeding, flocking European starlings (*Sturnus vulgaris*) is a type of play behavior [1]. In addition to meeting the criteria of play presented by Burghardt [244], it also functions as an opportunity for birds to prepare for social interactions with other birds and practice complex song "before it counts" for attracting a mate and maintaining a territory in the breeding season [2].

Both of these behaviors – play and gregarious song – are strongly associated with reward, as the performance of these behaviors is associated with development of a conditioned place preference [42, 247, 248]. In addition to their intrinsically rewarding nature, there are multiple neurobiological mechanisms in common between juvenile play and gregarious song, including the role of opioids in two highly conserved brain regions, the mPOA [66, 133] and nucleus accumbens [3, 17]. Despite this similarity, there have been no direct comparative studies investigating largescale neural similarities in the regulation of these two intrinsically motivated, gregarious behaviors.

In Chapter 3 of this dissertation, I identified potential candidates within the medial preoptic area (mPOA) that are likely involved in the production of gregarious song. Given the highly conserved nature of the mPOA, I hypothesized that many of these genes likely function to modulate intrinsically rewarded, gregarious behaviors across vertebrates due to their evolutionarily analogous function. In this chapter, I aim to test this hypothesis by performing a large-scale comparison of each species' transcriptome using RNA-sequencing. The goal is to determine if there is a conserved neurotranscriptomic profile associated with gregarious song and rough-and-tumble play in starlings and rats, respectively. An

additional goal is to identify novel, conserved modulators of these behaviors for future investigation.

Methods

All methods used to attain starling data are identical to those in Chapter 3 unless otherwise specified.

Behavioral testing

A total of 16 male rats 27-28 days old (PN27-28) upon arrival in our animal facility were used. Males were pair-housed and allowed to acclimate in our colony for 3 days. On PN31-32, animals were divided into two groups. For group 1 (n=8), rats remained in their home cages and behavior was recorded for 20 minutes for 5 consecutive days. For group 2 (n=8), rats from two cages (totaling four individuals) were introduced to each other and social play was recorded for 20 minutes for 5 consecutive days and then they were placed back in pairs. These two groups were used to avoid socially isolating rats, while ensuring one group played at a higher rate than the other. Play behaviors recorded for each animal included pouncing, pinning, wrestling, biting, and chasing. Immediately after the last behavioral test (PN35-36), rats were anaesthetized with isoflurane and decapitated.

Tissue preparation

Rat brains were flash-frozen on dry ice, and 200 μm coronal sections were sliced on a cryostat and stored at −80°C until processing. The mPOA (Bregma −0.24 to −0.48 mm) was collected according to *The Rat Brain in Stereotaxic Coordinates* [249]. RNA extraction and RNA-seq library preparation for the rats were identical to those used for the starling tissue in Chapter 3.

RNA-seq

All samples contained sufficient raw reads, so all 16 were included in downstream analyses. We used the 2014 Norway rat (*Rattus norvegicus*) NCBI release (GCA_000001895.4) for gene annotation. Identical to the starling, RNA expression was normalized using RSEM [174].

Differential gene expression analysis

All analyses below were performed in R-Studio (v.1.3) with R (v.4.2.1) [70].

Although differential gene expression analysis (DGE) for the starlings in Chapter 3 was performed using edgeR [175], I switched to use another popular tool, DESeq2 [250], for this study, given it has the highest power relative to the false discovery rate for our sample size [251].

One rat from housing group 1 exhibited a higher rate of play (177 bouts) than the median play behavior rate (145 bouts), while one rat from housing group 2 displayed a lower play rate (121 bouts). Due to my interest in the effect of rates of play instead of housing group, rats were separated by a median split of the sum of all 5 play behavior types across all 5 days, establishing a low-playing (n=8) and high-playing group (n=8) (Fig. 1). DESeq2 was performed, with the median split play groups and the original housing groups (group 1 v. group 2) as independent variables. This was done to statistically control for the initial social housing condition.

For starlings, DGE was re-run to ensure direct methodological compatibility for subsequent analyses. DESeq2 was performed using both a median split singing group and a median split locomotion group (a sum of flying, hopping, and switching to a new perch) as independent variables. I chose this approach to ensure that any identified similarities

between groups with high-play and high-singing behaviors could be attributed more convincingly to their social functions. This consideration was particularly important given the highly motorized nature of play behaviors in rats. Both male and female starlings were combined in the singing DGE comparison since the expression profiles of high and low singers were found to be strikingly similar between sexes, as discovered in Chapter 3.

Ortholog identification and rank rank hypergeometric overlap analysis

Ortholog identification was performed using OrthoDB v11 [252]. Given there have been multiple speciation events between starlings and rats, and to ensure that DGE results were comparable across species, I examined patterns of expression at the level of orthologous groups (OGs) instead of individual genes. An OG encompasses all descendants from a specific gene present in the last common ancestor of these two species. The most recent ancestor available in OrthoDB was at the Tetrapoda level. The DESeq2 output for singing starlings contained 21,721 genes, whereas that for rats contained 32,883 genes. After identifying shared OGs, this number was subsequently reduced to 10,038 OGs for starlings and 10,721 OGs for rats. It is important to note that some OGs contained multiple paralogs (duplicated genes within a species), while others only contained one specific gene.

To compare the expression profiles of high-singing starlings with high-playing rats, we performed rank rank hypergeometric overlap analysis (RRHO) to identify concordant and discordant patterns of gene expression between “highly-gregarious” and “less-gregarious” species. Similar to DGE, I adopted an updated variant of this method, called RRHO2 [253]. This differs from the original method in that it refines the heatmap visualization to provide a more intuitive interpretation of patterns of discordant gene expression (i.e., if there are no significant genes upregulated in high-singing birds and

down-regulated in high-playing rats, the top left and bottom right quadrants are completely uniform in color). RRHO2 required both gene lists being compared contained identical OGs, so each of the final inputs contained 9,329 OGs.

Due to multiple paralogs within some OGs, it was necessary that one gene was selected to represent its respective OG for each species for RRHO comparison (RRHO requires the log10 of the p-value of a singular gene given by DESeq2). Previous research conducting cross-species comparisons selected the gene with the highest differential expression in each OG for use in RRHO [38]. However, this method makes a major assumption that could be incorrect, as it is not guaranteed all paralogs within each OG differentially express in the same direction. To avoid this, I adopted a bootstrapping approach. The following was performed 200 times: 1) One paralog within each OG was randomly selected to represent that particular OG in the DESeq2 output that was used in RRHO2, 2) Genes with insufficiently low counts were removed from the DESeq2 results (p-value = NA), 3) Since RRHO2 requires input datasets to be identical, all OGs that did not match between the starling and rat dataset were removed, 4) RRHO2 was performed (stepsize = 100) and significant OGs in each quadrant were recorded. After bootstrapping, only significantly overlapped OGs found across 95% of the iterations (190/200) were reported. Since singular genes are required for enrichment analysis, I then took the most differentially expressed gene to represent remaining OGs in our results section for reported enrichments and heatmap visualizations.

Enrichment analysis

Enrichment analysis was performed on significantly overlapped genes identified by the RRHO bootstrapping method. For explanation of enrichment analysis, please see Chapter 3. STRING [184] was used as the exclusive tool for this chapter.

Results

High-singing starlings and high-playing rats will be referred to as highly-gregarious (HG), while low-singing and low-playing animals will be referred to as less-gregarious (LG).

The RRHO2 bootstrapping method identified 1098 OGs significantly and consistently upregulated in HG animals relative to LG animals, while 393 OGs were significantly and consistently downregulated. No significantly discordant OGs were identified between HG and LG animals (Fig. 2).

To reduce transcriptomic noise during enrichment analysis, only the top 500 most differentially expressed OGs that were upregulated in HG animals (~5% of OGs shared across species) were used. STRING enrichment analyses for upregulated genes showed significant enrichment for glutamatergic signaling across multiple ontologies, including 2 of the 3 subsets of Gene Ontology (Biological process and Cellular component) [182], along with activation of NMDA receptors for Reactome pathways [254]. These enrichments included the ionotropic glutamate receptor genes GRIN1, GRIN2A, GRIN3B, GRIA1, and GRIA3 (Fig. 3A). Additionally, there were multiple neurodevelopmental associated enrichments such as neurogenesis and neuron development that contained large proportions (78 and 42 genes respectively) of the 500 input genes. There were no other specific neurotransmitter or neuroendocrine enrichments outside of the glutamatergic synapse.

STRING also showed a cluster of genes associated with colony stimulating factor 1 (CSF1R), a gene in the brain almost exclusive to microglia. In STRING, CSF1R contained 18 direct connections with other genes, which means there is previously published literature indicative of strong protein-to-protein interactions between those gene products (Fig. 3B). 10 out of the 18 directly connected genes were highly expressed in microglia relative to other brain cells in humans as listed on the Brain RNA-seq website [255, 256].

For the 393 downregulated OGs in HG animals, STRING enrichments were largely related to basic cellular function such as eukaryotic cilia development and microtubule development, with no enrichment for specific signaling mechanisms. One notable gene that stood out was galanin peptide (GAL), although this was not nested within an enrichment.

Discussion

Utilizing DESeq2 and RRHO2, I have identified a shared transcriptomic profile in the mPOA between starlings and rats, representing genes associated with evolutionarily analogous, intrinsically rewarded, gregarious behaviors. Upregulated genes in this profile include multiple glutamate receptor genes, as well as several microglia-exclusive genes.

RRHO2 identified a shared transcriptomic profile of intrinsically-rewarding gregarious behaviors across starlings and rats

Despite the extensive evolutionary distance and the independent evolution of gregarious song and rat play, there was a clear and distinct shared expression profile between high-singing starlings and high-playing rats. This finding aligns with previous research that has found a conserved transcriptomic profile across vertebrates for monogamy despite its independent evolution [38]. It has also been found that even more

distantly related species, such as honeybees, stickleback fish, and mice, all have conserved "genetic toolkits" to regulate responses to social challenges [34, 264].

The mPOA is known to be a highly conserved region that is part of the vertebrate-wide social behavior network [27, 257]. Although studies independently studying juvenile rat play and gregarious song in starlings have shown similar mechanisms across the two behaviors independently, this is the first study to identify a conserved neurotranscriptomic profile for these behaviors.

Glutamate genes in the mPOA upregulated in highly-gregarious animals

An immediate standout in this data set is the prominence of glutamate receptors as part of the conserved genes upregulated in HG animals. Most notably, the emergence of GRIN1 and GRIA1 as consistently upregulated genes in HG is striking, given I identified them as likely candidates of modulating gregarious song in Chapter 3. This is also consistent with the other result that found significant enrichments for neurodevelopment, given glutamate receptors are often knocked down to model neurodevelopmental disorders associated with decreased social motivation such as autism spectrum disorder (ASD) [188, 189, 222]. The data reported here strongly supports the idea that these genes also modulate non-sexual affiliative behaviors. For full details regarding the role of glutamatergic genes in gregarious behaviors, please see the discussion of Chapter 3.

Multiple microglia-specific genes are upregulated in highly-gregarious animals

It was interesting to see that multiple microglia-specific genes were upregulated in HG animals relative to LG. Microglia have been well characterized in rodent models due to their possible role in a variety of neurodevelopmental and neurodegenerative diseases [258, 259], and there is recent research exploring microglia and microglia-like cells in

songbirds [260, 261]. Although microglia are well-known for their immune role as the "macrophages of the brain", there is a growing body of research explained below exploring how their interactions with neurons may influence social behaviors [262].

It has been shown in juvenile rats that the perinatal surge of testosterone that males receive in development interacts with the endocannabinoid system to promote microglia phagocytosis of astrocytes in the medial amygdala, which may be in part responsible for the increased play behavior seen in males [263, 264]. Furthermore, it has been shown that increased phagocytosis of neurons within the sexually dimorphic nucleus of the female rat POA is in part responsible for the reduced volume compared to males [265]. Microglia are also a common therapeutic target for neurodevelopmental disorders such as ASD [266]. Mice with reduced microglia (and therefore reduced synaptic pruning), show ASD-like phenotypes [267], as do mice with non-functional microglia that have reduced autophagy [262].

My RNA-seq findings are consistent with their identified role in social behavior, given that reduced levels of microglia-specific genes are associated with LG animals. Perhaps increases in the number of microglia or increases in activation of microglia within the mPOA can lead to changes in the propensity of vertebrates to participate in gregarious behaviors. It is important to note that the bulk RNA-seq data in this chapter have limitations. Single cell sequencing would be necessary to determine the degree to which these genes are in fact acting specifically within microglia, especially given the minimal characterization of microglia in the songbird brain. Additionally, there are robust differences in the number of microglia between males and females [268, 269], so it will be necessary to differentiate across sexes in future work.

Galanin peptide is downregulated in highly-gregarious animals

There were not any ontology enrichments of note for genes downregulated in HG, however, galanin peptide was downregulated in HG animals. Galanin has an important physiological role in glucose homeostasis, but has been shown to have roles in social behavior as well [270]. In mammals, galanin is implicated in parental behaviors [271], with activation of galanin-expression neurons in the POA leading to increases in pup-tending behaviors, while ablation of those neurons leads to parental deficits [272]. While social play and gregarious song are fundamentally different from parental behaviors, it is surprising to observe that the levels of galanin in the mPOA are not in concordance with the increases in gregarious behaviors. However, given the different effects that neuropeptides often have depending on the social context, it is plausible that galanin may act to generally inhibit gregarious behaviors. Experimental manipulation of galanin within the mPOA is necessary to determine its function in non-sexual affiliative behaviors.

Limitations

One limitation of this work is the discrepancy of the social conditions during play (pairs of 2 vs. groups of 4) to the actual low-playing and high-playing groups that were selected through a median split. Although this discrepancy was technically accounted for in the DESeq2 analysis, the variance introduced by differing housing groups within each play group is challenging to control for statistically. Performing a similar study with all animals in an identical housing condition would be ideal. This could be done with the existing data (e.g., comparing expression across low-players and high-players within a singular housing group), but this greatly decreases statistical power for DESeq2 as our sample size is reduced from eight rats in each group to four.

This study also used male and female starlings to compare against male only rats. Although low-singing and high-singing starlings' neurotranscriptomes were highly concordant across sexes, it is possible some male-only gene candidates were missed through this analysis. Conducting a behavioral assay that records female rats, enabling within-sex comparison of playing and singing, would bolster confidence in the existing conclusions and likely yield new results.

Conclusion

This study supports the hypothesis that there is a conserved neurotranscriptomic profile in the mPOA associated with intrinsically rewarded gregarious behaviors. Ionotropic glutamate receptors are likely powerful modulators of these behaviors, which is supported by previous work that highlighted their importance in influencing social behaviors in ASD models. A promising avenue of research also lies in understanding the possible conserved role of microglia phagocytosis in regulating intrinsically motivated social behavior. This work provides an exciting look at how analogous behaviors with diverse manifestations across taxa can be regulated by conserved neural mechanisms.

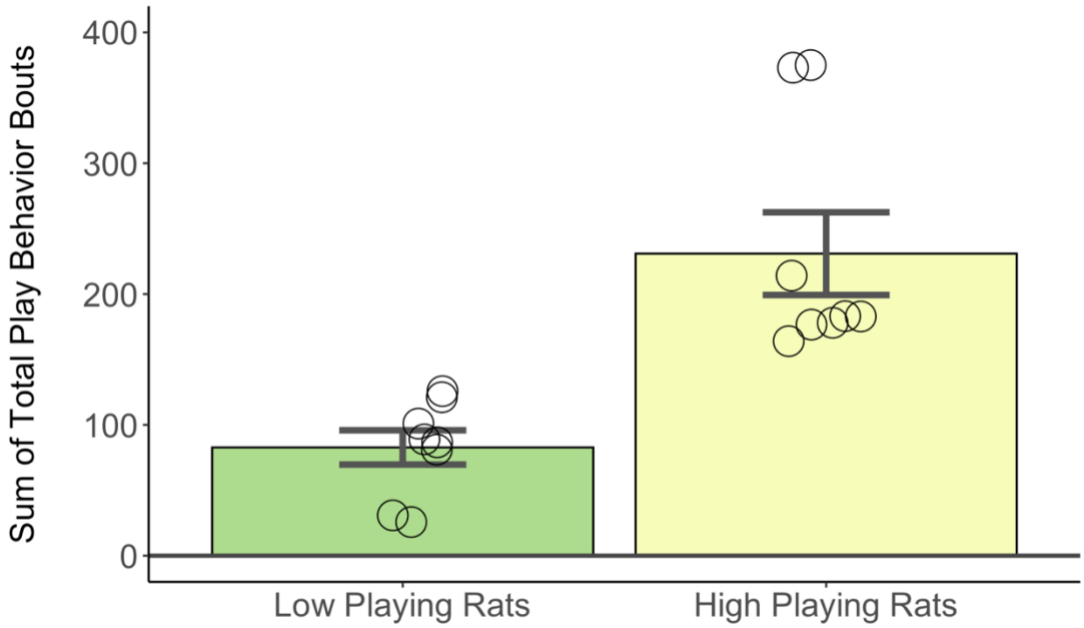

Figure 1. Shows total play bouts of individual rats used in differential gene expression. Low playing and high play groups were created by performing a median split based on the total bouts of play behavior (chasing, biting, pouncing, pinning, wrestling) summed over 5 days.

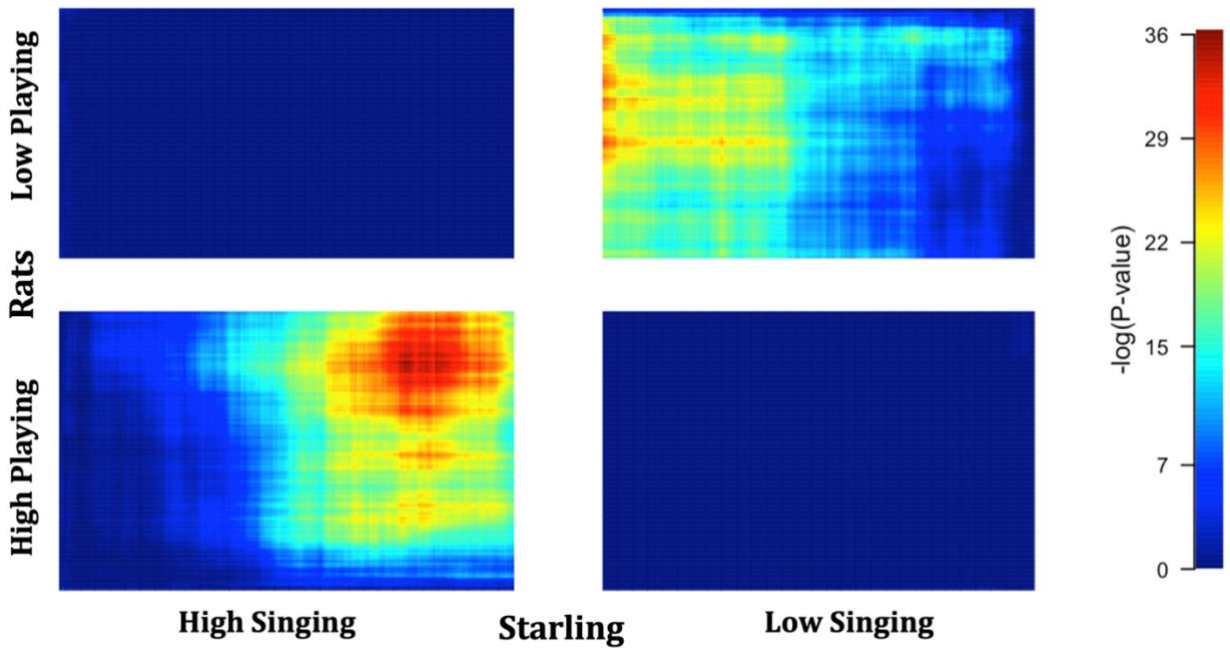

Figure 2. RRHO2 heatmap comparing orthologous groups (OGs) DESeq2 output of low v. high singing starlings and low v. high playing rats. OGs for this heatmap are represented by the most differentially expressed gene within each OG in each species. Warmer colors indicate higher overlap between lists of genes. There were 1098 significantly overlapped OGs upregulated in highly-gregarious animals (bottom left quadrant), and 393 OGs overlapped OGs downregulated in less-gregarious animals (top right quadrant). There no were significantly overlapped OGs between high-playing and low-singing groups (bottom right quadrant) or low-playing and high-singing groups (top left quadrant).

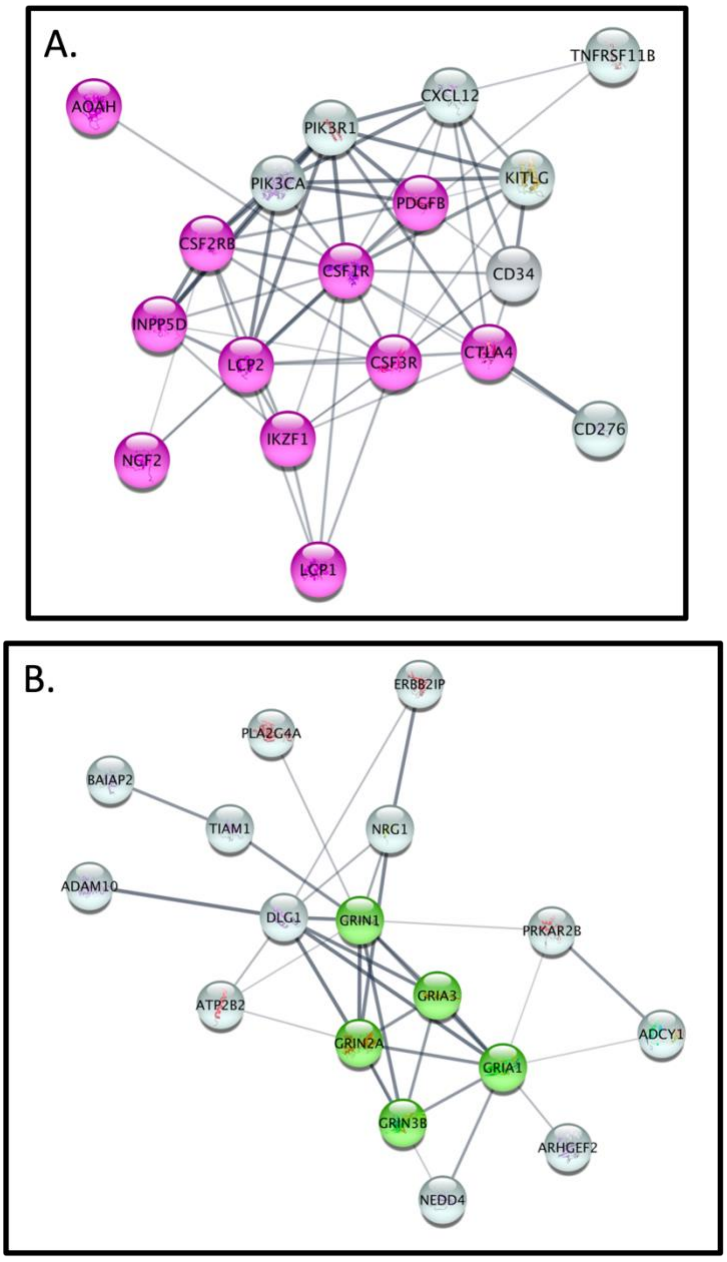

Figure 3. Groups of genes consistently upregulated in highly-gregarious animals. A) Genes associated with the glutamatergic synapse as identified by STRING. Green genes represent glutamate receptor genes. B) Genes with evidence for direct protein-to-protein interactions with CSF1R, a microglia-specific gene, as determined by STRING. Pink genes represent genes that are highly specific to microglia. The thicker the line between genes indicates stronger evidence for protein-to-protein interactions.

General Discussion

Throughout this dissertation, I have provided evidence demonstrating that both the NAC and mPOA regulate gregarious song in European starlings. Moreover, these regions are highly conserved, potentially indicating similar functional roles across vertebrates. In **Chapter 1**, I discovered that the NAC and its subregions (ACR, ACC, ACS) in male European starlings were similar to what is described in mammals, based on the relative labeling patterns of tyrosine hydroxylase and enkephalin, with differences present in neurotensin. In the second part of the study, we found a positive correlation between the activation of the immediate early gene FOS in all three subregions and the production of gregarious song. This correlation was absent with sexually-motivated song. Furthermore, we observed a positive correlation between ZENK and gregarious song exclusively in the ACR.

In **Chapter 2**, my hypothesis was that direct pharmacological stimulation of MORs within either the mPOA or rostral portion of the NAC would increase gregarious song and induce a positive affective state, as evidenced by CPP. The results demonstrated that MOR stimulation in both regions not only triggered singing behavior and established a CPP but also diminished beak wiping, which is indicative of stress behavior. It is important to note that complete singing bouts were only triggered by the stimulation of the NAC, suggesting functional differences between the NAC and mPOA in their roles in song production. I also confirmed that the NAC receives dopaminergic input from the VTA, providing further evidence of the location of the songbird NAC presented in chapter 1.

In **Chapter 3**, I utilized RNA-seq to examine the transcriptomic profile of the mPOA linked to song in both male and female starlings. I found that birds producing gregarious

song at high rates displayed similar profiles across both sexes. Utilizing a range of bioinformatics methods, I pinpointed a co-expressing cluster of glutamatergic genes, the expression of which correlates positively with the production of gregarious song. In **Chapter 4**, I compared the above RNA-seq results with a similar dataset representing the transcriptome of juvenile playing rats. This comparison exposed a conserved, neurotranscriptomic profile in the mPOA of intrinsically motivated behaviors across vertebrates that confirmed the likely importance of the previously identified glutamatergic genes. This discovery is particularly significant considering these behaviors evolved independently of one another.

Implications of findings and Future directions:

This dissertation explores the role of highly conserved, reward-associated brain regions in the regulation of non-sexual affiliative behavior, revealing several avenues for future research. Since there is comparative evidence for distinct subregions within the starling NAC, it will be important to explore potentially functionally distinct roles. There are technical difficulties in doing this given the small size of the starling ACC and ACS, and the direct adjacency they share to each other and the bed nucleus of the stria terminalis, another brain region known to be part of the social behavior network [26, 27]. Parallel pharmacological manipulations of the ACR, ACC, and ACS, akin to those detailed in Chapter 2, would be instrumental in unraveling the specific contributions each subregion makes in regulating gregarious song.

Although it is clear from Chapter 2 that opioids within the ACR and mPOA both play important roles in the intrinsic-reward associated with song, results suggest that there may be differences in the contribution of the mPOA and NAC to gregarious song. The logical next

step is to understand functionally distinct roles for these regions and what other neurotransmitters may be interacting with opioids in the mPOA and NAC to regulate distinct aspects of singing and other social behaviors. One obvious mechanism to explore is the interactions between MOR and glutamate receptors, given the RNA-seq findings in Chapter 3. Previous research has identified a significant amount of "cross talk" between the opioid and glutamatergic systems [273, 274]. These in-part regulate gregarious phenotypes. For instance, stimulating glutamatergic signals in mu opioid receptor null mice reduced autism-like behaviors [275]. Additionally, knockdown of MOR in the mPOA modifies glutamate receptor expression and suppresses social play in rats [66]. This methodology holds promise for future studies in songbirds. For instance, one could perform viral knockdown of one the glutamatergic hub genes (i.e. GRIN1, SHANK2) in the mPOA and explore how these manipulations impact opioid-induced song and place preference changes to gain insight into opioid-gluamate cross talk.

Comprehensive research to understand the conserved brain ciruitry responsible for controlling intrinsically rewarded social behaviors is important, given that deficits in this type of behavior are associated with multiple health disorders, such as depression and anxiety. Affiliative communication, such as conversational "chit-chat" with other people, can lose its reward value and indviduals may be less motivated to socialize with others [276, 277]. It is necessary to understand the neural pathways involved in the regulation of this behavior to identify possible therapeutic candidates. Given the patterns of gene expression shared with this affiliative behavior across two distantally related clades, this further underlies the liklihood of these conserved circuits being responsible for the regulation of this behavior across vertebrates, including humans. To further understood

the ubiquity of this conserved circuit, additional transcriptomic comparisons identifying intrinsically rewarded behaviors in other birds and mammals are necessary.

Conclusion

In *Descent of Man,* Charles Darwin proposed that birds may continue to sing after the breeding season "for their own amusement" [278]. His thoughts speak to the complexity of avian emotion and behavior, and that birds' reasons for song during the non-breeding season goes beyond drive for reproduction. Recent studies have substantiated this view [9, 36, 42], and the work presented here helps understand how the brain regulates the emotional expressiveness of positive, social affect in birds.

This dissertation provides novel insights into the relative contributions of multiple conserved brain regions in its production, and identifies region-specific candidate genes that may be responsible for modulation of this behavior. This opens up multiple promising avenues of research to understand the complexity of gregarious' song regulatory mechanisms. Futhermore, this work provides strong evidence that these systems are evolutionarily conserved, and function to control analagous behaviors across vertebrates. It emphasizes the importance of examining behaviors through their functional lens, although their manifestation within each species may be uniquely dictated by their natural history.

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