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Mu opioid receptor stimulation in the nucleus accumbens increases vocal-social interactions in flocking European starlings, *Sturnus vulgaris*

MOR in NAc increases vocal-social interactions in flocks

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Abstract

Social connections in gregarious species are vital for safety and survival. For these reasons, many bird species form large flocks outside the breeding season. It has been proposed that such large social groups may be maintained via reward induced by positive interactions with conspecifics and via the reduction of a negative affective state caused by social separation. Moreover, within a flock optimal social spacing between conspecifics is important, indicating that individuals may optimize spacing to be close but not too close to conspecifics. Mu opioid receptors (MORs) in the nucleus accumbens (NAc) are well known for their role in both reward and the reduction of negative affective states, suggesting that MOR stimulation in NAc may play a critical role in flock cohesion. To begin to test this hypothesis, social and non-social behaviors were examined in male and female European starlings (*Sturnus vulgaris*) in non-breeding flocks after intra-NAc infusion of saline and three doses of the selective MOR agonist D-Ala2, N-Me-Phe4, glycinol5-ENK (DAMGO). DAMGO in NAc dose-dependently increased singing behavior and facilitated social approaches while at the same time promoting displacements potentially used to maintain social spacing. These findings support the hypothesis that MORs in NAc promote social interactions important for group cohesion in non-sexual contexts and suggest the possibility that MOR in the NAc play a role in optimizing the pull of joining a flock with the push of potential agonistic encounters.

Significance Statement

Social interactions with conspecifics are vital for safety and survival. Group cohesion in gregarious species may be maintained by social reward and reduced negative affect, yet underlying mechanisms have not been well studied. Mu opioid receptors (MOR) in the nucleus accumbens (NAc) are strongly implicated in reward. Here, we demonstrate in flocks of European starlings that MOR stimulation in the NAc increases affiliative singing and approach behaviors as well as displacements. These results demonstrate a role for MOR in the NAc in both affiliative

behaviors and behaviors used to maintain social spacing. Findings suggest that opioids in the NAc may play a role in optimizing the pull of joining a flock with the push of potential agonistic encounters.

Introduction

In many species, social connections outside a breeding context are vital for both safety and survival. For instance, schooling allows teleost fish to avoid predators (Magurran, 1990) and coordinated hunting allows clans of spotted hyenas to more effectively hunt and defend large prey (Holekamp *et al.*, 1997). Many bird species form remarkable flocks, often outside the context of breeding, which offer protection from predators and increase foraging efficiency (Powell, 1974; Sullivan, 1984). It has been proposed that such large social groups may be maintained by rewarding positive interactions with conspecifics and by reducing negative states cause by social separation (Emlen, 1952; Riters *et al.*, 2019a). Moreover, within a flock optimal social spacing between conspecifics is important, indicating that individuals may optimize spacing to be close but not too close to conspecifics. Neuropeptides in the oxytocin and vasopressin family have been a major focus of research on non-sexual social bonding and flocking in birds (Goodson *et al.*, 2009). However, opioids also underlie social behavior and are well known for both inducing reward and reducing negative affective states (Panksepp *et al.*, 1980; Olds, 1982; Goeders *et al.*, 1984). This suggests that opioids may play a central role in the maintenance of social groups.

Across species, mu opioid receptor (MOR) stimulation facilitates non-sexual prosocial interactions. MOR activation is proposed to alleviate distress from maternal separation (Herman and Panksepp, 1978) and to reward social play (Normansell and Panksepp, 1990; Vanderschuren *et al.*, 2016). Opioids are also strongly implicated in social interactions that promote cohesion in large groups, such as schools of fish (Kavaliers, 1981) and songbird flocks. In zebra finches,

Taeniopygia guttata, administration of the non-selective opioid receptor antagonist naloxone suppresses undirected song—a type of song proposed to play a role in flock cohesion (Khurshid *et al.*, 2010). In European starlings, *Sturnus vulgaris*, MOR stimulation facilitates singing behavior in flocks, referred to here as gregarious song, as well as the positive affective state associated with gregarious song (Stevenson *et al.*, 2020). Additionally, gregarious singing behavior is associated with opioid-mediated analgesia (Kelm-Nelson *et al.*, 2012). These findings together suggest that opioids released in association with social interactions may act at MORs to promote social cohesion, by inducing reward and reducing the pain of being alone (Kelm-Nelson *et al.*, 2012; Riters and Stevenson, 2012; Riters *et al.*, 2014; Hahn *et al.*, 2017; Stevenson *et al.*, 2020).

Opioids act at MORs in numerous brain regions to induce reward and to alleviate negative affect. In songbirds, several studies demonstrate that the medial preoptic area (mPOA, commonly abbreviated POM in birds) is a crucial site in which MOR activation stimulates song in flocks and underlies song-associated reward (Riters *et al.*, 2005; Riters *et al.*, 2014; Stevenson *et al.*, 2020). In mammals, the majority of studies on the role of MOR in reward, including non-sexual social reward (e.g., social play (Vanderschuren *et al.*, 1997; Trezza *et al.*, 2011; Manduca *et al.*, 2016)), focus on the nucleus accumbens (NAc), an integral part of the mesolimbic pathway that underlies motivation and reward (Salgado and Kaplitt, 2015). A few correlational studies have examined a potential role for the avian NAc in social behaviors such as pair bonding and female responses to male courtship (Svec *et al.*, 2009; Earp and Maney, 2012; Riters *et al.*, 2013), but none have tested the effects of experimental manipulations of MOR in the NAc on avian social behavior.

It could be that MORs in the avian mPOA are important for facilitating non-sexual social interactions in flocks, and a separate system involving MORs in the NAc underlies similar behaviors in mammals. However, the mPOA is part of a circuit that accesses the NAc through

projections to the ventral tegmental area (Balthazart *et al.*, 1994; Riters and Alger, 2004), suggesting that studies of social interactions in birds and mammals may be focusing on separate parts of an evolutionarily conserved circuity. Recent evidence in support of this idea comes from a study in juvenile rats that demonstrates a role for MOR in the mPOA in social play (Zhao *et al.*, 2020). If MORs in the NAc are part of a conserved circuitry that stimulates social connections outside of a breeding context across vertebrates, then we predict that MOR stimulation in the NAc will facilitate prosocial behaviors in songbirds. To test this, we administered the selective MOR agonist DAMGO into the NAc of male and female European starlings (*Sturnus vulgaris*) and measured vocal-social behaviors in non-breeding flocks.

Methods

Animals and Housing

All animal procedures were performed in accordance with the [Author University] animal care committee's regulations and adhere to National Institute of Health (NIH) guidelines.

Fourteen European starlings (*Sturnus vulgaris*), eight males and six females, were included as experimental birds in this study. Additional birds were also included as unmanipulated flock mates to bring each flock to a total of either six or eight birds. The starlings were trapped from a local farm in a location which will be identified if the article is published and housed in same sex cages on a photoperiod of 18h light: 6h dark (lights on at 6:00am) until molting was complete. This photoperiod induces a condition referred to as photo-refractoriness that is characteristic of early fall when birds begin to sing in large mixed-sex flocks (Dawson *et al.*, 2001). Birds were then housed with flockmates for the duration of the study in indoor aviaries (2.13m x 2.4m x 1.98m) that were decorated with tree branches and supplied with food, drinking water, and bathing water ad libitum. Talk radio was playing outside the aviaries during daylight hours to habituate birds to sounds outside the room.

This study was conducted from September 2019 through March 2020. After birds were tested and removed from the aviaries, new birds were added into each aviary. A single observer watched the flock daily to identify singing birds for inclusion in the study. During observation periods, audio recordings of starling song were played to facilitate singing behavior (Marius Travell, YouTube). Once singing birds were identified and selected, they underwent surgery to implant a cannula guide targeting the NAc (detailed below). A maximum of two birds from each aviary were selected to be tested on alternate days, and there was always at least one unmanipulated singing bird present in the aviary to facilitate flock song.

NAc cannula surgery

The cannula targeted the location of the songbird NAc proposed by Reiner and colleagues (Reiner *et al.*, 2004). This region is in the rostral striatum located medially surrounding the ventral tips of the lateral ventricles, which in our sections appears in coronal sections in which Area X is relatively small and round (Fig 1). An 8 mm, 26 gauge cannula guide (C315G/SPC, Plastics One, Inc., Roanoke, VA) was placed unilaterally into either the left or right NAc following procedures similar to (Kelm-Nelson *et al.*, 2013). Birds were given an intramuscular injection of 0.10 mL ketofen (Zoetis, Inc., Parsippany, NJ), anesthetized with isoflurane/oxygen gas (isoflurane: Patterson Vet Supple, Inc., Greeley, CO; oxygen: Airgas, Inc., Randor, PA), and secured in a stereotaxic apparatus (Kopf Instruments 995) using ear bars in the most rostral position of the ear, with the beak approximately 45° below the plane of the ear bars. The dorsal head feathers were trimmed and a small incision was made in the skin to visualize the skull. The cannula guide tip was laterally zeroed on the midvein, in a position 1.2mm rostral to the ear bars. Then the guide was laterally rotated 4.5° and adjusted 1.0mm to the left or the right, and then vertical zero was again taken from the midvein. Three holes were drilled in the skull, one for the guide and two

superficial holes for the screws. The guide tip was lowered ventrally 6.1mm from the skull zero (the coordinate for dorsal NAc) and secured using screws and instant dental cement (Ortho-Jet™ dental cement powder and acrylic liquid; Lang Dental Manufacturing Company, Inc. Wheeling, IL). Once the dental cement was solidified, a dummy cannula (Plastics One, Inc., Roanoke, VA) was fitted into the guide. The animal was then allowed to recover under monitoring until alert, then transported and released into its home aviary. The birds were monitored that evening and again the following morning. Experimental treatments and observations began after the bird resumed singing in its flock, approximately seven days after surgery.

Pharmacological manipulations

The experimental birds were behaviorally tested on 4 days separated by at least one day. If two birds from an aviary were tested, they were tested on alternate days, such that only one bird per aviary was tested on a single day. A habituation treatment of vehicle (saline) was injected at least two days prior to the commencement of the treatment sequence to habituate birds to the procedure (details for the injection procedure are provided below). After this habituation injection, each treatment sequence lasted approximately three weeks, consisting of four treatments: vehicle (sterile saline, 0.85%; 0.50 µl) and three doses of D-Ala2, N-Me-Phe4, glycinol5-ENK (DAMGO) (cat#100929-53-1; low dose of 0.025 µg, intermediate dose of 0.25 µg, or high dose of 2.5 µg dissolved in 0.50 µl sterile saline). Each bird received each treatment once in a counterbalanced order and a minimum of 48 hours separated treatments. The treatments were color-coded by another researcher so that the observer would be blind to treatment conditions during all observations.

On each test day, injections began between 0930 and 1530 hrs. Experimental birds were swiftly caught in a net and anesthetized with isoflurane/oxygen using a nose cone. The cannula

dummy was removed and replaced with a 33-gauge cannula connected to PE50 tubing (C232CT, Plastics One) containing the color-coded treatment solution. The cannula extended 2 mm beyond the tip of the cannula guide. A Hamilton vacuum syringe (Hamilton Company, Reno, NV) connected to a Nanomite Syringe Pump (Harvard Apparatus, Holliston, MA) injected 0.50 µl of the treatment solution over a 120-second period. The cannula was left in place for 180 seconds to allow for equalization of pressure and diffusion from the cannula tip. Infusion volume was verified by following the movement of an air bubble in the tubing. The cannula was removed, the cannula dummy was replaced, and the bird was placed into a draped recovery cage for fifteen minutes before being released into its home aviary.

Behavioral observations

A single researcher observed each focal animal using a continuous sampling technique. The following behaviors were recorded for 40 minutes: *social approaches* (the focal individual approaches to within 10 cm of another bird and remains near that bird); *displacements* (the focal individual approaches to within 10 cm of another bird followed by the receiving bird leaving proximity within 1 sec), *gregarious singing* (sum of introductory whistles and song bouts separated by at least 1 sec), *calls* (sum of calls; however, we did not identify specific call types), *perch changes* (sum of movement along or between branches separated by at least 1 sec), *beak wipes* (beak wipes in starlings are not used as part of courtship and are considered to be a sign of stress) (Bauer *et al.*, 2011), and *feeding* (sum of bouts of feeding separated by at least 1 sec or a complete head lift). After the first 20 min observation period, a high-reward food (i.e., horse feed, which attracts large flocks in local barns) was added to examine the possibility that MOR stimulation in NAc would stimulate hedonic feeding, as observed in rats (Bakshi and Kelley, 1993; Zhang *et al.*, 1998); however, this food was ignored by the birds and will not be discussed further.

Cannula tip verification

Following the final treatments and observations, the birds were injected with 1.0 µl of Chicago Blue 6B dye (Fisher Scientific Company, Hampton, NH), or with a tract tracer using the same injection procedure as above, to identify the location of the tip of the cannulae. For the birds infused with blue dye, after infusion the birds were rapidly decapitated, and their brains were removed and frozen on crushed dry ice. They were stored at -80°C and then sectioned at 50 µm using a Leica CM1850 cryostat (Leica Biosystems, Wetzlar, Germany). Sections were mounted on slides, dehydrated, Nissl stained, and cover slipped. The slides were analyzed under a microscope to determine if the blue dye was contained within the NAc ("hit") or elsewhere ("miss"). For three birds, 0.20 µl of the neuronal tracer biotinylated dextran amine (BDA; 3,000 MW; NeuroTrace™; lot # 2089930) at 10% concentration was infused to confirm the site of injection (these infusions were part of a study not reported here). Ten days after infusion of the tracer, birds were perfused and the tracer was visualized as described in (Riters and Alger, 2004). Sex and non-breeding condition were verified immediately following death via presence and size of testes.

Statistical analysis

Statistical analysis was conducted using GraphPad Prism (GraphPad Prism version 8.0.0 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com). The proportion of each behavior produced on each day was used for analysis to control for high levels of individual variation in behavior across birds (untransformed descriptive statistics are reported in Table 1). Specifically, for each bird and each behavioral measure we took the sum of that behavior for each test day and divided it by the total sum of that behavior for all days. Difference scores between behavior frequency after treatments and behavior frequency under saline were also

examined and results were consistent with proportions of behavior; here we report the proportions. A General Linear Model was run for each behavior, with treatment entered as repeated measures, cannula location entered as a factor, and proportion of behavior entered as response variables, with separate analyses run for each behavior. Tukey's HSD Post hoc tests were run following significant ($p<0.05$) GLM results. Some of the misses targeted areas in which we expected MOR to regulate social behavior (see Discussion), which reduced the likelihood of seeing a significant interaction, potentially precluding post-hoc comparisons of doses within treatment groups. However, the goal of this study is to characterize the role of MOR specifically in the NAc in social behaviors. Thus, to provide additional insight, in cases where there was a dose effect but no dose-by-cannula interaction, we ran exploratory Dunnett's post hoc analyses on hits only to compare effects of the most effective dose of agonist (i.e., the highest dose) against the other treatments. A Levene's test was run to test the assumption of homogeneity of variance and a Lilliefor's test was run to test the assumption of normality. When assumptions were violated, analyses were run on log transformed data. For instances where there were zero occurrences of a behavior, a formula of $\log(x+0.05)$ was used. When transformation did not correct violation of assumptions, non-parametric Friedman's ANOVAs were used to examine treatment differences for hits and for misses. Effect sizes were calculated using η^2. Confidence intervals at 95% were determined using the untransformed proportions of each behavior. Pearson correlations for all behaviors at baseline (saline) were run to examine potential relationships between behaviors.

Results

The cannula tip was contained within the bounds of the NAc in 6 birds ("hits"; 4 males and 2 females) and outside the NAc in 8 birds ("misses"; 4 males and 4 females; Figure 1). For the misses, the cannula tips were contained within the striatum lateral to NAc (n=4), the lateral

hypothalamus (LH) (n=1), the medial preoptic area (mPOA) (n=1), the tractus septopallio-mesencephalicus (TSM) (n=1), the ventricle (n=1). There was not adequate statistical power to analyze sex, but results indicate that the same trends were observed in males and females, so males and females were combined, but figures indicate males and females (Stevenson *et al.*, 2020).

Effects of MOR stimulation on social behaviors

Singing behavior

Statistics were not conducted on singing behavior because most birds did not sing, driving variance down to zero for most doses (Figure 2A; Table 1 for untransformed descriptive statistics). Out of 6 hits, 3 birds (50%) sang when treated with highest dose of MOR agonist, two males and one female. Out of 8 misses, only 2 birds sang at any dose, one male and one female. The male sang at the lowest dose of the MOR agonist (0.025 μg DAMGO), and the cannula tip was located in the striatum lateral to NAc. The female sang at the highest dose (2.5 μg DAMGO), and the cannula tip was located in the mPOA.

Calling behavior

For calls, a GLM revealed a significant main effect for dose ($F_{3,36}$=6.95, p=0.013, η^2=0.317). No significant main effect was found for cannula location ($F_{1,12}$=1.37, p=1.0, η^2=0.0), but there was a significant dose-by-cannula location interaction ($F_{3,36}$=3.32, p=0.003, η^2=0.188). Post hoc analysis revealed that birds with a cannula tip in the NAc treated with the highest dose of MOR agonist called significantly more than when treated with the lowest dose of MOR agonist (p=0.023), but not with saline (p=0.064) or the intermediate dose of MOR agonist (p=0.15). There were no significant differences found between any dose for misses (p>0.05 for all comparisons; Figures 2B and 4A; Tables 1 and 2 for untransformed descriptive statistics and confidence intervals).

Social approaches

A GLM run on the log transformed approach data revealed a significant main effect for dose ($F_{3,36}$=4.84, p=0.007, η^2=0.233). No significant main effect was found for cannula location ($F_{1,12}$=0.17, p=0.689, η^2=0.001) and there was no dose-by-cannula location interaction ($F_{3,36}$=1.84, p=0.157, η^2=0.089. Post hoc analysis of the dose effect revealed that birds treated with the highest dose of MOR agonist approached significantly more than birds treated with saline (p=0.019). Post hoc analysis run on the hits demonstrates that the highest dose of intra-NAc MOR agonist was significantly higher than saline (p=0.032) and the lowest dose (p=0.034) but not the intermediate dose of MOR agonist (p=0.523; Figures 2C and 4B; Tables 1 and 2).

Displacements

A GLM run on the log transformed sum of displacements revealed a significant main effect for dose ($F_{3,36}$=5.33, p=0.009, η^2=0.248). No significant main effect was found for cannula location ($F_{1,12}$=0.14, p=0.712, η^2=0.001) or the dose-by-cannula location interaction ($F_{3,36}$=2.27, p=0.097, η^2=0.106). Post hoc analysis of the dose effect revealed that birds displayed a significantly higher proportion of displacements when treated with the highest dose of MOR agonist than treated with the low (p=0.015) or intermediate dose of MOR agonist (p=0.034). Post hoc analysis run on the hits demonstrates that the highest dose of intra-NAc MOR agonist yielded significantly higher proportion of displacements than saline (p=0.040) and the lowest dose (p=0.022) but not the intermediate dose of MOR agonist (p=0.088; Figures 2D and 4C, Tables 1 and 2).

Effects of MOR stimulation on non-social behaviors

Perch changes

For perch changes, a GLM revealed a significant main effect for dose ($F_{3,36}$=18.94, p<0.001, η^2=0.560). No significant main effect was found for cannula location ($F_{1,12}$=2.56, p=0.135, η^2=0.0), but there was a significant dose-by-cannula location interaction ($F_{3,36}$=4.70, p=0.007, η^2=0.139). Post hoc analysis revealed that birds with a cannula tip in the NAc treated with the highest dose of

MOR agonist had significantly higher proportion of perch changes than when treated with saline ($p=0.008$) or the lowest dose of MOR agonist ($p=0.003$), but not the intermediate dose of MOR agonist ($p=0.103$). There were no significant differences found between any dose for misses ($p>0.05$ for all comparisons; Figures 3A and 4D, Tables 1 and 2).

Feeding

A transformation of feeding data did not correct assumptions needed to run parametric statistics. Non-parametric Friedman's ANOVA tests yielded a significant effect for dose for hits (ANOVA Chi Sqr ($n=6$, $df=3$) $=7.48$, $p=0.048$) but not misses (ANOVA Chi Sqr ($n=8$, $df=3$) $=7.78$, $p=0.051$; Figures 3B and 4E, Tables 1 and 2). Post hoc analysis yielded no significant differences between doses for either hits or misses ($p>0.05$ for all comparisons), although for hits the highest dose compared to saline was $p=0.06$.

Beak wipes

For beak wipes, a GLM revealed a significant main effect for cannula location ($F_{1,12}=7.71$, $p=0.017$, $\eta^2=0.0$). No significant main effect was found for dose ($F_{3,36}=1.26$, $p=0.304$, $\eta^2=0.092$) or the dose-by-cannula location interaction ($F_{3,36}=0.29$, $p=0.831$, $\eta^2=0.020$; Figures 3C and 4F, Tables 1 and 2). However, Post hoc analysis yielded no significant difference between hits and misses at any dose ($p>0.05$ for all comparisons).

Correlation of behaviors

In addition, correlations were run for all behaviors listed above to determine if the base levels of any behavior correlated with another behavior, possibly indicating that an increase in one would drive an increase in another. However, we found no evidence of any correlations between these behaviors ($p>0.05$ for all comparisons; Table 3).

Discussion

This study is the first to demonstrate a causal role for the songbird NAc in the facilitation of social interactions in non-sexual gregarious contexts. Results demonstrate a role for MOR in NAc in behaviors considered important for group cohesion, including gregarious song, social approach, and displacements, with similar patterns observed in males and females. More broadly, these data provide evidence the NAc may be part of a conserved circuitry that promotes social cohesion in non-sexual contexts across vertebrates.

MOR stimulation in the NAc increased social spacing behaviors

The highest intra-NAc dose of the MOR agonist DAMGO facilitated social approach behaviors, offering support for a role for MOR in the NAc in prosocial behavior. Results of past studies in rodents also demonstrate a role for MORs in social proximity; however, in these studies peripherally administered MOR agonists decreased the amount of time rodents spent near conspecifics (Herman and Panksepp, 1978; Panksepp *et al.*, 1979). Although the findings were in the opposite direction from our study, these past results were interpreted to suggest that the agonist replaced the need for reward that would normally be induced by opioids released by social contact (Herman and Panksepp, 1978). Indeed, many rewarding behaviors (e.g., social play (Trezza *et al.*, 2010; Achterberg *et al.*, 2019) and hedonic feeding (Berridge, 2009; Gosnell and Levine, 2009)) are facilitated by low doses and inhibited by higher doses of MOR agonists. This suggests that the dose of agonist and site-specificity in our study may have been sufficient to facilitate social behavior, but not sufficient to fully replace social reward. Future studies are needed to test this hypothesis.

The highest intra-NAc dose of the MOR agonist DAMGO also stimulated displacements, a mildly agonistic behavior. This is similar to past findings which show that peripheral administration of a MOR agonist facilitates agonistic behaviors in response to an intruder in mice (Campbell Teskey and Kavaliers, 1988); however, relative to territorial defense, the agonistic

329 interactions in the present study were relatively non-threatening. Starlings do not maintain strong,
330 linear dominance hierarchies when in large flocks and are often observed sharing food sources.
331 Therefore the function of displacements does not appear to relate to dominance, but may play a
332 role in the maintenance of adequate social spacing (King, 1973). Starlings may space themselves
333 to maintain optimal levels of natural MOR stimulation and infusion of a MOR agonist may slightly
334 reduce the need for close social contact, as previously stated, perhaps triggering mildly agonistic
335 interactions to maintain social spacing. The finding that activation of MORs in the NAc stimulates
336 approach while at the same time increasing behaviors used to maintain social spacing suggests that
337 MOR in the NAc may play a role in optimizing the pull of joining the flock with the push of
338 potential agonistic encounters.

339 **MOR stimulation may stimulate vocal behaviors**

340 Although few birds sang, the administration of the highest intra-NAc dose of the MOR
341 agonist DAMGO initiated gregarious singing behavior in 50% of birds tested. Song in this context
342 is highly sensitive to stressors, and when a bird is caught and injected it stops singing (Stevenson
343 *et al.*, 2020), therefore, restoring singing behavior with intra-NAc MOR stimulation is noteworthy.
344 This type of gregarious singing behavior in a non-breeding context is associated with an intrinsic
345 reward state (Kelm-Nelson *et al.*, 2012; Riters and Stevenson, 2012; Riters *et al.*, 2014; Hahn *et*
346 *al.*, 2017; Stevenson *et al.*, 2020) and is proposed to be a form of play behavior that allows birds
347 to develop important social skills for use in more serious reproductive contexts (Riters *et al.*,
348 2019b). MOR stimulation in the NAc also dramatically stimulates and rewards social play in
349 rodents (Vanderschuren *et al.*, 1997; Trezza *et al.*, 2011; Manduca *et al.*, 2016). Thus, the present
350 study is the first to implicate MOR in the NAc in gregarious song, and it provides
351 neuropharmacological support for the hypothesis that playful behaviors involve neural systems
352 that are conserved across vertebrates.

353 In addition, the highest intra-NAc dose of the MOR agonist DAMGO stimulated calls.
354 Functionally distinct calls are used by starlings in flocks (Feare, 1984); however, we did not
355 distinguish between different call types. Therefore, although we can conclude that MOR
356 stimulation increased calls in this study, future studies are needed to explore the functional role of
357 opioids in calling behavior.

358 **MOR stimulation of non-social behaviors**

359 This is the first study to examine effects of MOR agonist administration in the NAc of
360 songbirds. The present study focused on the location of the songbird NAc proposed by Reiner and
361 colleagues (Reiner *et al.*, 2004). The functional homology of this site to the mammalian NAc has
362 not been well studied. To both determine the degree to which our focal area is functionally similar
363 to the mammalian NAc and to explore specificity of effects to social behavior, we measured motor
364 activity, feeding, and stress-related behaviors—all behaviors that are influenced by MOR agonist
365 infusion into the NAc in rodents.

366 The highest intra-NAc dose of the MOR agonist DAMGO increased motor activity, as
367 reflected in perch changes. Perch changes did not correlate with either approaches or social spacing
368 behaviors, therefore we do not consider motor activity to be driving the increase seen in either type
369 of behavior. This increase in motor activity is consistent with studies that show similar effects in
370 rodents (Vezina *et al.*, 1987; Cunningham and Kelley, 1992; Bakshi and Kelley, 1993). Intra-NAc
371 MOR stimulation also increased feeding, with the high dose compared to saline being just shy of
372 significance ($p=0.06$). Several studies in rats demonstrate that intra-NAc DAMGO administration
373 most powerfully increases hedonic feeding including the consumption of highly palatable, high fat
374 food (Bakshi and Kelley, 1993; Zhang *et al.*, 1998; Will *et al.*, 2003). Therefore, it is possible that
375 if the present study examined effects on a highly palatable food option, there may have been a
376 more substantial increase in feeding behavior. Although peripheral MOR administration alters

stress responses, the present data found that administration of MOR agonist in the avian NAc had no impact on beak wipes, which are considered an indication of stress in starlings (Bauer *et al.*, 2011). These data suggest that opioids may act on MOR in other brain regions outside the NAc in flocking songbirds to reduce stress.

Effects of MOR stimulation outside NAc

Although birds with cannula located outside the NAc were considered controls, for some of these birds the cannula tips were located in brain regions known to influence social and other behaviors. Sample sizes in most cases consist of a single bird so individual cases are not analyzed, rather, a few are highlighted here that are consistent with past research. Specifically, for one of the birds the cannula tip was located in the mPOA. For this bird, the highest dose of the MOR agonist DAMGO stimulated gregarious song, which is consistent with a recent study that showed that downregulation of MOR in the starling mPOA reduces gregarious singing (Stevenson *et al.* 2020). The highest dose of the MOR agonist DAMGO also stimulated feeding in birds with the cannula tip located in the striatum with a similar result observed for a bird with the tip located in the lateral hypothalamus, results that are similar to findings in rodents (Levine and Billington, 1989; Zhang and Kelley, 2000; Castro *et al.*, 2015; Ardianto *et al.*, 2016). The finding that displacement behaviors were higher for birds receiving high doses of MOR agonist in the lateral hypothalamus and mPOA is consistent with studies that implicate these regions in agonistic behavior (Panksepp, 1971; Hammond and Rowe, 1976; Schlinger and Callard, 1990; Zhang and Kelley, 2000; Nieh *et al.*, 2016). These results suggest sites in which MOR may act to influence these behaviors, thus, the misses support past research and suggest potential roles for MOR in areas outside the NAc in social and non-social behaviors that can be tested in future research.

Conclusions

The results of this study suggest that a region identified as the NAc in birds (Reiner *et al.*, 2004) is functionally homologous to the mammalian NAc, and that this region may be part of a core, conserved circuitry that underlies rewarding social behaviors across vertebrates. Future studies are now needed to examine proposed shell and core subdivisions of NAc in birds, which in mammals play distinct roles in reward (Kelley, 1999), as well as the extent to which the mesolimbic reward pathway is differentially involved in modulating social spacing behaviors seasonally in songbirds.

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553 **Legends**

554 **Figure 1. A-C**) Location of DAMGO infusion sites. Illustration of one hemisphere of starling
555 brain with "Hits" represented by filled-in shapes, and "misses" represented by open shapes. Males
556 are represented by squares and females are represented by circles. **D)** Photomicrograph of 2X
557 magnification of a Nissl-stained brain section that demonstrates a "hit" with the cannula tip located
558 in the NAc. Dotted line indicates boundaries of NAc. Scale bar represents 500 μm or 0.5 mm.
559 Abbreviations: AC=anterior commissure, mPOA=medial preoptic area, MSt=medial striatum,
560 NAc=nucleus accumbens, Rt=nucleus rotundus, TSM= tractus septopallio-mesencephalicus.

561 **Figure 2.** Effects of MOR stimulation in NAc on song, calls, social approaches, and displacements
562 (mean ± SEM). **A**) number of song bouts, **B**) number of calls, **C**) proportion of approaches, and
563 **D**) proportion of displacements in male (squares) and female (circles) starlings in which the
564 cannula tip missed the NAc (n=8) or hit the NAc (n=6). Analyses were run on log transformed
565 data for both **C** and **D** to correct for assumptions, but here we show untransformed proportions.
566 Dotted lines represent pairwise significance for intra-NAc dose effect. *p<0.05, **p<0.01,
567 ***p<0.001

568 **Figure 3.** Effects of MOR stimulation in NAc on non-social behaviors (mean ± SEM). Proportion
569 of **A**) perch changes, **B**) feeding, and **C**) beak wipes, in male (squares) and female (circles)
570 starlings in which the cannula tip missed the NAc (n=8) or hit the NAc (n=6). *p<0.05, **p<0.01,
571 ***p<0.001

572 **Figure 4.** Effects of MOR stimulation in locations in which the cannula tip was located outside
573 NAc (mean ± SEM). Proportion of **A**) calls, **B**) social approaches, **C**) displacements, **D**) perch
574 changes, **E**) feeding, and **F**) beak wipes in birds with cannula located in the medial preoptic nucleus
575 (mPOA), lateral hypothalamus (LHy), tractus septmesencephalicus (TSM), the lateral ventricle,
576 and striatum lateral to NAc (Figure 1). n=1 for each location except for the striatum (n=4) where
577 the mean ± SEM is given.

578

Table 1. Untransformed behavior measurements for each treatment and condition (mean ± SEM).

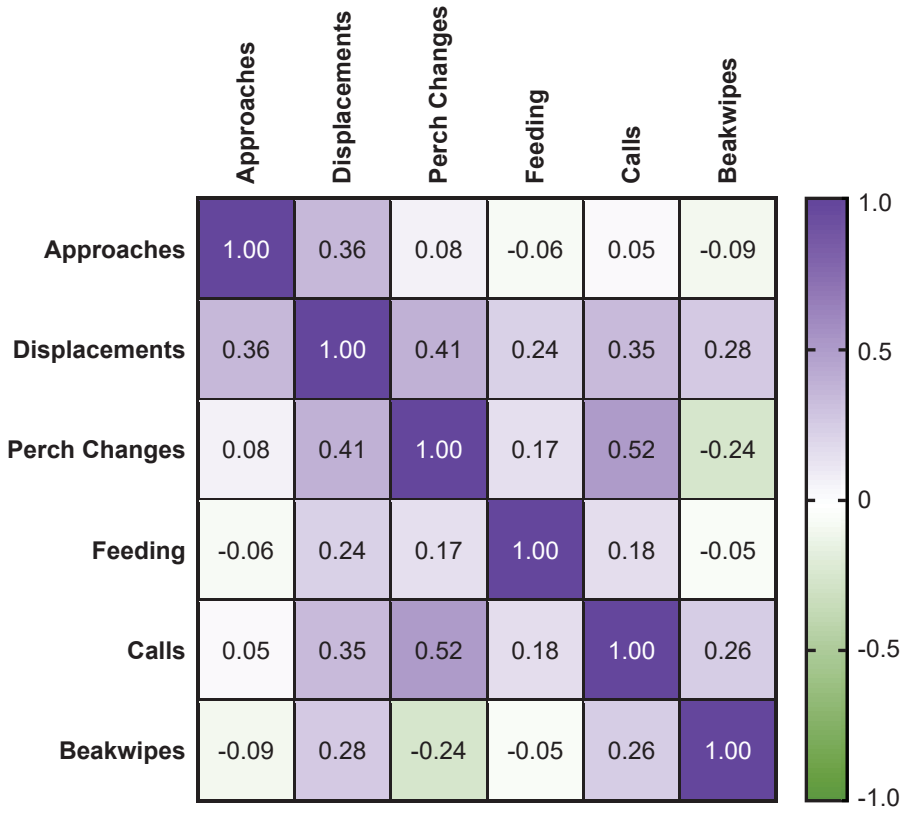
		Dose			
Behavior	Cannula	Saline	0.025µg DAMGO	0.25µg DAMGO	2.5µg DAMGO
Singing	Hit	0 ±0	0 ±0	0 ±0	26.17 ±15.86
	Miss	0 ±0	1.25 ±1.25	0.13 ±0.13	0.25 ±0.25
Calls	Hit	42.17 ±26.5	10.67 ±5.47	32 ±23.2	134.67 ±65.2
	Miss	21.75 ±12.23	17.5 ±9.31	21.38 ±10.91	28.63 ±8.55
Approaches	Hit	1.83 ±1.05	2.33 ±1.17	3.33 ±0.84	5.50 ±1.34
	Miss	4.88 ±2.96	3.88 ±1.23	2.63 ±0.96	4.63 ±1.41
Displacements	Hit	9.33 ±2.26	8.83 ±2.27	10.67 ±4.17	28.5 ±6.53
	Miss	6.25 ±1.46	7 ±2.17	5.25 ±1.71	9.63 ±2.68
Perch changes	Hit	87.17 ±21.89	105.67 ±17.86	158.67 ±33.05	359.67 ±74.1
	Miss	197 ±65.05	132.25 ±15.01	124.25 ±22	245.25 ±52.64
Feeding	Hit	6.67 ±3.3	9.67 ±1.8	12.17 ±5.99	16 ±5.94
	Miss	6.5 ±1.87	5.63 ±1.15	3.5 ±1.13	10.38 ±1.81
Beak wipes	Hit	28.5 ±8.25	34.83 ±7.32	33 ±3.51	34.33 ±8.28
	Miss	26 ±6.63	40.63 ±16.78	30.38 ±7.76	38.88 ±5.99

Table 2. 95% Confidence intervals based on the proportions of the occurrences for each behavior and condition.

	Dose

Behavior	Cannula	Saline	0.025µg DAMGO	0.25µg DAMGO	2.5µg DAMGO
Approaches	Hit	[-0.02, 0.256]	[0.011, 0.231]	[0.169, 0.507]	[0.375, 0.47]
	Miss	[0.055, 0.322]	[0.137, 0.38]	[0.062, 0.24]	[0.2, 0.605]
Calls	Hit	[0.038, 0.269]	[-0.004, 0.185]	[-0.012, 0.371]	[0.264, 0.889]
	Miss	[0.053, 0.348]	[0.006, 0.345]	[0.058, 0.293]	[0.242, 0.656]
Displacements	Hit	[0.053, 0.238]	[0.058, 0.201]	[0, 0.309]	[0.311, 0.83]
	Miss	[0.088, 0.575]	[0.108, 0.311]	[0.049, 0.276]	[0.162, 0.432]
Perch changes	Hit	[0.06, 0.194]	[0.12, 0.18]	[0.116, 0.341]	[0.371, 0.616]
	Miss	[0.279, 0.324]	[0.168, 0.253]	[0.12, 0.246]	[0.258, 0.452]
Feeding	Hit	[0.097, 0.156]	[0.14, 0.421]	[0.132, 0.347]	[0.238, 0.471]
	Miss	[0.118, 0.383]	[0.137, 0.307]	[0.041, 0.222]	[0.269, 0.523]
Beak wipes	Hit	[0.139, 0.226]	[0.159, 0.369]	[0.132, 0.361]	[0.242, 0.372]
	Miss	[0.097, 0.313]	[0.112, 0.445]	[0.162, 0.368]	[0.14, 0.363]

Table 3. Correlation matrix for behavioral proportions at baseline (saline) in combined hits and misses. r values are shown. All $p > 0.05$.



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