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Differences in dopamine and opioid receptor ratios in the nucleus accumbens relate to physical contact and undirected song in pair-bonded zebra finches

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Abstract

Long-term social bonds are critical for survival and reproductive success in many species. Although courtship and pair bond formation are relatively well-studied, much less is known about the neural regulation of behaviors that occur after pair bonding that reinforce the bond and contribute to reproductive success. Dopamine and opioids in the nucleus accumbens (NAc) alter motivational state and reward by binding to receptor subtypes that engage distinct and opposing second messenger systems, and there is evidence that receptor ratios may influence social behavior. We used quantitative real time PCR to explore relationships between mRNA ratios for dopamine D1 and D2 receptors (D1:D2) and mu and kappa opioid receptors (MOR:KOR) in NAc and behaviors implicated in reproductive investment and pair bond maintenance in established male-female zebra finch pairs. In males, D1:D2 expression in NAc related negatively, whereas MOR:KOR related positively, to undirected song production. D1:D2 receptors also related positively to physical contact with a female. For females, D1:D2 expression was lower in females exposed to high compared to low rates of the partner's undirected song, and MOR:KOR expression in females related positively to undirected song exposure and allopreening. Analyses of single genes did not yield the same results. These findings suggest that the ratio of D1 to D2 and MOR to KOR receptor signaling in NAc causes differences in behavior or that behavior (or the partner's behavior) causes receptor ratio changes to modulate behaviors that maintain pair bonds and promote reproductive investment.

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Author Note: Data and study materials are freely available upon request.

Keywords

undirected song; pair bond maintenance; nucleus accumbens; opioid receptors; dopamine receptors

Introduction

Long-term social bonds are critical for survival and reproductive success in many species. Although well studied in some primates (French, Cavanaugh, Mustoe, Carp, & Womack, 2018) and rodents (e.g., prairie voles, *Microtus ochrogaster* (Sadino & Donaldson, 2018)), attachments to mating partners are rare in mammals. In contrast, many birds, including zebra finches (*Taeniopygia guttata*), establish and maintain enduring, monogamous pair bonds that are characterized by ongoing contact such as allopreening and side-by-side physical contact (Mock & Fujioka, 1990; Prior & Soma, 2015). In addition to behaviors that likely function to reinforce the pair bond, sensory input from males impacts the female reproductive system. For example, in prairie vole pairs physical contact and chemical cues from male urine prepare the female's uterus for pregnancy (Carter, Getz, Gavish, McDermott, & Arnold, 1980). In songbirds, vocal signals can increase numbers of eggs laid and promote ovarian follicle development in canaries (Bentley, Wingfield, Morton, & Ball, 2000; Kroodsma, 1976), and male undirected song produced near the nest appears to increase female reproductive investment in pair bonded zebra finches (e.g., females spend more time in the nest and produce larger eggs with more orange yolks) (Bolund, Schielzeth, & Forstmeier, 2012).

In contrast to the numerous studies on courtship and the formation of pair bonds in both birds and mammals, less is known about the neural regulation of behaviors that occur after pair bonding that reinforce the bond and contribute to reproductive success (Aragona et al., 2006; Lowrey & Tomaszycki, 2014; Prior & Soma, 2015; Resendez, Kuhnmuench, Krzywosinski, & Aragona, 2012). A recent RNA-seq study on male zebra finches with long-term female partners indicated that male pair bond related behaviors and the behaviors of his partner related to a gene module in the male's ventral tegmental area (VTA) that contains many dopamine-related genes (Alger et al., 2020). The VTA sends dopaminergic projections to several areas, including the nucleus accumbens (NAc). Dopaminergic projections to the NAc are considered central to the regulation of motivated, reward-directed behaviors including mate- and offspring-directed behaviors in birds and mammals (Alger, Juang, & Riters, 2011; Aragona et al., 2006; Banerjee, Dias, Crews, & Adkins-Regan, 2013; Champagne et al., 2004).

In zebra finches and prairie voles, studies implicate dopamine in NAc in pair bond formation and maintenance (Alger, Larget, & Riters, 2016; Aragona et al., 2006; Banerjee et al., 2013). Dopamine in NAc binds to receptors in the D1 and D2 families (Missale, Nash, Robinson, Jaber, & Caron, 1998). These receptor subtypes are located in different subcellular components of the synapse (Benoit-Marand, Ballion, Borrelli, Boraud, & Gonon, 2011), activate opposing intracellular systems (Beaulieu & Gainetdinov, 2011; Soares-Cunha, Coimbra, Sousa, & Rodrigues, 2016), and differentially impact behavior, including pair

bond formation (Aragona et al., 2006; Aragona & Wang, 2007). In male prairie voles, pharmacological manipulations show that D1 receptors in NAc prevent pair bond formation; whereas, D2 receptors facilitate bonding. After bonding, D1 receptors in NAc upregulate and these receptors underlie selective aggression that is used to maintain bonds (Aragona et al., 2006; Lei, Liu, Smith, Lonstein, & Wang, 2017; Resendez et al., 2016). These results raise the possibility that an increase in D1 over D2 receptors in NAc after pairing may promote pair bond maintenance. It has also been proposed that an increase in D1 over D2 receptors in NAc may underlie rewarding effects related to paternal bonds with pups in mandarin voles (Fang & Wang, 2017; Lei et al., 2017). These findings suggest that the ratio of D1 over D2 in NAc may underlie important behaviors related to bond maintenance.

In contrast to the motivated, reward-directed behaviors stimulated by dopamine, opioids in NAc modulate affective responses to reward acquisition (Pecina, Smith, & Berridge, 2006). Mu and kappa opioid receptors (MORs and KORs, respectively) are found both pre- and post-synaptically in NAc; with the major effects of MOR on motivated behaviors attributed to postsynaptic activation, and the major effects of KOR attributed to presynaptic inhibition of dopamine release (Heijna et al., 1990; Mulder, Wardeh, Hogenboom, & Frankhuyzen, 1989; Svingos, Colago, & Pickel, 1999; Svingos, Moriwaki, Wang, Uhl, & Pickel, 1996). MOR and KOR engage distinct second messenger responses (Belcheva et al., 2005) and have distinct effects on affective state. MORs are well-known to induce reward (Le Merrer, Becker, Befort, & Kieffer, 2009; Wise, 1989); whereas, KORs generally induce depressed or aversive states (Bals-Kubik, Herz, & Shippenberg, 1989; Mucha & Herz, 1985; Shippenberg & Herz, 1986), but see (Castro & Berridge, 2014).

Numerous studies in birds and mammals implicate MORs in social attachment and reward (Herman & Panksepp, 1978; Khurshid, Jayaprakash, Shahul Hameed, Mohanasundaram, & Iyengar, 2010; Panksepp, Herman, Conner, Bishop, & Scott, 1978; Stevenson et al., 2020; Warnick, McCurdy, & Sufka, 2005). MOR activation in NAc is necessary for the establishment of partner preferences in prairie voles (Burkett, Spiegel, Inoue, Murphy, & Young, 2011; Resendez et al., 2013; Trezza, Damsteegt, Achterberg, & Vanderschuren, 2011). In prairie voles, pair bonding decreases KOR binding in NAc in males and KOR stimulation reduces dopamine release in NAc (Resendez et al., 2016). Although ratios of MOR over KOR in NAc have not been well-studied, the drop in KOR in NAc after bonding suggests that a change in the ratio of MOR over KOR in NAc may also underlie behaviors related to breeding success after pair bond formation.

The goal of this study was to begin to test the hypothesis that differences in the ratios of dopamine and opioid receptors in NAc correspond to important pair behaviors produced after bonding in male and female zebra finches. To do this, we used quantitative real time PCR (qPCR) to explore relationships between mRNA for D1:D2 and MOR:KOR in NAc and behaviors implicated in reproductive investment and pair bond maintenance in established male-female zebra finch pairs during a period of egg laying.

Method

Animals

Experimental birds in this study included 12 adult males that were housed with an assigned female partner and 9 adult females that were housed with an assigned male partner. Birds were bred at the University of Wisconsin-Madison and housed in stainless-steel cages in a single room on a photoperiod of 16 h light:8 h dark, humidity from 30–60% and temperature between 20–24 °C. After fledging, birds were housed in single sex groups until being paired for this study. Subjects were adults, ranging from 7–13 months of age at the time of pairing. Water and a pellet and seed mix were available ad libitum and diet was supplemented with vegetables and egg mixture twice a week. All experiments 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 testing

Subjects were each randomly assigned a non-related opposite-sex partner (the opposite sex partner was not a focal animal in this study). Each male-female pair was housed in a separate cage (56 X 58 X 57 cm^3) that included a nest box, perches, a water bottle, and cuttlebone. Food, water, and nesting material was available ad libitum. Prior to beginning the experimental observation period, pairs were housed together for 16 days, which is a sufficient amount of time for pair bond formation to occur in zebra finches (Silcox & Evans, 1982). Each day for 20 min a single observer sat in a chair approximately 1.5 m from the pairs to habituate them to her presence and to confirm the observation of allopreening, clumping and nest building as indicators of pair bond formation. The focus of this study was on pair bond maintenance behaviors, so eggs were removed daily to prevent the initiation of parental behavior.

The observer conducted daily 20 min observations of each established pair in a random order for 5 days (days 17–21 after pairing). We selected this observation frequency and duration based on our past studies in European starlings and zebra finches in which 20 min observations across 4–5 days revealed strong correlations between behavior and neuronal markers, including mRNA (e.g., (Alger et al., 2011; Alger et al., 2020; DeVries, Cordes, Stevenson, & Riters, 2015; Kelm, Forbes-Lorman, Auger, & Riters, 2011)) and a survey of observation durations in zebra finches which range from 5 to 15 to 30 minutes in different studies (Banerjee et al., 2013; Bolund et al., 2012; Tomaszycki & Adkins-Regan, 2006). Observations began between 30min and 5h 30min after the lights turned on. The observer noted pair allopreening and clumping behaviors, which are indicators of pair bond maintenance. Pair allopreening was calculated as the number of allopreen bouts performed by the experimental bird plus those of its partner. Pair clumping was calculated as the number of times birds were observed to be in bodily contact with one another. This frequency measurement correlated positively with the duration of clumping behavior ($r = 0.70$, $p < 0.001$) measured with scan samples, which indicates that a score of 1 reflects a low rate of clumping and not a single long bout of clumping. Although clumping is a combined male-female measure, it differs across our male and female subjects because they were paired with non-focal partners, not with one another. Separate bouts of behavior

were separated by 2 secs. Undirected songs (songs produced by birds facing away from potential recipients) were quantified in males only (female zebra finches do not sing). We additionally counted the number of times males and females produced calls; however, we did not measure antiphonal calling, quiet calling or identify specific call types that are proposed to maintain pairs (e.g., (D'Amelio, Trost, & Ter Maat, 2017; Elie et al., 2010; Gill, Goymann, Ter Maat, & Gahr, 2015). Sexual and agonistic behaviors, including female directed songs, were also quantified but occurred too infrequently to analyze, likely because pairs during our observation period were well established. The sums of each measurement for the 5 days of observation were used for analyses.

Tissue collection and processing

Experimental birds were rapidly decapitated on day 22 (the day after behavioral observations were completed). Brains were removed immediately, frozen on dry ice and stored at −80 °C. Brains were sectioned coronally at 200 μms using a cryostat at −15 ºC. Sections were thaw mounted onto glass slides and a 2 mm punch over the midline containing the rostral nucleus accumbens (NAc; Figure 1), identified based on (Reiner et al., 2004) was collected using a sample core (FST 18035–02, Foster City, CA, USA) under a dissection microscope over dry ice. Punches were stored at −80 °C until qPCR was performed. For consistency, a single researcher collected all tissue punches and a single researcher ran qPCR. All samples included in this study were similar in RNA concentration and purity.

Quantitative real time PCR (qPCR)

Tissue punches were homogenized and total RNA was extracted using a Bio-Rad Aurum Total RNA Fatty and Fibrous Tissue Kit (Catalog No. 732–6830; Bio-Rd, Hercules, CA, USA). A Nanodrop system (Thermo Scientific, Wilmington, DE, USA) was used to measure total RNA. All samples had an A260/A280 ratio greater than 1.8. DNase treated RNA (100 ng/uL per cDNA reaction) was converted into single-stranded cDNA using the Invitrogen SuperScript III First-Strand Synthesis System (Catalog No. 18080–05; Invitrogen, Carlsbad, CA, USA).

After conversion, relative gene expression for mu opioid receptor (OPRM1), kappa opioid receptor (OPRK1), dopamine receptor 1 (D1) and dopamine receptor 2 (D2) was determined for each brain region as a normalized ratio to reference genes. Primers for all genes were designed (NCBI Gene Database, Primer-Blast) using the zebra finch (*Taeniopygia guttata*) genome for qPCR analysis (Table 1). The qPCR reaction product was sequenced using Sanger sequencing with both forward and reverse primers at the University of Wisconsin Biotechnology Center (Table 2). Using NCBI BLAST these sequences match the intended targets. Two reference genes (Peptidylprolyl Isomerase A [PPIA]); phosphoglycerate kinase [PGK1]) were also analyzed to normalize mRNA levels across samples. These reference genes were selected because expression has been found to be similar in songbirds (ie., starlings) tested in different seasons and associated hormone conditions, and the primers used for reference genes were previously shown to match the intended targets (using Sanger sequencing) in starlings (Riters, Cordes, & Stevenson, 2017).

Samples were prepared in qPCR reaction tubes containing sample cDNA, nuclease free water, forward and reverse primers (5 μM; University of Wisconsin), and SsoFast EvaGreen Supermix (Catalog No. 172–5201; Bio-Rad, Hercules, CA). Five standards were run with each plate of samples (1:10 serial dilution, starting concentration at 500 ng/μl), along with a negative control (nuclease free water substituted for cDNA). Standards and samples were run in triplicate on each plate and all plates were read with the BioRad CFX96 Touch Real-Time PCR Detection System (Catalog No. 185–5195; Bio-Rad, Hercules, CA). Each qPCR run consisted of the following: an initiation step at 95 °C for 30 s, followed by 40 cycles of 95 °C for 5s, a 30 s annealing phase, a 30 s elongation phase at 72 °C, and a melt curve from 60 °C to 92 °C, 0.5 ° for each 5 s step. Plates were read following each elongation and melt curve step. Criteria for inclusion in the dataset were based on MIQE guidelines: run efficiencies between 90 and 110%, an R^2 of at least 0.990, and a melt curve displaying a single peak indicative of primer specificity. Individual data points for each gene were calculated using the PFAFFL method and reported as expression relative to the two reference genes. We focus our analyses on ratios of OPRM1:OPRK1 and DRD1:DRD2 mRNA measurements in NAc based on the premise developed in the introduction, but report p values for the same analysis of individual genes to demonstrate that in this study ratios were most relevant.

Analysis

We examined data in two ways. To explore the extent to which relationships between ratios and behaviors were linear, we used correlation analyses. To explore the extent to which relationships may be categorical, we also ran general linear models (GLMs). Because the study is correlational, it could be that receptor ratios cause differences in behavior or it could be that behavior (or the partner's behavior) causes receptor changes. Thus, it is not possible to know whether the appropriate predictor variable would be the expression ratio or the behavior. During preliminary GLM analyses, we divided birds into two groups based on a median split for MOR:KOR and D1:D2 (low vs high groups) with the idea that expression ratios may predict behavior; however when divided in this way there were extreme statistical outliers, variance was not homogenous between groups, and points above and below the median split were quite close together. In contrast, when we divided birds into two groups using a median split for behavior (low vs high behavior, based on the sum of behavior across the 5 observation days), variance between groups was more similar and there were cleaner differences between individuals in the two categories. We thus present here only GLMs for which low vs high behavior is entered as a categorical variable. Specific details for each analysis are detailed below. Data and study materials are freely available upon request.

Results

Evidence of pair bonding

In the five days of behavioral observation, all but two subjects were observed to clump and/or allopreen with their partners, and the two subjects that were not observed to allopreen or clump were found to build nests and produce eggs. Furthermore, all pairs except one produced a nest and/or eggs, and the one pair that did not produce a nest or eggs was found to both allopreen and clump. Thus, although pairs demonstrated differences in the degree

to which they displayed bonding related behaviors (shown below), all pairs showed some evidence of bonding.

Analyses of sex differences

With the exception of singing behavior, which is performed by male zebra finches only, no significant sex differences were identified for any of the behaviors or mRNA measurements ($p > 0.05$ for all comparisons; Table 3). No significant sex differences were identified for single genes (D1: $p = 0.276$, D2: $p = 0.157$, MOR: $p = 0.126$, KOR: $p = 0.346$).

Undirected song

Male NAc and undirected song production—For each male, total undirected song produced across the 5 observation periods was categorized as low or high based on a median split (median = 33; 33 or below = low). A GLM with low vs high song entered as an independent variable and both D1:D2 and MOR:KOR mRNA measurements in male NAc entered into the same model as dependent variables revealed a significant song x mRNA interaction ($F_{1,10} = 8.06$, $p = 0.0176$; Figure 2). Fisher's LSD posthoc analyses revealed that D1:D2 was significantly lower in high compared to low singers ($p = 0.0024$), whereas MOR:KOR was significantly higher in high compared to low singers ($p = 0.0257$). No significant main effects were observed for song ($F_{1,10} = 1.01$, $p = 0.3397$) or mRNA ($F_{1,10} = 0.81$, $p = 0.3889$). Lilliefors tests indicated that data were normal; however, a Levene's test indicated that D1:D2 measurements violated assumptions of homogeneity of variance. Log transformation corrected this violation. Analysis of log transformed or untransformed data yielded the same results. We report the untransformed results here for transparency (i.e., so that actual song numbers are clear). The same analysis with single genes entered in place of ratios yielded a significant gene by song rate interaction ($F_{3,30} = 8.09$, $p = 0.0004$), but post hoc Fisher's LSD analysis did not reveal any differences between high and low singers for any individual genes (D1: $p = 0.472$, D2: $p = 0.494$, MOR: $p = 0.395$, KOR: $p = 0.779$).

Correlation analyses revealed no significant linear relationships between male song and MOR:KOR ($r = 0.46$, $p = 0.1306$). For the D1:D2 analysis residual analysis revealed a statistical outlier (Standard residual > 2sd, shown in Figure 2). Without this outlier, a significant negative correlation was found between male song and D1: D2 ($r = 0.62$, $p = 0.0408$). In addition, after removal of this outlier a second point (1.8 for D1: D2) became an outlier, however retaining or removing this point did not impact the outcome so it remains in the analysis. All assumptions required for parametric statistics were met. The same analysis run on single genes did not reveal any significant correlations (D1: $p = 0.466$, D2: $p = 0.233$, MOR: $p = 0.202$, KOR: $p = 0.956$; Figure S1).

Female NAc and song production by her male partner—For females, total undirected song produced by each male partner was categorized as low or high based on a median split (median = 19; 19 or below = low). A GLM with low vs high partner song entered as an independent variable and both MOR:KOR and D1:D2 mRNA measurements in female NAc entered into the same model as dependent variables revealed a significant song x mRNA interaction ($F_{1,7} = 10.58$, $p = 0.0140$; Figure 3). Fisher's LSD posthoc analyses revealed that D1:D2 was significantly higher in females with partner's that sang at low

compared to high rates (p = 0.0047), whereas MOR:KOR tended to show the opposite patterns (p = 0.0659). No significant main effects were observed for partner song ($F_{1,7}$ = 1.81, p = 0.2207) or mRNA ($F_{1,7}$ = 2.98, p = 0.1280). Lilliefors and Levene's tests revealed that data did not violate any assumptions. The same analysis with single genes entered in place of ratios yielded a significant gene by song rate interaction ($F_{3,21}$ = 5.81, p = 0.0047), but post hoc Fisher's LSD analysis did not reveal any differences between high and low singers for any genes (D1: p = 0.059, D2: p = 0.243, MOR: p = 0.189, KOR: p = 0.430).

Correlation analyses revealed a significant positive correlation between female MOR:KOR and song produced by a female's partner (r = 0.70, p = 0.0339) and a nearly significant negative relationship for D1:D2 (r = 0.67, p = 0.0500; Figure 3). All assumptions required for parametric statistics were met. The same analysis run on single genes did not reveal any significant correlations (D1: p = 0.183, D2: p = 0.094, MOR: p = 0.124, KOR: p = 0.098; Figure S1).

Male NAc and clumping—For each male, total clumping was categorized as low or high based on a median split (median = 2.5). A GLM with low vs high clumping entered as an independent variable and both D1:D2 and MOR:KOR mRNA measurements in male NAc entered into the same model as dependent variables revealed no significant main effects (clumping: $F_{1,10}$ = 2.80, p = 0.1251; mRNA: $F_{1,10}$ = 1.36, p = 0.2702) or clumping x mRNA interaction ($F_{1,10}$ = 2.32, p = 0.1588). The D1:D2 data violated normality so the analysis was run on the square root transformed data. The analysis with single genes entered in place of ratios did not reveal any significant main effects or interaction (p > 0.241 in all cases).

Correlation analyses revealed a positive correlation between male clumping and D1:D2 (r = 0.63, p = 0.0276; Figure 4) but no significant linear relationships between male clumping and MOR:KOR (r = 0.49, p = 0.107). The same analysis run on single genes did not reveal any significant correlations (D1: p = 0.126, D2: p = 0.397, MOR: p = 0.305, KOR: p = 0.824: Figure S1).

Female NAc and pair clumping—For females, total clumping was categorized as low or high based on a median split (median = 2; 2 or below = low). A GLM with low vs high clumping entered as an independent variable and both D1:D2 and MOR:KOR mRNA measurements in female NAc entered into the same model as dependent variables revealed no significant main effects (clumping: $F_{1,7}$ = 2.78, p = 0.1391; mRNA: $F_{1,7}$ = 1.65, p = 0.2399) or clumping x mRNA interaction ($F_{1,7}$ = 0.44, p = 0.5299). The analysis with single genes entered in place of ratios did not reveal any significant main effects or interaction (p > 0.062 in all cases).

Correlation analyses also revealed no significant linear relationships between clumping and D1:D2 (r = 0.27, p = 0.4883) or MOR:KOR (r = 0.02, p = 0.9598) in females. The same analysis run on single genes did not reveal any significant correlations (D1: p = 0.566, D2: p = 0.764, MOR: p = 0.451, KOR: p = 0.441; Figure S1). All statistical assumptions were met.

Male NAc and pair allopreening—For males, total pair allopreening was categorized as low or high based on a median split (median = 9.5). A GLM with low vs high pair

allopreening entered as an independent variable and both D1:D2 and MOR:KOR and mRNA measurements in male NAc entered into the same model as dependent variables revealed no significant main effects (pair allopreening: $F_{1,10} = 0.74$, $p = 0.4104$; mRNA: $F_{1,10} = 0.50$, $p = 0.4969$) or pair allopreening x mRNA interaction ($F_{1,10} = 1.07$, $p = 0.3261$). The analysis with single genes entered in place of ratios did not reveal any significant main effects or interaction ($p > 0.369$ in all cases).

Correlation analyses also revealed no significant linear relationships between pair allopreening and male MOR:KOR in NAc ($r = 0.32$, $p = 0.3046$). The D1:D2 residual analysis revealed the same two statistical outliers (standard residual > 2sd) that were identified in the analysis of undirected song. Results were not significant with or without these outliers (with all data, $r = 0.12$, $p = 0.7097$; without outliers, $r = 0.16$, $p = 0.6649$). All statistical assumptions were met. The same analysis run on single genes did not reveal any significant correlations (D1: $p = 0.490$, D2: $p = 0.397$, MOR: $p = 0.215$, KOR: $p = 0.309$; Figure S1).

Female NAc and pair allopreening—For females, total pair allopreening was categorized as low or high based on a median split (median = 7; 7 = low). A GLM with low vs high pair allopreening entered as an independent variable and both D1:D2 and MOR:KOR mRNA measurements in female NAc entered into the same model as dependent variables revealed no significant main effects (pair allopreening: $F_{1,7} = 0.19$, $p = 0.6743$; mRNA: $F_{1,7} = 1.90$, $p = 0.2102$) or pair allopreening x mRNA interaction ($F_{1,7} = 2.95$, $p = 0.1296$). The analysis with single genes entered in place of ratios did not reveal any significant main effects or interaction ($p > 0.088$ in all cases).

For correlation analyses, residual analysis revealed a statistical outlier for MOR:KOR (standard residual > 2sd, shown in Fig 5). Without this outlier a significant positive linear relationship was found between pair allopreening and female MOR:KOR in NAc ($r = 0.86$, $p = 0.0061$; Figure 5). The correlation between D1:D2 in NAc and pair allopreening was not significant ($r = 0.31$, $p = 0.4238$). All statistical assumptions were met. The same analysis run on single genes did not reveal any significant correlations (D1: $p = 0.282$, D2: $p = 0.628$, MOR: $p = 0.319$, KOR: $p = 0.210$; Figure S1).

Discussion

The results of this study demonstrate distinct relationships between ratios of dopamine and opioid receptor expression in NAc and physical contact as well as the production of undirected song in zebra finch males and exposure to undirected song in zebra finch females. Analyses of single genes did not yield the same results, indicating that ratios of dopamine and opioid receptor gene expression in NAc, rather than single gene expression, may be more biologically relevant to pair bond maintenance. These findings suggest that the balance of D1 and D2 receptor signaling and MOR and KOR receptor signaling in NAc may contribute to individual differences or be altered by individual differences in behaviors proposed to maintain long-term pair bonds and to promote female reproductive investment.

D1:D2 expression in NAc of males relates positively to clumping and negatively to undirected song

Past studies in voles suggest that after the establishment of a pair bond, an increase in D1 relative to D2 signaling in NAc may facilitate pair bond maintenance behaviors (Aragona et al., 2006; Fang & Wang, 2017; Lei et al., 2017; Resendez et al., 2016). Consistent with this prediction, we report a positive linear correlation between D1 over D2 expression ratios in NAc and clumping behavior in males. A past conditioned place preference study showed that in mandarin voles a drop in D2 receptor expression in NAc appears to underlie rewarding effects related to the paternal bond with pups (Fang & Wang, 2017). If this mechanism that underlies paternal-pup bonds generalizes to pair bonds in songbirds, this suggests that lower D2 relative to D1 in NAc in male zebra finches may underlie rewarding effects of male physical contact with his partner.

In contrast to clumping, we found a negative relationship between D1 over D2 mRNA ratios in NAc and undirected song production. Whereas female-directed song in zebra finches is known to be critical for mate attraction (Riebel, 2009; Tomaszycki & Adkins-Regan, 2005), the function of undirected song in zebra finches remains elusive. Undirected song is produced at higher rates by unpaired males than by paired males (Bolund et al., 2012) and in paired males is positively correlated with extra-pair directed song but not partner-directed song (Dunn & Zann 1996), suggesting a role in mate attraction. However, undirected song during the period of egg laying does not appear important for attracting extra-pair or within-pair females (Bolund et al., 2012; Tomaszycki & Adkins-Regan, 2006). The vast majority of zebra finch song output produced while the female mate is in the nest is undirected song (Dunn & Zann, 1996). Females can recognize their mate’s song (Miller, 1979) and pair bonds can be maintained through auditory contact when visual and tactile contact is lost (Silcox & Evans, 1982). Furthermore, females with partners that produce high levels of undirected song remain in the nest longer (Dunn & Zann 1996) and produce larger eggs with more orange yolks (Bolund et al., 2012), indicating a higher reproductive effort. Despite this evidence in support of the role of undirected songs in pair bond maintenance, the surgical alteration or elimination of song does not significantly affect established pair bonds (Tomaszycki & Adkins-Regan, 2006). Thus, the role of undirected songs in zebra finches may be context dependent.

Although effects of D2-specific manipulations on song have not been well-studied, peripheral D1 receptor agonists stimulate male courtship song that is used to attract mates in male starlings (Schroeder & Riters, 2006). Dopamine levels in the striatal area X in zebra finches increase during both directed and undirected song, but they are lower during production of undirected song (Sasaki, Sotnikova, Gainetdinov, & Jarvis, 2006). D2 receptors are proposed to be more sensitive to continuous dopamine release; whereas, D1 receptors are preferentially sensitive to phasic burst activity of dopamine (Dreyer, Herrik, Berg, & Hounsgaard, 2010). It is thus possible that a larger pulse of dopamine activates more D1 relative to D2 receptors to facilitate directed song, while a lower pulse would engage relatively fewer D1 compared to D2 receptors to facilitate undirected song. Consistent with this possibility, blocking D1 receptors in striatal area X in male zebra finches causes song to become structurally more like undirected song (Leblois, Wendel, &

Perkel, 2010). This suggests that higher expression of D2 over D1 in NAc observed in the present study may play a role in reducing courtship song when it is not necessary during egg laying. Studies involving site-directed manipulations of D1 and D2 receptors in striatal NAc are now needed to determine the degree to which the correlations identified in this study reflect causal relationships.

MOR:KOR expression is higher in high compared to low singing males

In this study, we found that MOR predominated over KOR in the NAc of high compared to low singing males. Given the role of MOR in NAc in reward, this suggests that males with greater MOR relative to KOR signaling in NAc may experience a positive state that facilitates undirected song or that female responses or the act of producing song induce a positive state. MOR agonism stimulates undirected song in male starlings (Stevenson et al., 2020), and studies of male zebra finches and starlings demonstrate that males develop a strong preference for a place that had been paired with their own production of undirected song (Hahn et al., 2017; Riters & Stevenson, 2012; Riters, Stevenson, DeVries, & Cordes, 2014). Although in those studies undirected song was not produced in the context of egg laying, the results suggest that undirected singing at least in some contexts is tightly associated with a positive, intrinsic reward state. The extent to which this is the case in males singing undirected song to promote female reproductive investment has not been examined; however, because undirected song results in no obvious extrinsic reinforcement (e.g., it does not immediately result in copulation or repel a territorial intruder) it is logical to suggest that singing outside the nest may be intrinsically rewarding.

D1:D2 expression is lower in females exposed to high compared to low rates of male song

In this study, females with partners that produced high rates of undirected song had higher D2 relative to D1 expression in NAc compared to females exposed to low rates of undirected song. In zebra finches, male undirected song is thought to encourage female nesting behavior (Dunn & Zann, 1996), and female zebra finches develop preferences for the songs of their mates over other males (Coleman, Saxon, Robbins, Lillie, & Day, 2019; Tokarev et al., 2017; Woolley & Doupe, 2008). In female starlings, labeling for tyrosine hydroxylase in NAc correlated positively with nesting behavior (Pawlisch, Kelm-Nelson, Stevenson, & Riters, 2012), suggesting dopamine in this area may facilitate reproductive investment. In female zebra finches, a recent study demonstrated that D2 but not D1 activation induced female preferences for male song and blocking D2 but not D1 receptors abolished song preferences in paired females (Day et al., 2019). Although causal relationships must be tested, our findings raise the possibility that exposure to male undirected song results in higher D2 expression which may influence nesting behavior and / or the development and maintenance of female preferences for the song of her mate.

MOR:KOR expression in females relates positively to allopreening and undirected song exposure

In females, the positive linear relationship identified between pair allopreening and MOR over KOR mRNA ratios in NAc is consistent with numerous past studies implicating MOR in contact comfort (Herman & Panksepp, 1978; Panksepp et al., 1978). This finding suggests that MOR in NAc may facilitate and reward social contact with mates. A positive

linear correlation was also found between higher MOR over KOR expression in NAc and exposure to undirected song. Past studies show that immediate early gene expression in NAc increases in females in response to playback of male song (Earp & Maney, 2012) and that females will spend time near speakers playing mate song (Woolley & Doupe, 2008), perform operative responses to hear mate song (Coleman et al., 2019) and develop conditioned place preferences for male song (Riters et al., 2013). These findings indicate that male song can serve as a reward, and the present findings suggest that a predominance of MOR over KOR signaling in NAc may play a role.

Conclusion

Given the role of dopamine and opioids in NAc in motivation and reward respectively, the associations identified in this study suggest that differences in receptor ratios may modulate the affective state of males and females to promote physical contact, undirected singing behavior in males, and responses to undirected singing in females. Causal relationships are suggested by past studies; however, it is now necessary to test the hypotheses generated here with site specific manipulations of D1, D2, MOR, and KOR receptors in NAc. Furthermore, dopamine and opioids can bind to receptors on the same neurons (Spool, Merullo, Zhao, & Riters, 2018) and can act at G protein-coupled receptor (GPCR) heteromers, which consist of two or more functionally interacting GPCR subunits that are activated by different ligands (Ferre et al., 2009; Fujita, Gomes, & Devi, 2014). For example, MOR-D1 heteromers appear to mediate locomotor sensitization to opioids (Tao et al., 2017). Understanding these actions will also be critical in future research.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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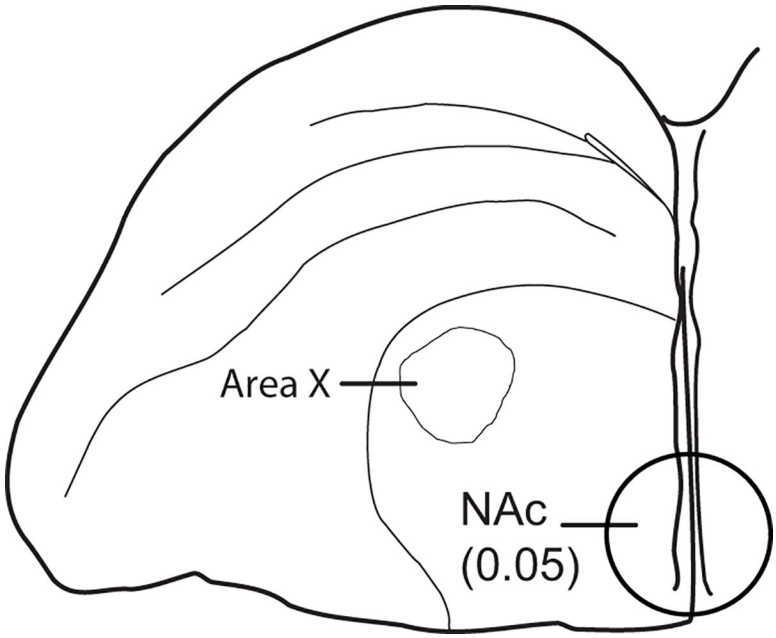

Figure 1.
Illustration of the approximate location of the 2mm diameter tissue punch taken from NAc in a coronal section of brain.

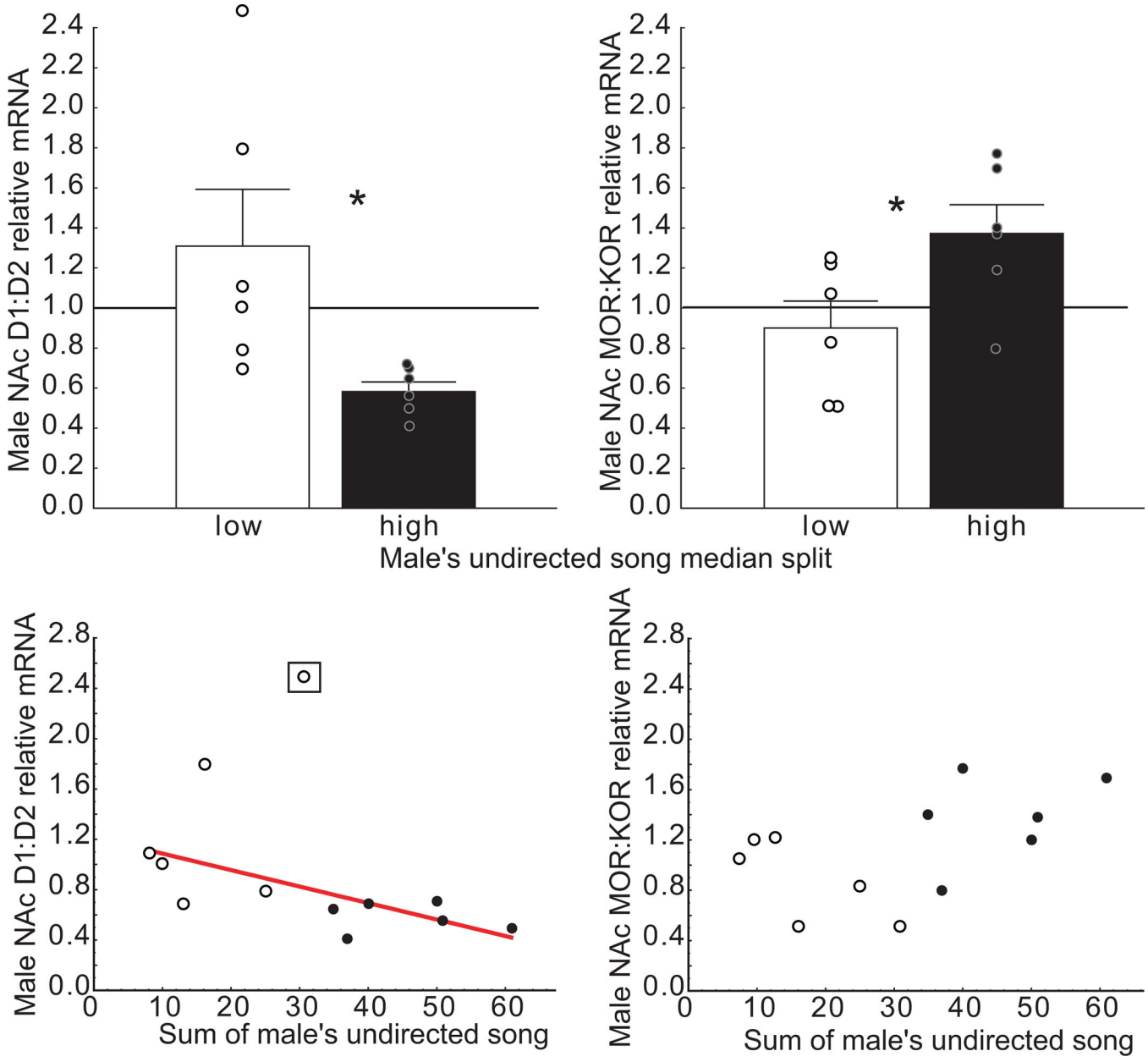

Figure 2.

Dopamine and opioid receptor mRNA ratios in NAc differ in males that produce low versus high rates of undirected song. Top row -- Mean relative mRNA ratios + sem for D1:D2 (left) and MOR:KOR (right) in males that produce low (open bars) or high (filled bars) rates of song as determined using a median split. The point at which ratios diverge from 1:1 is indicated by a horizontal line. Bottom row -- Scatterplots illustrating relationships between dopamine and opioid receptor mRNA ratios in the male's NAc and undirected song in males. The x axis illustrates male undirected song rate, the y axis indicates relative mRNA ratios for D1:D2 (left) and MOR:KOR (right) with each dot representing an individual male. Open dots indicate low and filled dots represent high singers in bar graphs. An outlier that was removed from the analysis is indicated by the box (Note: the categorical analysis in

row 1 was significant with or without this point). The regression line indicates a significant correlation ($p < 0.05$).

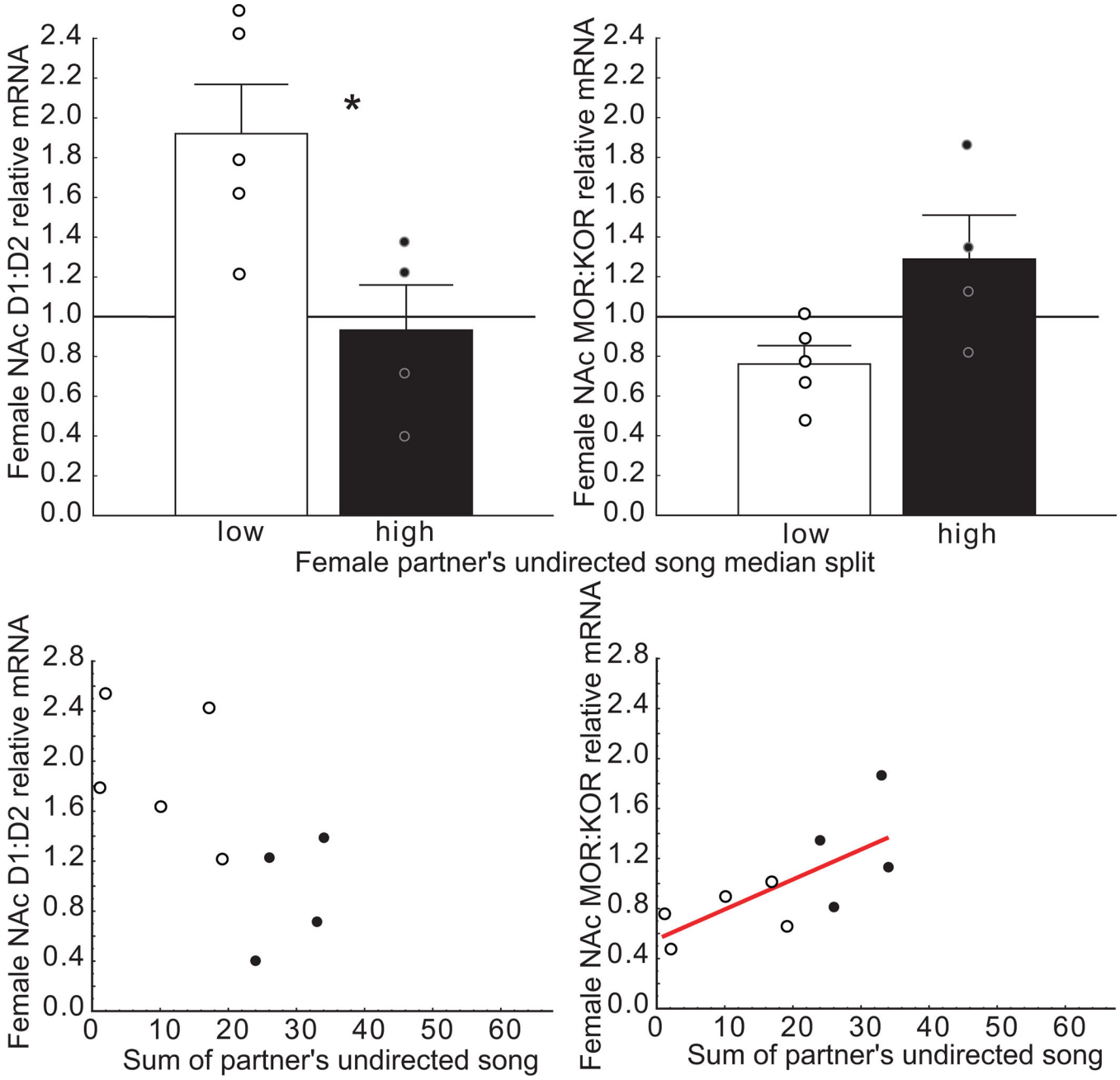

Figure 3.

Dopamine and opioid receptor mRNA ratios in NAc differ in females whose partners produced low versus high rates of undirected song. Top row -- Mean relative mRNA ratios + sem for D1:D2 (left) and MOR:KOR (right) in females paired with males that produced low (open bars) or high (filled bars) rates of song as determined using a median split. The point at which ratios diverge from 1:1 is indicated by a horizontal line. Bottom row -- Scatterplots illustrating relationships between dopamine and opioid receptor mRNA ratios in the female NAc and the number of undirected songs produced by her partner. The x axis illustrates male undirected song rate, the y axis indicates relative mRNA ratios for D1:D2

(left) and MOR:KOR (right) with each dot representing an individual female. Open dots indicate females with male partners that sang at low and filled dots represent partners that sang at high rates in bar graphs. The regression line indicates a significant correlation ($p < 0.05$).

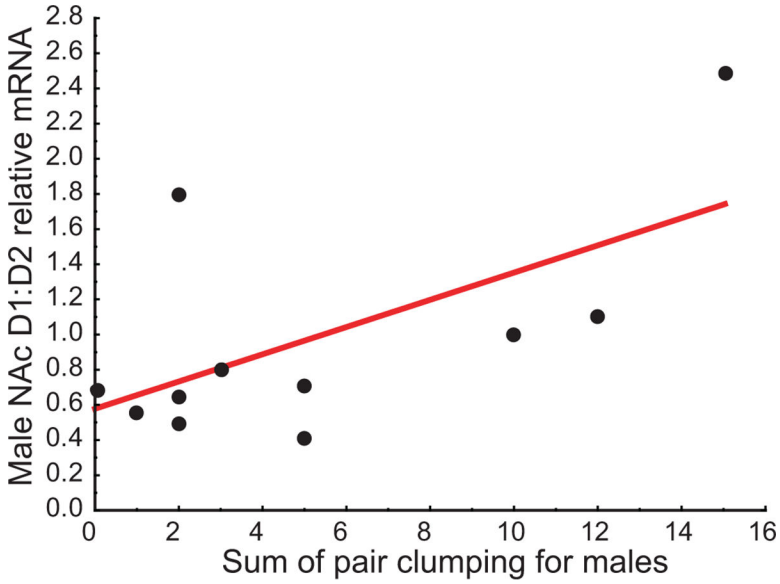

Figure 4.

Scatterplot illustrating relationship between the D1:D2 receptor mRNA ratio in the male NAc and the number of clumping events within the pair. The x axis illustrates pair clumping and the y axis indicates relative mRNA ratios for D1:D2 with each dot representing an individual male. The regression line indicates a significant correlation ($p < 0.05$).

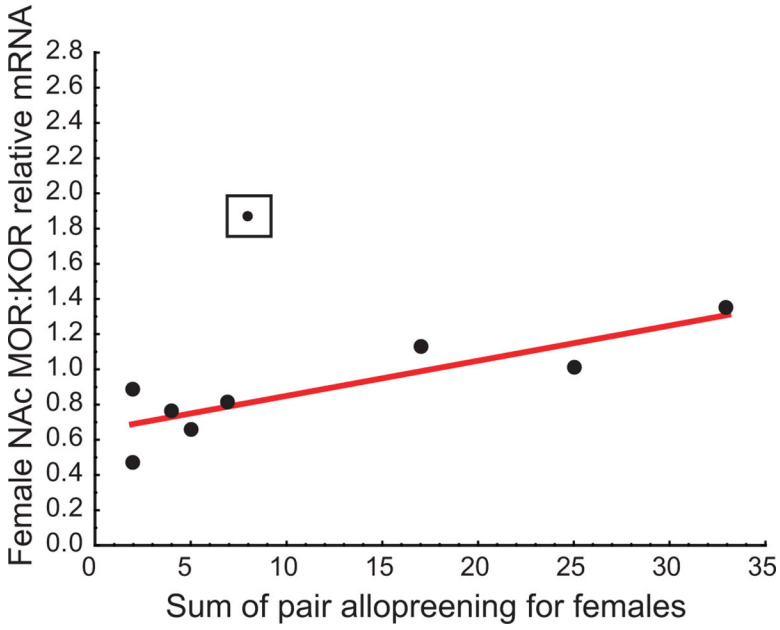

Figure 5.

Scatterplot illustrating relationship between the MOR:KOR receptor mRNA ratio in the female NAc and the number of allopreening events within the pair. The x axis illustrates pair allopreening and the y axis indicates relative mRNA ratios for MOR:KOR with each dot representing an individual female. The regression line indicates a significant correlation ($p < 0.05$), after the removal of the outlier indicated by the box.

Table 1.

Primers sequenced using the zebra finch genome

Gene	Accession	Forward Primer	Reverse Primer	Target length	Tm
OPRM1 (gene name for MOR)	XM_002187352	GCAGATGCCCTAGCAACAAG	CACGTAGCGATCCACACTCA	165	57
OPRK1 (gene name for KOR)	XM_012571983.1	GATGAACTCCTGGCCCTTTG	AGCAGTATCTTCCCTGACTTTGG	259	60
DRD1 (gene name for D1)	NM_001243833.1	ACGAGAGGAAAATGACCCCC	GTTGTAGCCTTGTGCCAGTT	112	58
DRD2 (gene name for D2)	XM_002191611.2	TACCAGTCCCCCTGAGAAAG	GTAGAGTTGTTGCCCCGATT	96	58
PPIA	NM_001245462.1	AGACAAGGTCCCGAAGACAG	CCATTGTGGCGTGTGAAGT	138	61
PGK1	XM_002199475.2	AAAGTTCAGGATAAGATCCAGCTG	GCCATCAGGTCCTTGACAAT	167	60

Table 2.

PCR products amplified

Gene	Sequence (Sanger)
OPRM1	GGATACTTGAGGGACGTGGCCATTCGGTACCATCCTTTGTAAGATTGTTATATCCATAGACTACTACAATATGTTCACCAGTATCTTCACACTCTGCACCA
OPRK1	TGATTTCTATTGACTATTACAACATGNTTTACCAGCATTTTCACACCTCACCATGATGAGTGTTGATCGATACATTGCCTGTGTGTCACCCTGTGAAGGCT
DRD1	GAGGAANACACGGGACAAAGGTCCACGGCCACGCCTGGATCATGGATGAAGGGCTGCCTTGGGGGGTCATTTTCCCTCTT
DRD2	GGGGNTNNTTGGTGCTGTGGGNTTTGAAGGGCCGCTTTCTCAGGGGGACCTGGATAA
PPIA	GTGAGAAGGGATTTGGCTACAAGGGGTCCTGCTTCCACAGAATCATTCCTGGGTTCATGTGCCAGGGTTGGTGACTTCACACGCCACAATGGA
PGK1	GTCATGAGATGATCATTGGGTGGTGGAATGGCATTCACCTTTCCTCAAAGGTGCTCAACAACATGGNAGATTGGCAACTCTCTGTTTTGATGAANAGG

Table 3.

Comparisons of behavior and mRNA ratios for males and females.

	Mean (sd) male (n=12)	Mean (sd) female (n=9)	t-value	df	p
Allopreening	4.58 (4.03)	4.22 (5.21)	0.18	19	0.86
Clumping	4.75 (4.96)	4.56 (5.68)	0.08	19	0.934
MOR:KOR	1.14 (0.41)	1.00 (0.41)	0.78	19	0.447
D1:D2	0.95 (0.61)	1.48 (0.71)	−1.86	19	0.078