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Newly paired zebra finches have higher dopamine levels and immediate early gene Fos expression in dopaminergic neurons

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Abstract

Most birds are socially monogamous, yet little is known about the neural pathways underlying avian monogamy. Recent studies have implicated dopamine as playing a role in courtship and affiliation in a socially monogamous songbird, the zebra finch (*Taeniopygia guttata*). In the present study, we sought to understand the specific contribution to pair formation in zebra finches of the mesolimbic dopaminergic pathway that projects from the midbrain ventral tegmental area to the nucleus accumbens. We observed that paired birds had higher levels of dopamine and its metabolite 3,4-dihydroxyphenylacetic acid in the ventral medial striatum, where the nucleus accumbens is situated, than unpaired birds. Additionally, we found that the percentage of dopaminergic neurons expressing immediate early gene Fos, a marker of neuronal activity, was higher in the ventral tegmental area of paired birds than in that of unpaired birds. These data are consistent with a role for the mesolimbic dopaminergic pathway in pair formation in zebra finches, suggesting the possibility of a conserved neural mechanism of monogamy in birds and mammals.

Introduction

Although the majority of avian species are socially monogamous (Black & Hulme, 1996), the neural mechanisms underlying the formation of these long-term pair bonds are not well understood. The zebra finch is an avian species that forms permanent pair bonds in the wild, and is used as a laboratory model for social monogamy (Zann, 1996; Adkins-Regan, 2009; Pedersen & Tomaszycki, 2012; Klatt & Goodson, 2013). On the basis of the role of the mesolimbic dopaminergic pathway in the processing of reward linked to stimuli such as food, sex, addictive drugs, and pair formation in mammals (Young *et al.*, 2005; Alcaro *et al.*, 2007), we hypothesised that this pathway plays a key role in pair formation in zebra finches.

The activation of the mesolimbic dopaminergic pathway, consisting of dopaminergic neurons projecting from the midbrain ventral tegmental area (VTA) to the nucleus accumbens, results in individuals desiring or expecting a pleasurable stimulus. Dopaminergic signaling in the nucleus accumbens leads to the development of incentive value for previously neutral stimuli, as well as the coupling of reward-predicting cues and behaviors associated with reward, which is important both for motivation that precedes and reinforcement that arises from earning a reward (Blackburn *et al.*, 1992; Ikemoto & Panksepp, 1999; Kalivas & Nakamura, 1999; Adinoff, 2004; Wise, 2004; Alcaro *et al.*, 2007; Arias-Carrion & Poppel, 2007; Carlezon & Thomas, 2009).

Dopamine has been previously implicated in courtship behaviors such as directed song, and in affiliative behaviors such as clumping and allopreening in zebra finches (Bharati & Goodson, 2006; Goodson *et al.*, 2009; Alger *et al.*, 2011). In the current study, we hypothesised that specific activation of the mesolimbic dopaminergic pathway occurs in response to pairing in zebra finches. Our first prediction was that paired zebra finches would have higher levels of dopamine in the nucleus accumbens than unpaired birds. We measured dopamine and its metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) in the ventral medial striatum (henceforth referred to as the medial striatum), where the nucleus accumbens is situated (Nixdorf-Bergweiler & Bischof, 2007; Alger *et al.*, 2011), the hyperpallium apicale (situated dorsal to the nucleus accumbens, and therefore serving as a control region), and the VTA. We also measured neuromodulator levels in the preoptic area, as dopaminergic signaling in this region has been implicated in copulatory behaviors (Hull & Dominguez, 2007; Kleitz-Nelson *et al.*, 2010). In order to compare differences in activation of the mesolimbic dopaminergic pathway between birds that were courting and those that were in pair bonds, we included an additional experimental group of birds performing courtship behaviors but that were not in pair bonds.

Our second prediction was that the percentage of dopaminergic neurons in the mesolimbic dopaminergic pathway that expressed

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Fos would be greater in paired than in unpaired birds. Therefore, we compared percentages of Fos-expressing dopaminergic neurons, in the VTA and substantia nigra (situated adjacent to the VTA, and therefore serving as a control region), between paired and unpaired birds. We also included other dopaminergic regions involved in copulation and directed song (courtship) in our analysis.

Materials and methods

Animals and housing

All male and female zebra finches in this experiment were sexually naïve, and were housed in single-sex aviaries (0.94 × 0.76 × 0.94 m) prior to the onset of the experiment. Seed and water were provided *ad libitum*. All housing, testing and killing procedures were in accordance with Federal and State regulations, and were approved by the Cornell University IACUC.

Aviary design for courtship and pairing experiments

Courtship experiment for high-performance liquid chromatography (HPLC) study

For the courtship experiment, there were four groups: two control groups (one of each sex), and two experimental groups (one of each sex). The control male group consisted of four sexually naïve male birds (subjects) with four sexually experienced male birds (stimuli); the experimental male group consisted of four sexually naïve male birds (subjects) with four sexually experienced female birds (stimuli). The control female group consisted of four sexually naïve females (subjects) with four sexually experienced females; the experimental female group consisted of four sexually naïve females (subjects) with four sexually experienced males. Each group of subject (n = 4) and stimulus (n = 4) birds was placed in an individual aviary (0.94 × 0.76 × 0.94 m) along with nest boxes and nesting materials. Individuals in each group were allowed to interact for 30 min, after which the subjects from the control groups and the subjects from the experimental groups that engaged in courtship behaviors were immediately killed. This experiment was repeated so that there was a maximum of seven courting subjects in each group.

Pairing experiments for HPLC and tyrosine hydroxylase (TH) + Fos studies

For the pairing experiments, there were four groups: two control groups (one of each sex), and two experimental groups (one of each sex). The control male group consisted of four sexually naïve male birds (subjects) with four sexually experienced male birds; the experimental male group consisted of four sexually naïve male birds (subjects) with four sexually experienced female birds. The control female group consisted of four sexually naïve females (subjects) with four sexually experienced females (stimuli); the experimental female group consisted of four sexually naïve females (subjects) with four sexually experienced males (stimuli). Each group of subject (n = 4) and stimulus (n = 4) birds was placed in an individual aviary (0.94 × 0.76 × 0.94 m) along with nest boxes and nesting materials. Individuals in each group were allowed to interact for 2–4 days, after which the subjects from the control groups and the subjects from the experimental groups that formed pairs were killed. The earliest day on which pairs were observed was day 2, and the remaining subjects formed pairs on day 3 or 4. Therefore, subjects were killed between day 2 and day 4, on the first day that pairing behaviors were observed. Unpaired birds from the experimental male group and experimental female group were not used as subjects. This experiment was repeated such that there was a maximum of seven paired subjects in each group. This paradigm was then repeated for the TH + Fos experiment, so as to generate a maximum of seven paired subjects in the control group and in the experimental group for both sexes. In both courtship and pairing experiments, the stimulus birds were experienced, to ensure that the maximum number of naŏve subjects (male or female birds) would form pairs in the experimental group, as experienced birds tend to pair faster.

Behavioral observations

Behaviors in control and experimental birds were observed for 15 min during 30 min of interactions (courtship experiment) prior to the birds being killed. For the pairing experiments (both HPLC and TH + Fos), birds were observed once a day from day 1 until the day on which they were killed (days 2–4) for 15 min on each day, and only the observations made on the last day prior to the birds being killed are reported. For both courtship and pairing experiments, birds were killed within 15 min after observations were completed.

The following behaviors, as described in a previously published study (Tomaszycki *et al.*, 2006), were recorded during each 15-min observation period by an observer using custom-designed software (Goldstein & Brodsky, 2006).

Directed song bouts (recorded as number of bouts of song in 15 min): a salient male courtship behavior in which the male sings to a target bird while perched close to the target. Males produce several repetitions of the song before stopping, marking the end of the song bout.

Undirected song bouts (recorded as number of bouts of song in 15 min): singing that is not obviously directed at another bird.

Beak wipes (recorded as number of beak wipes in 15 min): a courtship behavior performed by either a male or female when it wipes its beak on a perch near another bird.

Aggression (recorded as number of bouts in 15 min): one bird attacking another. Birds in the process of pairing show elevated aggressive behavior as they compete for a mate.

In nest box (recorded as duration in seconds): birds in the process of pairing spend substantial time in the chosen nest box together. Although behaviors such as clumping and allopreening are characteristic of paired birds, we have reported time spent in nest box together as the most salient behavior indicative of two birds being paired and maintaining a nest together.

Tissue collection for HPLC analysis

Along with dopamine, we measured levels of its metabolite DOPAC. When dopamine is released into a synapse, it either binds to its receptors or it is taken up into presynaptic neurons by dopamine transporters, where it is oxidised by mitochondrial monoamine oxidase to form DOPAC (Giros & Caron, 1993). We report both dopamine and DOPAC levels. Subjects from each experiment were decapitated, and their brains were rapidly dissected, frozen on dry ice, and stored at −80 °C until the time of sectioning. With a cryostat (Microm HM 500 OM), 200-μm coronal sections were mounted onto Superfrost Plus slides (Erie Scientific, Portsmouth, NH, USA). Sections were then rapidly frozen with a cooling block set at −20 °C (Physitemp Instruments, Clifton, NJ, USA), and the brain regions of interest (VTA, preoptic area, hyperpallium, and medial

striatum) were dissected with a 300-μm-diameter micropunch. Tissue samples from each bird were assayed independently of each other, and not pooled. The punched tissue was stored in 70 μL of an ice-cold homogenisation solution, which consisted of a mixture of 60 μL of homogenisation buffer [0.1 M perchloric acid (Sigma-Aldrich, St. Louis, MO, USA) containing 347 μM sodium bisulfate (Sigma-Aldrich, St. Louis, MO, USA) and 134 μM EDTA disodium salt (Fluka, St. Louis, MO, USA)], and 10 μL of 100 nM epinine internal standard (Sigma-Aldrich). Tissue samples in homogenisation solution were frozen at −80 °C overnight, and thawed after 24 h. The thawed samples were centrifuged at 16 873 × ***g*** at 4 °C for 20 min, after which the supernatant was collected and used for HPLC analysis. The protein content in the resulting pellet was determined by resuspending and agitating the pellet in 45 μL of ice-cold 0.3 M NaOH for 24 h at 4 °C, and then performing a modified Bradford assay (Pierce Biotechnology, Rockford, IL, USA).

HPLC analysis

Levels of dopamine and its metabolite DOPAC were determined by HPLC with electrochemical detection, with modifications of Bai *et al.* (1999), by H.-H. Lo in the CRED Analytical Instrumentation Facility Core (UT-Austin). Briefly, 50 μL of sample was injected into an HPLC system that comprised a Shimadzu SCL-10A system controller, an LC-10AD pump, and an SIL-10A auto-sampler (Shimadzu, Columbia, MD, USA), coupled with a four-channel CoulArray electrochemical detector (ESA, Chelmsford, MA, USA). The isocratic mobile phase contained 4 mM citric acid, 8 mM ammonium acetate, 120 μM 1-octanesulfonic acid sodium salt, 60 μM EDTA disodium in water, and 5% MeOH, pH 3.5. The flow rate of the mobile phase was maintained at 1 mL/min. Separation was achieved with a 4.6 × 80-mm reverse-phase HR-80, 3-μm particle size column (ESA). The potentials of channels 1–4 of CoulArray were set at −50, 0, 300 and 400 mV, respectively. Peak area (nC) dopamine and DOPAC at the corresponding retention time on the chromatogram resulted from 300 mV, and was used to quantify the amount based on the standard curve of each neurotransmitter. Recovery of internal standard was consistently high across all experimental runs (95–100%). Levels of dopamine and DOPAC were expressed as picograms per microgram of protein in the microdissected tissue extract.

Tissue collection for TH + Fos immunohistochemistry

Subjects from each group were deeply anesthetised with pentobarbital (Nembutal). Following this, they were perfused transcardially with 0.9% saline followed by 4% paraformaldehyde solution. The brains were removed and post-fixed overnight in 4% paraformaldehyde. They were then placed in a 30% sucrose solution for 2–3 days, following which they were embedded in gelatin and stored in a 30% sucrose and 10% formaldehyde solution until they were sectioned. Brains were sectioned at a thickness of 40 μm with a freezing microtome, and stored at 4 °C until being used for immunohistochemistry.

TH + Fos immunohistochemistry

Double labeling for TH and Fos was performed as follows [adapted from Bharati & Goodson (2006)]. Tissue was subjected to two 10-min rinses in phosphate-buffered saline (PBS), and then placed for 1 h in blocking serum (PBS, 5.0% normal goat serum, 0.3% Triton-X). Tissue was then incubated for 40–48 h at 4 °C in mouse anti-TH (Immunostar, Hudson, WI, USA) and rabbit anti-Fos (Santa Cruz Biotechnology, Santa Cruz, CA, USA), the former diluted 1 : 10 000 and the latter 1 : 1000 in PBS, 2.5% normal goat serum, and 0.3% Triton-X. This was followed by two 30-min rinses in PBS, and incubation for 2 h in donkey anti-rabbit secondary antibody conjugated to Alexa Fluor 594 and donkey anti-mouse antibody conjugated to Alexa Fluor 488 in PBS, 2.5% normal goat serum, and 0.3% Triton-X. Alexa Fluors were purchased from Invitrogen (Eugene, OR, USA). Sections were then rinsed in PBS, mounted on superfrost slides (VWR, Radnor, PA, USA), and coverslipped with SlowFade Light containing 4′,6-diamidino-2-phenylindole nuclear stain (Molecular Probes, Grand Island, NY, USA).

Confocal imaging and cell counting for TH + Fos immunohistochemistry

All confocal imaging was performed at the Microscopy and Imaging Facility, a part of the Life Sciences Core Facility Center at Cornell University, with a Leica SP2 scanning laser confocal microscope. Dopaminergic (TH-expressing) neurons were visualised with a × 20 oil immersion lens, and were manually counted on a computer attached to the microscope. TH-expressing, Fos-expressing and TH + Fos-expressing neurons were counted manually in each dopaminergic area of a section while scanning along the *Z*-axis, and *Z*-stack images were obtained. For each region, cells were counted in four to seven sections of both hemispheres from each brain. For each subject, cell numbers (TH + Fos-expressing neurons and TH-expressing neurons) were then averaged, following which TH + Fos-expressing neurons were reported as a percentage of all TH-expressing neurons per region. Cells were counted in the VTA (we did not differentiate between the rostral and caudal VTA), the substantia nigra in the same sections as the VTA, A11, and the preoptic area. Regions were delineated on the basis of a previous study and the zebra finch atlas (Bharati & Goodson, 2006; Nixdorf-Bergweiler & Bischof, 2007).

Statistical analysis

Behavioral data were analysed with Kruskal–Wallis tests followed by Dunn's multiple comparison tests. Data from the HPLC and TH + Fos experiments were analysed with mixed linear model analysis after correcting for repeated sampling of different brain regions from the same individual. Dopamine and DOPAC levels, and percentages of TH + Fos-expressing neurons, were analysed with region and treatment as fixed factors, and individual subject as a random factor, to account for repeated sampling from the same subject. Data from the HPLC and TH + Fos experiments were natural log-transformed to satisfy assumptions for equality of variances and normality. For mixed linear model analyses, *P*-values are described as significant if they are lower than the Bonferroni-corrected alpha.

Results

Courtship experiment behaviors in male and female zebra finches

During 30 min of interaction, male subjects in the experimental male group performed more bouts of directed song and beak wipes to other birds than subjects in the control male group (Table 1; $P < 0.05$). Subjects in the experimental female group received greater numbers of beak wipes and bouts of directed song than

TABLE 1. Courtship experiment behaviors observed in male and female zebra finches (mean ± SEM) during 30 min of interaction (behaviors scored for 15 min) in aviaries with males or females; male and female subjects were in different experiments, and those observations are independent of each other

Behavior	Duration (s) or frequency		Significance
	Control birds	Experimental birds	
Male subjects			
Directed song	2.57 ± 1.66	15.57 ± 1.96	$P \leq 0.05$
Beak wipe	0	5 ± 1.41	$P \leq 0.05$
Undirected song	0.14 ± 0.14	1.14 ± 0.40	$P > 0.05$
Aggression	0.42 ± 0.29	0.28 ± 0.18	$P > 0.05$
Female subjects			
Directed song (from males)	0	31.43 ± 6.90	$P \leq 0.05$
Beak wipe (from males)	0	3.28 ± 0.96	$P \leq 0.05$
Aggression	0	3.57 ± 1.55	$P > 0.05$

TABLE 2. Pairing experiment on behaviors of male and female zebra finches (mean ± SEM) observed for 15 min during 2–4 days of interaction with males or females (HPLC experiment); male and female subjects were in different experiments, and those observations are independent of each other

Behavior	Duration (s) or frequency		Significance
	Control birds	Experimental birds	
Male subjects			
Directed song	0.33 ± 0.21	1.16 ± 0.83	$P > 0.05$
Undirected song	1.33 ± 0.98	3.5 ± 3.3	$P > 0.05$
Aggression	1.33 ± 0.55	13 ± 4.44	$P > 0.05$
Time spent in nest with female	0	1062 ± 295.6	$P \leq 0.05$
Female subjects			
Aggression	5.75 ± 3.66	3.75 ± 2.42	$P > 0.05$
Time spent in nest with male	0	676.10 ± 421.9	$P \leq 0.05$

females in the control female group (Table 1; $P < 0.05$). Pairing behaviors were not observed during 30 min of interaction.

Pairing experiment behaviors in male and female zebra finches

After 2–4 days of interaction, in both the HPLC and the TH + Fos experiments, subjects in the experimental male group spent more time in nests with other birds, and in the TH + Fos experiment, subjects showed more aggressive bouts than subjects in the control male group (Tables 2 and 3; $P < 0.05$). After 2–4 days of interaction, in both the HPLC and the TH + Fos experiments, subjects in the experimental female group spent more time in their nests with other birds than subjects in the control female group (Tables 2 and 3; $P < 0.05$). All male and female birds that were used for the experiment formed pairs within 2–4 days of being placed in the experimental aviary. Same-sex pairs were not observed in the control aviaries. Although courtship behaviors were observed, they were not significantly different between control and experimental groups, and fewer bouts were performed than in the 30-min initial interaction period in the courtship experiment.

TABLE 3. TH + Fos experiment on behaviors of male and female zebra finches (mean ± SEM) observed for 15 min during 2–4 days of interaction with males or females; male and female subjects were in different experiments, and those observations are independent of each other

Behavior	Duration (s) or frequency		Significance
	Control birds	Experimental birds	
Male subjects			
Directed song	0	0.50 ± 0.34	$P > 0.05$
Undirected song	13 ± 4.86	3.66 ± 2.07	$P > 0.05$
Aggression	0	5.66 ± 1.94	$P \leq 0.05$
Time spent in nest with male	0	244.20 ± 62.53	$P \leq 0.05$
Female subjects			
Aggression	0.85 ± 0.40	1.00 ± 0.57	$P > 0.05$
Time spent in nest with male	0	191.70 ± 117	$P \leq 0.05$

Dopamine and DOPAC levels in male and female zebra finches in response to courtship and pair bond formation

Courtship experiment

After 30 min of courtship interaction (Table S1), overall there was no significant interaction between treatment and region in males for either dopamine ($F_{3,35} = 0.872$, $P = 0.465$, $n = 6$–7/group) or DOPAC ($F_{3,35} = 0.908$, $P = 0.447$). In females, once again there was no significant interaction between treatment and region for dopamine ($F_{3,36} = 0.775$, $P = 0.515$, $n = 7$/group) or DOPAC ($F_{3,36} = 1.9$, $P = 0.147$, $n = 7$/group). There were no significant differences in dopamine or DOPAC levels between control and experimental groups in any region of the brain ($P > 0.05$).

Pairing experiment

In male birds, for dopamine there was a significant interaction between treatment and region ($F_{3,27} = 70.859$, $P = 0.029$, $n = 5$–6/group), and significant main effects of both treatment ($F_{1,9} = 6.58$, $P = 0.03$) and region ($F_{3,27} = 70.85$, $P < 0.001$). After 2–4 days of interaction (Fig. 1), dopamine levels were significantly higher in male subjects of the experimental male group than in the control male group in the medial striatum ($P < 0.001$), but not in the hyperpallium apicale ($P = 0.773$). No significant differences between groups were observed in the preoptic area ($P = 0.318$) or VTA ($P = 0.867$). DOPAC levels in the medial striatum were significantly higher in male subjects of the experimental male group than in the control male group ($P = 0.004$), and there were no significant differences in the hyperpallium apicale ($P = 0.924$; Fig. 1). No significant differences were observed in dopamine and DOPAC levels in the preoptic area ($P = 0.316$) or VTA ($P = 0.932$) between unpaired and paired birds.

In female birds, overall there was a significant interaction between treatment and region ($F_{3,24} = 10.117$, $P < 0.001$, $n = 4$/group), and significant main effects of treatment ($F_{1,24} = 8.69$, $P = 0.007$) and region ($F_{3,24} = 48.51$, $P < 0.001$). After 2–4 days of interaction (Fig. 1), dopamine levels were significantly higher in the medial striatum ($P < 0.001$), but not in the hyperpallium apicale ($P = 0.995$), of female subjects of the experimental female group than in the control female group. There were no significant group differences (data not shown) in the preoptic area ($P = 0.842$) or VTA ($P = 0.892$). DOPAC levels were also significantly higher in the medial striatum of female subjects of the

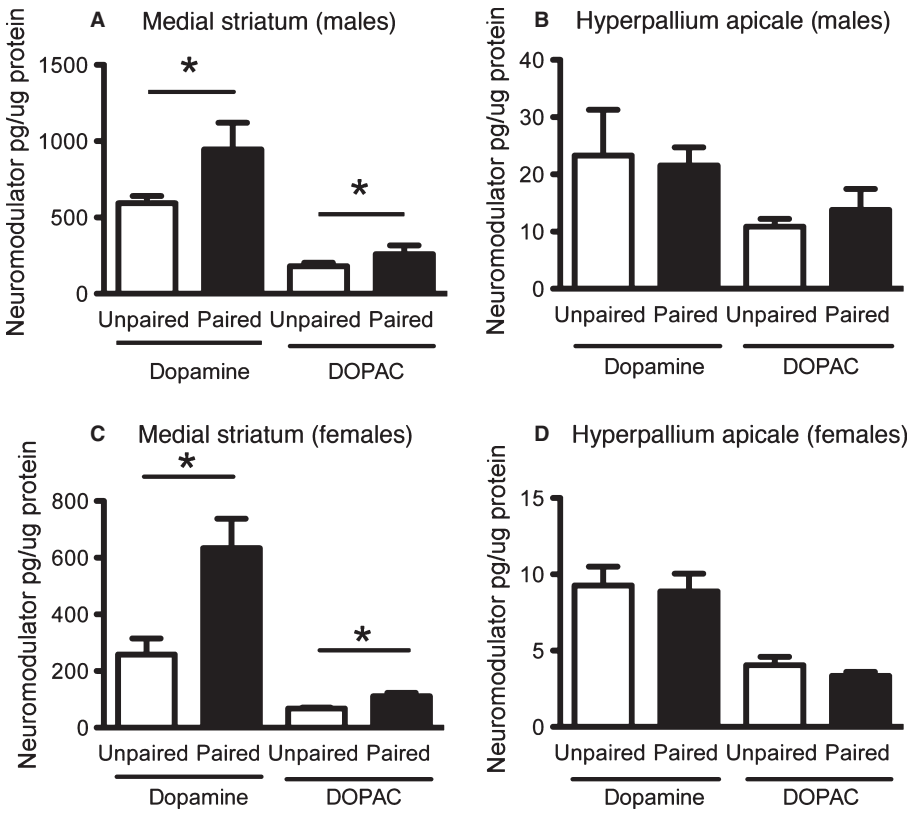

FIG. 1. Levels of dopamine and DOPAC as measured by HPLC in unpaired and newly paired male zebra finches after 2–4 days of interaction in (A) the medial striatum and (B) the hyperpallium apicale, and in female zebra finches in (C) the medial striatum and (D) the hyperpallium apicale. *$P < 0.05$, $n = 4$–6/group.

experimental female group than in the control female group ($P < 0.001$), whereas there were no significant differences in DOPAC levels in the hyperpallium apicale ($P = 0.913$; Fig. 1). No significant differences in dopamine and DOPAC levels were observed in the preoptic area ($P = 0.77$) or VTA ($P = 0.891$) between groups (data not shown).

TH + Fos expression in control and experimental male zebra finches

Overall, there was no significant interaction between treatment and region ($F_{4,38.96} = 0.832$, $P = 0.513$, $n = 4$–7/group). There were, however, significant main effects of treatment ($F_{1,12.34} = 14.30$, $P = 0.002$) and region ($F_{4,38.96} = 35.21$, $P < 0.001$). Percentages of TH + Fos-expressing neurons (Fig. 2) were higher in the ventral medial striatum ($P = 0.002$), caudal A11 nucleus ($P = 0.031$) and rostral A11 nucleus ($P = 0.048$) of subjects in the experimental male group than in subjects of the control male group. Percentages of TH + Fos-expressing cells did not differ between subjects of both groups in the preoptic area ($P = 0.343$) or substantia nigra ($P = 0.265$). Cell counts are reported in Table S2, and images of TH-expressing and Fos-expressing cells are shown in Fig. 3.

TH + Fos expression in control and experimental female zebra finches

Overall, there was no significant interaction between treatment and region ($F_{4,37.90} = 1.22$, $P = 0.318$, $n = 4$–7/group). There was a trend towards a main effect of treatment ($F_{1,20.23} = 4.035$, $P = 0.058$) and there was a significant main effect of region ($F_{4,35.68} = 1.221$, $P < 0.001$). Percentages of TH + Fos-expressing neurons (Fig. 2) were higher in subjects in the experimental female group than in the control female group in the VTA ($P = 0.010$) but not in the preoptic area ($P = 0.662$), rostral A11 ($P = 0.242$), caudal A11 ($P = 0.574$), or substantia nigra ($P = 0.426$). Cell counts are reported in Table S2.

Discussion

In the current study, we have demonstrated that the mesolimbic dopaminergic pathway is specifically activated in response to pairing in zebra finches. We define paired birds as those that demonstrate pairing behaviors, such as spending time in a nest together exclusively. This behavior is highly specific to paired birds, and does not occur when birds are merely courting opposite-sex birds. We observe that dopamine levels in the medial striatum are higher in newly paired than in unpaired birds, but not in birds performing or receiving courtship behaviors prior to pair formation. Additionally, numbers of immediate early gene Fos protein-expressing neurons are greater in newly paired male and female zebra finches in the VTA than in unpaired birds. Our results point to the involvement of the mesolimbic dopaminergic pathway in pair formation in zebra finches. Other dopaminergic areas previously implicated in courtship behaviors, such as the A11 dopaminergic nuclei, also show enhanced activation in newly paired male birds, suggesting that these regions might also contribute to pair formation.

Neuroanatomical studies suggest that the mesolimbic dopaminergic pathway is conserved between birds and mammals. In birds such

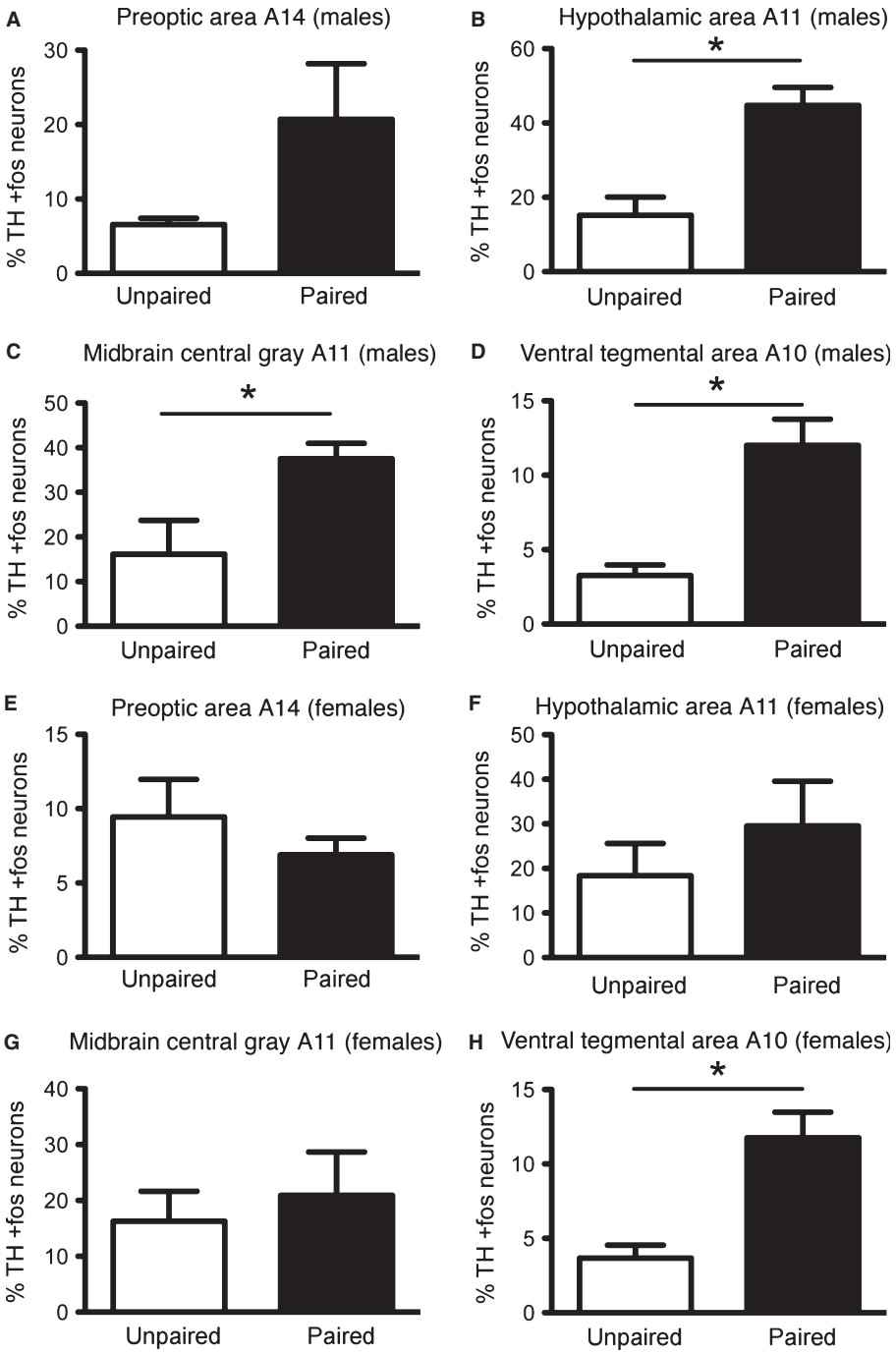

FIG. 2. Percentage of TH + Fos-expressing neurons in unpaired and newly paired male zebra finches in (A) the preoptic area, (B) the hypothalamus, (C) the midbrain central gray, and (D) the VTA, and in female zebra finches in (E) the preoptic area, (F) the hypothalamus, (G) the midbrain central gray, and (H) the VTA. *$P < 0.05$, n = 4–7/group.

as chickens and pigeons, dopaminergic neurons from the midbrain VTA and substantia nigra project into the striatum, where the nucleus accumbens is situated. In chickens, the nucleus accumbens has been shown to have core and shell divisions, as observed in mammals, and obvious hodological similarities between the nucleus accumbens of pigeons and mammals have been demonstrated recently. Additionally, previous work using TH immunostaining has shown the presence of dopaminergic nuclei in the VTA of zebra finches. These studies have demonstrated that the nucleus accumbens is situated in the ventral region of the medial striatum (Bottjer, 1993; Wynne & Gunturkun, 1995; Durstewitz *et al.*, 1999; Jarvis *et al.*, 2005; Balint & Csillag, 2007; Husband & Shimizu, 2011). Overall, both the homologies between the mammalian and avian reward system and the neurochemical boundaries of the main regions in this pathway have been well established (O'Connell & Hofmann, 2011, 2012).

Courtship behaviors in birds and reptiles involve mesolimbic dopamine. In geckos, interfering with dopaminergic signaling with receptor antagonists resulted in a decrease in courtship displays. Additionally, higher levels of dopamine in the nucleus accumbens

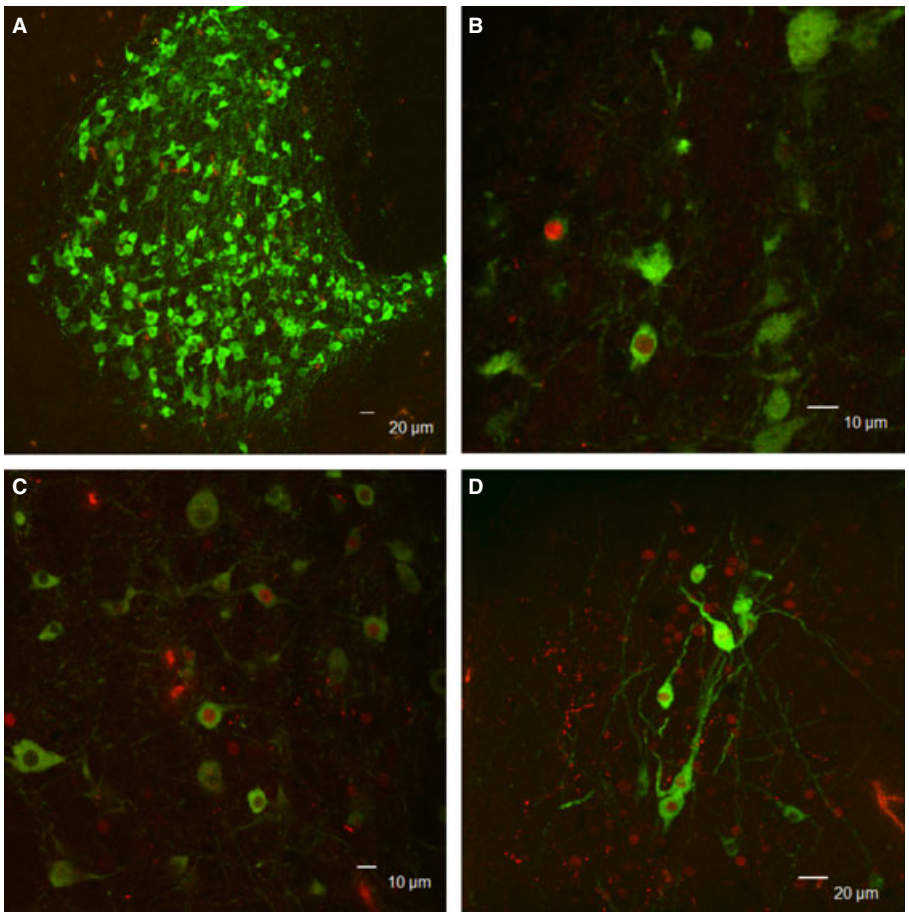

FIG. 3. Immunohistochemistry for TH-expressing (green) and Fos-expressing (red) neurons in (A and B) the VTA of an unpaired male zebra finch, (C) the VTA of a newly paired male zebra finch, and (D) the rostral A11 nucleus of a newly paired male zebra finch.

were observed in naive gecko males from male-biased clutches than in naöve males from female-biased clutches when they encountered a receptive female conspecific across a barrier (Woolley *et al.*, 2004; Dias *et al.*, 2007). In zebra finches, TH labeling in the rostral VTA is positively related to the amount of courtship received from a partner (Alger *et al.*, 2011). In addition, neural activity as measured by Fos expression is increased in a group of caudal VTA neurons during courtship song (Goodson *et al.*, 2009), and neural activity indicated by early growth response protein 1 expression is increased in GABAergic interneurons in the VTA of male zebra finches during directed song in comparison with undirected song (Hara *et al.*, 2007). Finally, glutamatergic synaptic currents to the VTA were enhanced in male zebra finches after males sang to females as compared with when they sang alone (Huang & Hessler, 2008). Dopaminergic drugs have been found to modulate sexually motivated singing. In starlings (*Sturnus vulgaris*), the administration of a drug that increased levels of dopamine in synapses increased singing behavior, whereas the systemic administration of a D1-like receptor antagonist decreased singing behavior (Schroeder & Riters, 2006). The systemic administration of a combined D1-like and D2-like receptor antagonist to male zebra finches decreased male courtship song, in addition to decreasing the frequencies of other courtship displays, such dancing, beak wiping, and female following (Rauceo *et al.*, 2008). Given the findings of these studies, it is perhaps surprising that we did not observe increased dopamine levels in the mesolimbic dopaminergic pathway in birds that were courting prior to pairing. One possibility is that we may have needed to kill the birds after a shorter or longer time interval after the initiation of courtship to detect enhanced dopamine levels in the nucleus accumbens. Another reason could be that the short (30 min) time allowed for courtship was not sufficient to result in the successful attraction and copulation that would be reflected in the dopamine levels in the nucleus accumbens (Riters, 2012; Riters & Stevenson, 2012).

Pair formation in a socially monogamous rodent, the prairie vole, involves mesolimbic dopamine. Mating with a female resulted in a 33% increase in dopamine turnover in male prairie voles that was followed by the development of a partner preference for the female (Aragona *et al.*, 2003). Activation of the D1-like dopamine receptor prevented partner preference formation, whereas activation of the D2-like dopamine receptor facilitated this process in male and female prairie voles (Aragona *et al.*, 2003, 2006; Liu & Wang, 2003). The VTA has also been implicated in the formation of pair bonds in voles. Administration of a GABA or a glutamate receptor antagonist to the VTA induced partner preference in male prairie voles in the absence of mating (which is normally important for the formation of a partner preference in males), owing to a reduction in inhibition within the VTA, ultimately leading to increased dopamine release in the nucleus accumbens (Curtis & Wang, 2005). These studies indicate that dopaminergic neurotransmission in the nucleus accumbens of prairie voles is critical for partner preference formation. As we observed higher levels of dopamine and DOPAC in the medial striatum of paired than of unpaired birds of both sexes, we

proceeded to investigate whether dopamine-synthesising neurons in the VTA were more active in paired birds than in unpaired birds. We observed that paired male and female birds had a higher percentage of dopaminergic neurons that expressed Fos in the VTA than unpaired birds. Greater immediate early gene activity in VTA dopaminergic neurons confirmed that recent neural activity was enhanced in the mesolimbic dopamine pathway in paired versus unpaired birds, and that the enhanced dopamine levels that we observed in the nucleus accumbens in paired birds were not merely attributable to the duration of interaction with an opposite-sex conspecific. Therefore, both HPLC and TH + Fos experiments point to the involvement of the mesolimbic pathway in pair formation in male and female zebra finches.

In addition to playing a role in pair bonding, nucleus accumbens dopamine is involved in sexual behaviors (Pfaus, 2009). In rats, the release of dopamine at the level of the nucleus positively regulated the anticipatory/motivational phase of copulatory behavior (Giuliano & Allard, 2001). When Japanese quail (*Coturnix japonica*) were injected with the dopamine D1-like and D2-like receptor agonist apomorphine, copulatory behaviors were inhibited (Absil *et al.*, 1994). Another study showed that a D1-receptor agonist stimulated whereas a D1-receptor antagonist inhibited consummatory sexual behavior in Japanese quail. In contrast, a D2-receptor agonist inhibited and a D2-receptor antagonist facilitated this behavior in the same species (Balthazart *et al.*, 1997). As paired birds engage in copulation, it is possible that this behavior contributes to enhanced levels of dopamine in the medial striatum of newly paired birds.

In the TH + Fos experiment, we observed that paired males had a higher percentage of Fos-expressing cells in the A11 region. A11 dopaminergic neurons play a role in courtship singing in male zebra finches (Bharati & Goodson, 2006; Goodson *et al.*, 2009), and show increased activation in response to copulatory activity in male Japanese quail (Charlier *et al.*, 2005). Therefore, it is possible that sexual behavior and courtship behaviors in paired male zebra finches contributed to the enhanced Fos expression in this region.

In our experiments, paired males not only showed longer durations of pairing behaviors towards females, but were also more aggressive towards other birds. Aggression towards unfamiliar conspecifics is also observed in paired male prairie voles, and dopamine and vasopressin in limbic regions are involved in this behavior (Gobrogge *et al.*, 2007). Similarly, increased activation of dopaminergic neurons of the VTA in response to fighting with conspecifics has been observed in zebra finches (Bharati & Goodson, 2006). Therefore, it is possible that dopamine levels in the medial striatum and Fos expression in VTA dopaminergic neurons are also associated with increased aggression in paired male zebra finches. Overall, we have not correlated each behavior observed with either dopamine levels or TH + Fos expression, but we suggest that the combination of all these pairing behaviors observed during 2–4 days of mixed-sex interaction is correlated with enhanced mesolimbic dopamine function. A shorter duration of mixed-sex interaction, wherein pairing behaviors were not observed, did not result in activation of this pathway at the time point that we observed (30 min after interactions). Therefore, it is likely that the onset of pairing is correlated with and involves activation of the mesolimbic dopaminergic pathway.

Our data suggest that there are no sex differences in the involvement of the VTA and nucleus accumbens with respect to dopamine/DOPAC levels in the nucleus accumbens and the percentages of TH + Fos-expressing cells in the VTA. In mammals, on the other hand, sex differences in the activation of the mesolimbic dopaminergic circuit have been observed that are probably attributable to differences in its neuroendocrine regulation by ovarian, placental and lactational hormones in females (Becker, 2009). Female zebra finches, on the other hand, do not have an estrous cycle, and the regulation and activation of the mesolimbic pathway in relation to pairing and sexual behavior is therefore likely to be similar to that in male zebra finches. Further evidence supporting this argument is that estrogens also do not affect the colocalisation of TH-labeled and Fos-labeled cells in zebra finches (Kabelik *et al.*, 2011). Finally, because both male and female zebra finches, and the males of many other birds, contribute to raising their young (unlike most male mammals), it is likely that the reward circuit is activated to the same extent by pairing and nesting in both sexes.

In summary, our study has shown that the mesolimbic dopaminergic pathway is activated by an extended duration of opposite-sex interactions primarily involving pairing behaviors, consistent with a contribution to pair formation in male and female zebra finches as in a socially monogamous mammal, the prairie vole. Further study is needed to understand the role of specific dopamine receptors and downstream signaling pathways in reward mediation and pair formation in this species.

Supporting Information

Additional supporting information can be found in the online version of this article:

Table S1. Dopamine and DOPAC levels (mean ± SEM) after 30 min of interaction with male or female zebra finches (courtship experiment).

Table S2. Numbers of TH-expressing and TH + Fos-expressing cells (mean ± SEM) in unpaired and paired zebra finches.

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Abbreviations

DOPAC, 3,4-dihydroxyphenylacetic acid; HPLC, high-performance liquid chromatography; PBS, phosphate-buffered saline; TH, tyrosine hydroxylase; VTA, ventral tegmental area.

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