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SOCIAL AFFILIATION RELATES TO TYROSINE HYDROXYLASE IMMUNOLABELING IN MALE AND FEMALE ZEBRA FINCHES (*TAENIOPYGIA GUTTATA*)

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Abstract

The catecholamines dopamine and norepinephrine are implicated in affiliative behaviors, yet few studies have addressed the extent to which affiliative behaviors within distinct social settings rely upon similar or distinct catecholaminergic mechanisms. To explore the role of catecholamines in affiliative behavior within distinct long-term social contexts, we examined the density of the catecholamine synthetic enzyme tyrosine hydroxylase (TH) in brain regions within both the mesolimbic dopaminergic system and “social behavior network” in male and female zebra finches (*Taeniopygia guttata*) paired for 21 days with either a same- or opposite-sex conspecific. On days 16–21 after pairing, members of both same- and mixed-sex pairs produced similar rates of affiliative behaviors. Measures of affiliation related to TH labeling in the ventral tegmental area (VTA), nucleus accumbens (Ac), medial preoptic nucleus (POM), and ventromedial nucleus of the hypothalamus (VMH). Relationships between TH labeling density and specific measures of affiliative behavior differed in rostral compared to caudal subregions of Ac and VTA, suggesting distinct roles for these subregions in the regulation of affiliative behavior. Finally, TH labeling density in the VMH and rostral VTA were positively related to the amount of courtship received from the partner and TH labeling in Ac was denser in opposite-sex pairs compared to same-sex pairs, indicative of socially-induced brain plasticity. Overall, results highlight a complex region- and behavior-specific role for catecholamines in vertebrate affiliation.

Keywords

mesolimbic dopamine system; norepinephrine; catecholamines; nucleus accumbens; ventral tegmental area; social behavior network

1. INTRODUCTION

Affiliative behaviors are central to social relationships, including group- and kin-alliance, sexual, pair bond, and parent-offspring relationships. Despite the importance of affiliative interactions to survival and reproductive success in social species, the neural mechanisms

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involved in the regulation of affiliative behaviors have not been fully elucidated. Furthermore, affiliative behaviors are observed within multiple social contexts (e.g. sexual, parental, and group maintenance), both between and within sexes. Few studies have addressed the extent to which affiliative behaviors within distinct social settings rely upon similar or distinct neural mechanisms.

Affiliative behavior involves social recognition and directing positive approach, feeding, grooming, and/or protective behaviors towards preferred conspecifics. The catecholamine dopamine (DA), acting within the mesolimbic system, has long been associated with reward-related processes, including associative learning and motivated behavioral responses to incentives such as food or an opposite-sex conspecific (Berridge *et al.*, 2009; Schultz, 2007). In songbirds, peripheral pharmacological manipulations implicate DA in male courtship song and other female-directed behaviors (Rauceo *et al.*, 2008; Schroeder and Ritters, 2006). In both birds and rodents, DA activity in the ventral tegmental area (VTA; one of the major sources of mesolimbic DA projections) has been shown to be important in affiliative and appetitive sexual behaviors (Balfour *et al.*, 2004; Bharati and Goodson, 2006; Charlier *et al.*, 2005; Heimovics and Ritters, 2008; Huang and Hessler, 2008; Yanagihara and Hessler, 2006; Zhang *et al.*, 1994). Dopaminergic cells in the VTA project onto multiple limbic forebrain areas essential to the regulation of affiliative behavior, including the nucleus accumbens (Ac), a principal target of the mesolimbic system, and the bed nucleus of the stria terminalis (BST), a component of the “social behavior network” (discussed below; Balthazart and Absil, 1997; Bjorklund and Dunnett, 2007; Durstewitz *et al.*, 1999). Studies of prairie voles provide evidence that dopaminergic neurons in the BST respond to social and sexual interactions with conspecifics (Cavanaugh and Lonstein, 2010b; Northcutt and Lonstein, 2009) and DA in the Ac facilitates mating-induced partner preference, reflected in an enhancement of mating partner-directed affiliative behaviors (Aragona *et al.*, 2003; Aragona *et al.*, 2006; Gingrich *et al.*, 2000). In rats, DA in the Ac promotes affiliative maternal behaviors, such as pup approach, retrieval, and grooming (Numan *et al.*, 2005; Stolzenberg *et al.*, 2007). Although anatomical and physiological attributes of DA systems are similar in mammals and birds (Durstewitz *et al.*, 1999; Reiner *et al.*, 2004), the precise anatomical location and subdivisions of the Ac in birds are still being determined (Balint and Csillag, 2007; Csillag *et al.*, 2008; Husband and Shimizu, 2010; Roberts *et al.*, 2002).

The catecholamine norepinephrine (NE) has also been implicated in affiliative behavior, particularly with respect to learning and memory of preferred individuals (Nelson and Panksepp, 1998). For example, offspring recognition in mother mice and ewes (Calamandrei *et al.*, 1992; Levy *et al.*, 1990) and mother recognition in mouse pups (Raineke *et al.*, 2010) are regulated by NE. NE also plays a role in the recognition of the male with which a pregnant female mouse has mated (Rosser and Keverne, 1985). Although not as extensively studied, NE also plays other roles in affiliative behavior. In rats, evidence suggests that NE influences juvenile play behavior (Vanderschuren *et al.*, 1997), social interactions between unfamiliar adults (Kask *et al.*, 2000), and the maintenance of maternal behaviors (Moffat *et al.*, 1993). In humans, Reboxetine, a selective NE reuptake inhibitor, was found to increase cooperative social behavior and social drive towards strangers (Tse and Bond, 2003).

DA and NE and their synthetic enzymes and receptors are found in the mesolimbic system and in an interconnected group of steroid-sensitive limbic fore- and midbrain regions (Bailhache and Balthazart, 1993; Ball *et al.*, 1995; Bottjer, 1993; Heimovics *et al.*, 2009; Heimovics and Ritters, 2008; Kubikova *et al.*, 2010; Mello *et al.*, 1998; Moons *et al.*, 1995; Moons *et al.*, 1994; Ritters and Ball, 2002) implicated in a variety of social behaviors, sometimes referred to as the “social behavior network” (Goodson, 2005; Newman, 1999). It has been argued that all social behaviors involve activity within this network but that different patterns of activity underlie different social behaviors. The role of catecholamines

in affiliation has been examined in a few of these areas. For example, data in zebra finches, quail and prairie voles demonstrate differential responsiveness of neurons containing tyrosine hydroxylase (TH; the rate-limiting enzyme in catecholamine synthesis) to a variety of social stimuli in several brain regions involved in social behavior, including the preoptic area, midbrain central gray, bed nucleus of the stria terminalis, and medial amygdala (Bharati and Goodson, 2006; Charlier *et al.*, 2005; Goodson *et al.*, 2009; Northcutt and Lonstein, 2009). These neurons are likely to be dopaminergic, because although dopamine- β -hydroxylase (DBH, a NE synthetic enzyme) immunoreactive fibers and varicosities can be found throughout these social behavior brain regions, DBH-positive cell bodies are restricted to the caudal midbrain, pons, and caudal portion of the medulla (Mello *et al.*, 1998). Furthermore, DA receptor activation in the medial preoptic nucleus of female rats stimulates maternal behavior (Stolzenberg *et al.*, 2007) and the density of D1-like DA receptors in the medial preoptic nucleus in male starlings positively correlates with the production of song used to maintain social flocks (Heimovics *et al.*, 2009). Data also implicate NE activity in the ventromedial nucleus of the hypothalamus in female starling approach responses to male song playback (Riters *et al.*, 2007; Riters and Pawlisch, 2007). These data suggest that catecholaminergic cells in areas involved in social behavior play a role in mediating context-specific affiliative behaviors.

In gregarious species, affiliation can be observed not only between members of mating pairs but also between females or between males that are members of the same social group. The role of catecholamines in affiliative behavior in social contexts outside of pair bond formation and maternal behavior has not been extensively examined. The zebra finch (*Taeniopygia guttata*) is a highly gregarious avian species that lives in large social flocks, forms long-lasting monogamous pair bonds and has among the lowest known rates of extra-pair fertilizations (Griffith *et al.*, 2010; Zann, 1996). Males and females display affiliative behaviors directed towards both opposite- and same-sex flock members, providing different social contexts in which the neural regulation of affiliation can be examined. Here we use zebra finches as a model system to examine the extent to which differences in affiliative behavior in same- and opposite-sex conspecific pairs relate to immunolabeling for TH within brain regions involved in incentive motivation and social behavior. The goal of the present study is to fill gaps in the current understanding of catecholamines and affiliative behavior by examining the distribution and density of TH in brain regions comprising both the mesolimbic system and “social behavior network” in male and female zebra finches displaying affiliative behavior within distinct social contexts.

2. METHODS

2.1 Animals

Adult zebra finches (7-13 months of age) were obtained from our breeding colony at the University of Wisconsin - Madison. After fledging and until the onset of the study, all birds were housed indoors in stainless steel cages in single-sex groups on a light cycle of 16 hours of light: 8 hours of dark with humidity ranging from 30-60% and temperature ranging from 20-24°C. Birds were in visual, but not acoustic isolation from the opposite sex. The birds were provided water and pellet and seed mix diet *ad libitum* and vegetables and egg mixture twice weekly. 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.

2.2 Behavioral Testing

2.2.1 Test Cage Set-up and Pairing—Animals were divided into four treatment groups: subject males paired with a female partner (n=12), subject males paired with a male

partner (n=12), subject females paired with a male partner (n=12), and subject females paired with a female partner (n=12). Partners were not biologically related to subjects. Subjects and their partners were introduced into separate ($56 \times 58 \times 57 \text{ cm}^3$) cages that each included a nest box, perches, cuttlebone, and food, water and nesting material *ad libitum*. Animals were housed in their treatment cage for 16 days prior to observations, a time period sufficient for pair bond formation in this species (Silcox and Evans, 1982). Nest boxes were weighed prior to cage set up and again after the observation days to determine approximate nest weight, if present. To prevent any potential expression of parental behavior, all eggs laid were removed daily.

2.2.2 Behavioral Testing—Zebra finch subjects were observed for 20 minutes each in a random order on each of days 16-21 after pairing. Behavior measures (Zann, 1996) recorded for all birds included: courtship sequence (including courtship dancing, crouch and quiver, exaggerated greeting, and mounting), nest-directed behaviors (including nest material gathering, looking in the nest box, and entering the nest box), clumping (sitting in side-by-side contact), synchronized flights (flights at the same time in the same direction as partner), and allopreening. Additional behavior measures recorded for subject males included undirected singing (song produced by subjects facing away from the partner) and directed singing (song produced by subjects facing the partner). Among these behaviors, clumping, allopreening, and synchronized flights are considered to be affiliative behaviors seen at high rates between pair bonded individuals and often seen between both same-sex and opposite-sex individuals of the same flock (Zann, 1996). The average number of behavior bouts (at least two seconds apart) expressed over all five observation periods was recorded.

2.3 Immunocytochemistry

Zebra finches were sacrificed on day 22 by rapid decapitation. Brains were removed, frozen with dry ice and preserved in a -80°C freezer. In preparation for immunocytochemistry, brains were removed from the freezer and submersion fixed overnight in a 5% acrolein solution, cryoprotected in 30% sucrose for two days, then frozen with dry ice, and stored at -80°C . Frozen brains were cut in coronal sections at $30 \mu\text{m}$ using a cryostat and every third section was collected for TH immunocytochemistry. Brains were divided into two batches with equal numbers of each treatment group in each batch for immunocytochemistry processing. The sections were rinsed four times in phosphate buffered saline (PBS, pH=7.4), incubated in 0.5% hydrogen peroxide solution for 10 minutes, rinsed four times in PBS, incubated in 0.5% sodium borohydride solution for 15 min, rinsed four times in PBS, rinsed once in PBS with 0.2% triton-X (PBS-T), and incubated in 5% normal goat serum (NGS, made in PBS-T) for one hour. Sections were then incubated in 2% NGS anti-TH primary antibody solution (made in mouse, Cat#22941, 1:20,000; Immunostar, Inc., Hudson, WI, USA) overnight at room temperature. The following day, sections were rinsed seven times in PBS and incubated in 2% NGS biotinylated goat anti-mouse secondary antibody solution (1:1000; Vector Laboratories, Burlingame, CA, USA) for 90 min at room temperature. The sections were rinsed seven times in PBS, incubated in avidin-biotin solution (Vector ABC kit) for one hour, rinsed seven times in PBS, and the avidin-biotin complex was visualized using DAB (Sigma-Aldrich, St. Louis, MO, USA). Sections were float mounted on gel-coated slides, dehydrated, and cover slipped.

The fixation process reduced the integrity of cellular structure, but likely did not change the overall quantity of TH in each region (Figure 4); therefore we measured optical density (OD; in log-scale) within each brain region but did not include measures of the area of labeled fibers or cell counts. OD was quantified using a Spot camera (SPOT Imaging Solutions, Sterling Heights, MI, USA) attached to a Nikon microscope (Nikon Instruments Inc., Melville, NY, USA) and a computer using MetaVue software (Molecular Devices

Corp., Downingtown, PA, USA). Brain nuclei analyzed included eleven brain regions implicated in affiliative and sexual behavior (the rostral and caudal subregions of the ventral tegmental area [VTA], rostral pole of the nucleus accumbens [AcR], nucleus accumbens shell [AcS], nucleus accumbens core [AcC], substantia nigra [SN], medial preoptic nucleus [POM], ventromedial nucleus of the hypothalamus [VMH], midbrain central gray [CG], nucleus taeniae of the amygdala [TnA], and bed nucleus of the stria terminalis [BST]). Measurements were made within ovals centered within the regions of interest (Figure 1) and OD was determined by MetaVue in each of three serial sections in both hemispheres of each brain region for each bird. The placements of measurement ovals within social behavior regions were based on previous immunocytochemistry studies (Alger *et al.*, 2009; Bottjer, 1993). Ac subregions were based on studies that combined immunocytochemistry with tract tracing techniques (Balint and Csillag, 2007; Csillag *et al.*, 2008; Husband and Shimizu, 2010; Roberts *et al.*, 2002). VTA subregions were based on work by Goodson (Goodson *et al.*, 2009). Measurements were averaged for each brain region for each bird. In cases of extensive tissue damage, the individual was dropped from analysis for affected brain areas.

2.4 Statistics

Data were analyzed using the statistical software program Statistica (StatSoft 2001, Tulsa, OK). Q-Q plots and Lilliefors tests were used to check normality assumptions and Brown-Forsythe tests were used to check homogeneity of variance. Behavior measures were log-transformed with $\ln(x+0.05)$ to improve normality. TH OD measures were statistically corrected for batch effect. Type III ANOVAs were used to assess the effect of sex and group (opposite-sex partner or same-sex partner) on behavior and brain measures and Fisher LSD post-hoc tests were used where appropriate.

Multiple linear regression analyses were used to assess relationships between TH densities and behaviors. Multiple linear regressions were conducted using log-transformed behavior measures (clumping, synchronized flights, courtship, nest-directed behaviors, and allopreening) as dependent variables and sex, group, and TH OD in the eleven brain regions as independent variables. Multiple linear regressions were also conducted for males only using log-transformed directed song and undirected song as dependent variables and group and TH OD measures as independent variables. We also investigated the ability of sex, group and log-transformed behavior measures (clumping, synchronized flights, courtship, nest-directed behaviors, and allopreening) to explain TH OD in each brain area using multiple regression analyses. Forward, backward and standard stepwise regressions were conducted and models were chosen based on adjusted R^2 , model simplicity and residual plots. All variables in the final models and associated p-values are reported. The Benjamini-Hochberg method was used to control the false discovery rate in the assessment of significant variables in the models.

3. RESULTS

3.1 Effects of sex of subject and sex of partner on behavior and brain

There were significant effects of both sex and group on courtship behavior, such that males showed more courtship behavior than females and birds housed with opposite-sex partners showed more courtship behavior than birds housed with same-sex partners (effect of sex: $F_{1,44}=21.26$, $p<0.001$; effect of group: $F_{1,44}=6.71$, $p=0.013$; interaction: not significant (NS); significant LSD post-hocs: female with a male partner (F-M) vs. male with a female partner (M-F) $p<0.001$, female with a female partner (F-F) vs. M-F $p<0.001$, F-F vs. male with a male partner (M-M) $p=0.038$, M-F vs. M-M $p=0.005$; Figure 2A). There were significant effects of sex, but not group on nest-directed behaviors, such that males showed more nest-directed behaviors than females (effect of sex: $F_{1,44}=5.04$, $p=0.030$; effect of

group: NS; interaction: NS; significant LSD post-hocs: F-F vs. M-F $p=0.014$; Figure 2B). There were no group effects on directed or undirected song nor were there sex or group effects on affiliative behaviors (i.e., clumping [Figure 2C], synchronized flight, and allopreening). Chi-square tests also revealed that the proportion of birds to clump, allopreen, build a nest (as indicated by increased nest box weight), and synchronize flight was no different between birds housed with opposite-sex partners and birds housed with same-sex partners or between males and females.

Birds of both sexes had a higher TH OD in AcS when housed with an opposite-sex partner compared to a same-sex partner (effect of sex: NS; effect of group: $F_{1,41}=4.10$, $p=0.049$; interaction: NS; no significant LSD post-hocs; Figure 3C). Birds of both sexes showed a trend of a higher TH OD in AcC when housed with an opposite-sex partner compared to a same-sex partner (effect of sex: NS; effect of group: $F_{1,41}=3.72$, $p=0.061$; interaction: NS; no significant LSD post-hocs; Figure 3B). No other significant effects of the sex of the subject or the partner on TH OD were observed.

3.2 Contribution of TH to behavior

Results of multiple regression analyses with behaviors entered as dependent variables and sex, group and TH density measures entered as predictor variables indicated that different patterns of TH density best explained behaviors (all results presented in Table 1). TH labeling in the POM and VMH significantly explained and TH labeling in the rostral VTA contributed to variation in clumping behavior (Figures 4A, 5C and 5D). TH density in AcR significantly explained variation in synchronized flights and TH density in AcC and AcS significantly explained variation in allopreening. TH density in the caudal VTA contributed significantly to both courtship behavior in both sexes and directed song in males (Figures 4B, 6A and 6B). TH density in the BST contributed significantly to undirected song in males. Nest-directed behaviors were only predicted by the sex of the subject and both the sex of the subject and of the partner contributed to courtship behavior.

3.3 Contribution of behavior to TH densities

Multiple regression analyses with TH OD entered as dependent variables and social group, sex, and all of the behavioral measures entered as predictor variables indicated that different behavior variables best explained TH labeling for each brain region (all results presented in Table 2). Multiple regression results indicated that subjects housed with opposite-sex partners had significantly higher TH densities in both AcC and AcS compared to those housed with same-sex partners (as also seen in the ANOVA analyses, Figures 3B and 3C). However, multiple regression results did not reveal any effects of the sex of the subject on TH densities in any brain region measured. Clumping behavior contributed to TH labeling in AcR, AcC, rostral VTA and POM, and all of these relationships were negative (Figures 4A, 5A-D). Nest-directed behaviors contributed to TH labeling in AcS, and this relationship was also negative. No other affiliative behaviors (allopreening and synchronized flight) significantly explained variation in TH OD in any brain region measured. However, the amount of courtship produced by an individual contributed to TH labeling in caudal VTA and the amount of courtship produced by an individual's partner contributed to TH labeling in VMH and rostral VTA (Figures 4C, 6B, 6C and 6D); all of these relationships were positive.

4. DISCUSSION

The present data implicate catecholamines in the mesolimbic system and social brain regions in affiliation between both opposite- and same-sex adults. Furthermore, they demonstrate that social experience can influence catecholaminergic circuitry. The results

suggest functional differences between subregions of VTA and Ac and demonstrate that similar catecholaminergic circuits underlie affiliation across vertebrates, indicating that highly conserved neuronal circuits regulate these crucial behaviors.

4.1 TH densities are influenced by social environment

Birds of both sexes had higher TH densities in AcS and AcC when housed for three weeks with an opposite-sex partner compared to birds housed for three weeks with a same-sex partner, suggesting that the social environment may influence DA synthesis. Furthermore, TH labeling in the VMH and rostral VTA related positively to the amount of courtship received from the partner. Although courtship measures were recorded in the final five days of observation, these behaviors were likely consistent for the duration of cohabitation, as zebra finch courtship levels have been found to be comparable at observation points one month apart (Goodson *et al.*, 2009). These findings suggest that the long-term social environment affects DA synthesis in the mesolimbic system, consistent with evidence of socially-induced plasticity in TH densities observed in female prairie voles and male golden hamsters (Cavanaugh and Lonstein, 2010b; Wommack and Delville, 2002).

The mechanisms for such socially-driven brain plasticity are not known. One possibility is that social interactions affect circulating steroid hormone concentrations and the neural expression of Fos, an immediate early gene (IEG), which in turn influence TH synthesis. This idea is supported by data showing the induction of *c-fos* gene transcription to coincide with TH gene transcription (Icard-Liepkalns *et al.*, 1992). Furthermore, in male prairie voles, cohabitation and mating with a female result in both increased circulating gonadal hormone concentrations and Fos expression in TH-positive neurons in social brain regions. Circulating gonadal hormones are known to affect the number of TH-positive cells in these same brain regions (Cavanaugh and Lonstein, 2010a, b; Northcutt and Lonstein, 2009; Northcutt *et al.*, 2007; Wang *et al.*, 1994). As in prairie voles, exposure to an opposite-sex conspecific results in gonadal hormone release in both male and female songbirds (Dufty and Wingfield, 1986; Gwinner *et al.*, 2002; Kroodsmas, 1976; Pinxten *et al.*, 2003; Silverin and Westin, 1995). Additionally, increased expression of Fos proteins within TH-positive VTA and VMH neurons is observed following sexual behavior in rodents and birds (Balfour *et al.*, 2004; Bharati and Goodson, 2006; Charlier *et al.*, 2005; Heimovics and Ritters, 2006; Kollack-Walker and Newman, 1995) and following exposure to a conspecific of either sex in male zebra finches (Bharati and Goodson, 2006). Thus, this mechanism may influence the upregulation of TH in the VTA and VMH of zebra finches cohabitating with partners that court at high rates. Finally, although the Ac does not contain TH-positive cell bodies (Bailhache and Balthazart, 1993; Bottjer, 1993), the innervations of the Ac are considered to mainly originate in the VTA and the majority of VTA neurons that project to Ac contain TH (Balthazart and Absil, 1997; Dominguez and Hull, 2005). Thus, socially-induced steroid hormones and Fos-associated proteins in TH-positive VTA neurons may increase TH synthesis and immunocytochemically observable cell bodies in VTA and fibers in both VTA and Ac.

4.2 Affiliative behaviors relate to TH densities within the mesolimbic system and social behavior areas

4.2.1 Affiliative behaviors relate differently to TH densities in rostral VTA and caudal VTA—The results of the present study confirm and elaborate upon prior studies implicating DA in affiliative behaviors. TH labeling in the rostral VTA, and not the caudal VTA, was negatively related to clumping behavior and positively related to the receipt of courtship from the partner. In contrast, TH density in the caudal VTA, but not the rostral VTA, related positively to both the production of directed song in males and the production of courtship behaviors in both sexes. These findings are consistent with past studies that

show that activity in dopaminergic VTA neurons in male rats, quail, zebra finches and starlings increases in response to female exposure and female-directed behavior (Balfour *et al.*, 2004; Bharati and Goodson, 2006; Charlier *et al.*, 2005; Goodson *et al.*, 2009; Heimovics and Riters, 2008; Huang and Hessler, 2008; Yanagihara and Hessler, 2006) and during exposure to a conspecific of either sex in male zebra finches (Bharati and Goodson, 2006). The rostral and caudal subregions of the VTA are morphologically distinct (Goodson *et al.*, 2009; Lammel *et al.*, 2008; Shabat-Simon *et al.*, 2008) and rostral-caudal distinctions in VTA function have been observed recently in male rats (Shabat-Simon *et al.*, 2008) and zebra finches (Goodson *et al.*, 2009). Consistent with the present results, a previous study showed that male zebra finches that court have more TH-positive cell bodies in the caudal VTA, but not rostral VTA, than do males who do not court. Furthermore, song number positively correlated with the percentage of TH-positive neurons that expressed Fos in caudal VTA, but not rostral VTA (Goodson *et al.*, 2009). Past data also showed that more affiliative finch species had higher numbers of TH-positive neurons in the caudal VTA, but not rostral VTA, compared to less affiliative species (Goodson *et al.*, 2009). The present results are consistent with data suggesting functional differences for DA in rostral and caudal VTA and specifically suggest that DA in caudal VTA is strongly associated with the production of courtship behavior, whereas DA in rostral VTA may be more closely associated with the receipt of a partner's affiliative behavior. These functional distinctions must be examined in future work. Finally, the present results show that the relationship between DA in VTA and courtship is not limited to males.

4.2.2 Affiliative behaviors relate differently to TH densities in Ac subregions—

Significant relationships were also found between TH density in the Ac and several affiliative behaviors. Interestingly, similar to results in rodent studies (Aragona *et al.*, 2006; Champagne *et al.*, 2004; Faure *et al.*, 2008), relationships between affiliative behaviors and DA in Ac subregions were distinct. Specifically, TH density in AcR was positively related to synchronized flights and negatively related to clumping behavior, TH density in AcC was negatively related to both allopreening and clumping behavior, and TH density in AcS was positively related to allopreening and negatively related to nest-directed behaviors. Although birds housed with an opposite-sex partner had more TH immunolabeling in AcS and AcC (but not AcR) compared to birds housed with a same-sex partner, we did not see any relationships between the amount of courtship by the partner and TH density in Ac, similar to observations made by Svec and colleagues (Svec *et al.*, 2009). Overall, these data support previous findings in prairie voles and rats that DA in the Ac plays a role in a variety of affiliative behaviors (Aragona *et al.*, 2003; Aragona *et al.*, 2006; Gingrich *et al.*, 2000; Numan *et al.*, 2005; Stolzenberg *et al.*, 2007).

4.2.3 TH densities in social brain regions relate to song in a context-dependent manner—

Relationships between TH densities and male song production were contextually-dependent. Specifically, the number of directed songs was best predicted by a positive relationship with TH density in the caudal VTA, whereas the number of undirected songs was best predicted by a negative relationship with TH density in the BST. VTA neuronal activity is modulated more during directed than undirected song production in male zebra finches (Yanagihara and Hessler, 2006) and in male starlings, TH density in VTA positively correlates with female-directed sexually motivated, but not nonsexually motivated song (Heimovics and Riters, 2008). The VTA is one of the primary sources of dopaminergic inputs to song control region Area X (Lewis *et al.*, 1981), and DA levels in Area X are also higher during directed than during undirected singing in male zebra finches (Sasaki *et al.*, 2006). Thus, dopaminergic projections from the VTA to Area X appear to play a role in the regulation of female-directed birdsong (Hara *et al.*, 2007). Less is known about the relationship between DA in the BST and song that is not female-directed, although the

present results in combination with evidence of negative linear relationships between male-directed singing behavior and D1-like receptor density in BST (Heimovics *et al.*, 2009), suggest that this relationship deserves further study.

4.2.4 Clumping behavior relates to TH densities in VTA, Ac, POM and VMH—

Clumping behavior, one of the most commonly used measures of affiliation in zebra finches, related to TH densities in POM and VMH, in addition to rostral VTA, AcR, and AcC (discussed above). Converging evidence implicates catecholamines in the POM and VMH in social responses to conspecifics. For example, the densities of TH-immunoreactive fibers and D1-like receptors in the POM negatively correlated with male starling song produced in a sexual context, whereas D1-like receptor density in the POM positively correlated with male starling song used to maintain social flocks (Heimovics *et al.*, 2009; Heimovics and Ritters, 2008). Also, DA receptor activity in the medial preoptic area is important for the regulation of maternal behavior in female rats (Miller and Lonstein, 2005). The present study expands our understanding of the role of catecholamines in the POM to include affiliative behaviors outside of immediate sexual and parental contexts. Furthermore, past studies implicate NE in VMH in female sexual behavior in rats (Etgen and Morales, 2002; Vathy and Etgen, 1989) and female responses to male courtship song in songbirds (Ritters *et al.*, 2007; Ritters and Pawlisch, 2007). In combination with the present findings that TH-immunolabeling in VMH was positively correlated with both clumping behavior and the amount of courtship performed by the partner in both males and females, the role of catecholamines in VMH in responses to opposite-sex conspecifics merits further investigation.

4.2.5 TH densities in many brain regions related negatively to several affiliative behaviors—

Positive relationships were identified between DA markers and affiliative behaviors in the prairie vole, rat, and songbird studies reviewed above. Although in the present study many relationships between affiliative behaviors and TH densities were positive, relationships were negative between clumping behavior and TH density in the POM, rostral VTA, AcR, and AcC. Relationships between nest-directed behavior and TH density in AcS and between allopreening and TH density in AcC were also negative. OD measurements of TH immunolabeling represent both labeled cell bodies and processes and it is not clear what proportion of the immunolabeled TH is active or stored (Cooper *et al.*, 2003). Furthermore, the majority of studies investigating relationships between catecholamines and social behaviors have collected behavior and brains within the first day, and often within an hour, of exposure to a conspecific, a time usually associated with mate attraction and social anticipation. In contrast, this study examined the relationship between catecholamines and affiliative behavior after three weeks, a time sufficient for the establishment of pair bonds in this species (Silcox and Evans, 1982). Mesolimbic DA transmission initially promotes motivated seeking behavior, but is diminished after the attainment of the reward (Alcaro *et al.*, 2007). Thus, the low density of TH-immunolabeling in birds that clumped at high rates may reflect low DA activity associated with affiliative behavior in long-term zebra finch pairs. An alternate interpretation is that chronic high rates of DA synthesis and release in these brain areas has depleted TH stores and/or resulted in end product inhibition of TH. In rats, chronic administration of cocaine and methamphetamine, drugs that increase extracellular DA concentrations, decreased mesolimbic TH-immunolabeling (Trulson *et al.*, 1987a; Trulson *et al.*, 1987b), consistent with end-product inhibition of TH by continually high DA concentrations. Further studies are needed to elucidate the relationships between catecholamines and long-term affiliative relationships.

4.3 Affiliative behaviors occurred at equal rates between both opposite- and same-sex pairs

Zebra finches are a popular model for pair bonding due to the fact that they form pair bonds quickly, maintain them throughout the year, remain highly genetically monogamous, and sustain the pair bond until the apparent death of one partner (Griffith *et al.*, 2010; Zann, 1996). Data here highlight that high frequencies or durations of certain behaviors commonly used to define bonded pairs, such as clumping (Dunn and Zann, 1997; Tomaszewski and Adkins-Regan, 2005), or even the occurrence of nest building, are not exclusive to opposite-sex zebra finch pairs. This could be indicative of pair bond formation among some same-sex pairs, as observed in other studies (Adkins-Regan, 2002). Alternatively, this could be a result of the experimental paradigm in which only two birds of a highly gregarious species that typically form large flocks (Zann, 1996) are placed in a cage for a long duration. Assignment of a pair bonded status should be made with caution, particularly in socially-limited environments.

4.4 Conclusion

The present results implicate catecholamines in the mesolimbic system and social brain areas in affiliative behaviors directed towards both opposite-sex and same-sex adult conspecifics. These results also demonstrate that social environment may influence catecholamine synthesis. Similar catecholaminergic circuits appear to underlie affiliation across vertebrates, indicating that these crucial behaviors are regulated by highly conserved neuronal circuits. It is important to note that both DA and NE are released in all brain areas investigated here and that TH is the rate-limiting enzyme in the synthesis of both. Thus, further studies will be needed to differentiate the actions of each catecholamine in affiliative behavior.

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Abbreviations

AC	anterior commissure
Ac	nucleus accumbens
AcC	nucleus accumbens core
AcR	rostral pole of the nucleus accumbens
AcS	nucleus accumbens shell
BST	bed nucleus of the stria terminalis
CG	midbrain central gray
DA	dopamine
DBH	dopamine- β -hydroxylase
NE	norepinephrine
NIII	third cranial nerve
OD	optical density
POM	medial preoptic nucleus

SN	substantia nigra
TH	tyrosine hydroxylase
TnA	nucleus taeniae of the amygdala
V	ventricle
VMH	ventromedial nucleus of the hypothalamus
VTa	ventral tegmental area

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Research Highlights

- Tyrosine hydroxylase (TH) labeling was examined in same- and mixed-sex finch pairs
- TH in nucleus accumbens (Ac) was denser in members of mixed-sex pairs
- TH density in mesolimbic and social brain areas related to affiliative behaviors
- Behavior/TH correlations for Ac and ventral tegmental area were subregion specific
- Affiliative behavior was similar in same- and mixed-sex zebra finch pairs

Figure 1.

Regions in which TH OD was quantified and their respective areas in mm². See text for abbreviations.

Figure 2.

Barplots of ANOVA results indicating effects of sex of individual and partner on (A) courtship behaviors and (B) nest-directed behaviors. Main effects are indicated in the top left corner of each plot and lowercase letters above bars indicate significant post-hoc differences. Sample sizes indicated in plot (C) are identical to those in plots (A) and (B). Despite the common use of clumping as a measure of pair bonding status, there is no effect of sex of subject or partner on (C) clumping. There were also no group effects on directed or undirected song, synchronized flight, or allopreening (not shown).

Figure 3.

Barplots of ANOVA results indicating main effects of sex of partner on TH OD in (A) AcR, (B) AcC, and (C) AcS. Main effects less than $p=0.10$ are indicated in the top left corner of each plot. There were no significant post-hoc differences. Sample sizes are indicated at the base of each bar.

Figure 4.

Photomicrographs illustrating relationships between behaviors and TH density: (A) TH labeling in Ac and rostral VTA in a male who did not clump with his female partner (left) and in a male who clumped with his female partner at high rates (15 times over five 20 minute sessions; right); (B) TH labeling in caudal VTA in a male who courted his female partner at low rates (once; left) and in a male who courted his female partner at high rates (8 times; right); and (C) TH labeling in VMH in a female who was not courted by her male partner (left) and in a female who was courted by her male partner at high rates (8 times; right). All scale bars indicate 0.5 mm². See text for abbreviations.

Figure 5. Scatterplots showing negative correlations between clumping behavior and TH density in (A) AcR, (B) AcC, (C) rostral VTA and (D) POM. Closed circles represent data from animals housed with an opposite-sex conspecific; Crosses represent data from animals housed with a same-sex conspecific. R^2 and p-values are shown for each correlation.

Figure 6.

Scatterplots showing positive relationships between (A) directed song and TH density in caudal VTA, (B) courtship behavior and TH density in caudal VTA, (C) the partner's courtship behavior and TH density in rostral VTA and (D) the partner's courtship behavior and TH density in VMH. Closed circles represent data from animals housed with an opposite-sex conspecific; Crosses represent data from animals housed with a same-sex conspecific. R^2 and p-values are shown for each correlation.

Table 1

Regression models showing brain areas in which TH density explains variation in behavior.

Behavior	Model Statistics	Variables in Model	Beta	p-values
Clumping	adjusted $R^2=0.46$, $F_{3,37}=12.20$, $p<0.001$ *	POM VMH Rostral VTA	-2.46 3.52 -1.32	0.001 * 0.002 * 0.051
Synchronized Flight	adjusted $R^2=0.09$, $F_{1,39}=5.14$, $p=0.029$ *	AcR	0.65	0.029 *
Allopreening	adjusted $R^2=0.10$, $F_{2,38}=3.20$, $p=0.052$	AcC AcS	-7.88 7.18	0.017 * 0.021 *
Nest-Directed Behavior	adjusted $R^2=0.10$, $F_{1,39}=5.60$, $p=0.023$ *	Sex	0.38	0.023 *
Courtship	adjusted $R^2=0.65$, $F_{3,37}=25.29$, $p<0.001$ *	Caudal VTA Group Sex	2.51 -0.29 0.49	<0.001 * <0.001 * 0.006 *
Directed Song (Males Only)	adjusted $R^2=0.30$, $F_{1,18}=9.16$, $p=0.007$ *	Caudal VTA	1.90	0.007 *
Undirected Song (Males Only)	adjusted $R^2=0.31$, $F_{1,18}=9.34$, $p=0.007$ *	BST	-9.24	0.007 *

* P-values marked with an asterisk indicate variables with a significant ability to explain behavior variation after Benjamini-Hochberg correction. P-values without an asterisk indicate variables that contribute to the ability of the model to explain behavior variation but do not explain significant variation after Benjamini-Hochberg correction. See text for abbreviations.

Table 2

Regression models showing behaviors that explain variation in TH density for each brain area measured.

Brain Area	Model Statistics	Variables in Model	Beta	p-values
Rostral VTA	adjusted $R^2=0.18$, $F_{2,43}=6.00$, $p=0.005$ *	Clumping Partner's Courtship	-0.08 0.05	0.003 * 0.034 *
Caudal VTA	adjusted $R^2=0.37$, $F_{1,44}=27.35$, $p<0.001$ *	Courtship	0.14	<0.001 *
AcR	adjusted $R^2=0.10$, $F_{1,40}=5.62$, $p=0.026$ *	Clumping	-0.08	0.026 *
AcC	adjusted $R^2=0.18$, $F_{2,42}=5.83$, $p=0.006$ *	Clumping Group	-0.07 -0.08	0.010 * 0.020 *
AcS	adjusted $R^2=0.15$, $F_{2,42}=4.75$, $p=0.014$ *	Group Nest-Directed Behavior	-0.09 -0.08	0.018 * 0.032 *
POM	adjusted $R^2=0.08$, $F_{1,42}=4.85$, $p=0.033$ *	Clumping	-0.05	0.033 *
VMH	adjusted $R^2=0.13$, $F_{1,44}=7.43$, $p=0.009$ *	Partner's Courtship	0.04	0.009 *
CG	No models returned	(none)		
BST	No models returned	(none)		
TnA	No models returned	(none)		
SN	No models returned	(none)		

* P-values marked with an asterisk indicate variables with a significant ability to explain TH density variation after Benjamini-Hochberg correction. See text for abbreviations.