A Thesis for the Degree of Ph.D. in Science

Variability of inter-individual communication during songbirds' pair formation period

-Modulation of vocal pathway and physical distance-

October 2014

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Chapter 1: Purpose of this study

The goal of this study is to characterize neural activity which differentially modulates inter-individual communication in animal society, using a monogamous songbird, zebra finch. In this species, males and females form a lifelong pair bond that includes both durable close physical proximity and interaction, and occasional sexual courtship leading to copulation (Zann.1996). Concerning those, we treated two topics in this study.

1.1 Singing-related activity in anterior forebrain of male zebra finches reflects courtship motivation for target females

The first study is more related to copulation-leading sexual courtship. We tried to elucidate distinctions between male courtship singing and associated neural activity during exposure to either familiar or unfamiliar females (Chapter2).

Previous study reported that male courtship songs directed to female and the ones undirected to female (=sung alone) are acoustically very similar, but female-directed song has a (1)lower level of variability in fine frequency control, and (2)higher singing tempo (Sossinka et al.1980, Kao et al. 2005). Importantly, females prefer to approach directed songs(Woolley et al.2008).

For (1)the variability in frequency control, anterior forebrain pathway (AFP) is responsible. When a female bird is visible to the male, stronger activation of dopaminergic (DAergic) neurons in ventral tegmental area (VTA) causes denser dopamine release in AFP nuclei, and it makes AFP neural variability lesser (Leblois et al. 2010), then next, this AFP activity is reflected in vocalization via neural projection from AFP to RA (a component of motor pathway). Because DAergic neurons in VTA is considered to evaluate natural reward (Stuber et al. 2008), if a female of male's singing target is more valuable for him, it may cause stronger VTA activation resulting in much lesser AFP variability, then ultimately its vocal acoustic frequency may become less variable.

To test this hypothesis, for each male bird, we showed a female who had been co-housed with him for 1-2 weeks (operationally "paired" female), and another female who was a complete stranger ("non-paired" female). Then we compared the variability of acoustic feature of male song between the two female targets, and the same comparison was applied for the variability of AFP neural activity (Area X; a part of the songbirds' basal ganglia).

In addition to acoustic fine frequency control, previous study reported that, female-directed song has shorter motif duration, and the number of motifs per bout is higher compared to the song produced when male was alone. However, those characteristics were not attenuated by injection of dopamine D1 receptor (D1R) blocker to Area X (Leblois et al.2012) suggesting that AFP dissociates from song speed control. Instead, for (2)song

speed control, a component of motor pathway, HVC (not RA) seems responsible. According to the previous study, presentation of females causes elevation of HVC temperature possibly as a by-product of elevation of body temperature, resulting in higher singing tempo (Aronov et al.2012).

Such distinction in song tempo between female-directed and undirected songs may exist between singing to paired and unpaired female as well if one female drives higher arousal in the male. Although we did not record from HVC this time, for each female presentation, we calculated the latency of the first song motif produced the number of motifs produced, (2)the tempo of motif renditions (motifs/sec) and the acceleration of motif renditions (motifs/sec^2).

1.2 Sexually dimorphic activation of dopaminergic areas depends on affiliation during courtship and pair formation

The second study is more related to development of durable physical proximity characteristic of pair bonding. We investigated the possibility of midbrain catecholaminergic contribution to male-female pair affinity development (chapter 3 and chapter 4).

Ethological study reported that when male bird initiates copulation-leading courtship, female shows a copulation solicitation display involving a horizontal posture and tail quivering only in case she agrees to complete copulation (Morris, 1954). Perhaps, during the courtship interaction, both individuals make ongoing judgments of their valence regarding the other, and produce behavioral signals reflecting these decisions. In order to investigate brain activation modulated by the valence of interaction, we wished to examine behavioral interactions in a more natural setting than a choice chamber, so that both birds have the opportunity to complete approach or withdrawal.

For this purpose, we developed a system for characterizing male and female behaviors during free interaction sessions. Positions of both birds during 3 daily 30 minute sessions were automatically recorded with a 3D video-tracking system we developed. The male-female pair affinity (valence) was quantified by a principal component analysis (PCA) based on five parameters from the tracking system.

Because most bird pairs committed “toward-copulation” ritualized courtship during their first meeting (day1), we focused on the behavior on day 3 which is after intense courtship period blew over and their relationship seems to start settling down. Additionally, we examined if the valence score judged on day 3 is related to any birds’ behavior during the copulation-leading courtship occurred on day 1; singing frequency, singing duration, copulation attempt, complete copulation, or aggressive behavior.

Next we examined if there is any correlation between the valence scores and concurrent c-Fos induction level within several catecholaminergic neural subpopulations (A10 in VTA, A11 in CG, A11 in SCI, A14, A15 (Appeltants et al. 2001) of both male and female midbrain. Among the areas, VTA and CG were found to be active during songbird courtship in previous studies, so they could reflect the birds' valence level.

For example, Yanagihara et al. (2006) reported that A10 DAergic neurons in VTA are active when male zebra finches sing to court females or just sees females without singing, but are not when they sing alone. Huang et al. (2008) also reported that the strength of synapses onto DAergic but not non-DAergic neurons in VTA increased following courtship singing or just seeing females without singing, but did not following singing alone. In monogamous mammal, prairie voles, D1R binding level within nucleus accumbens (NAc) increases after 2 weeks of male vole exposure to female, and the binding level has a causal relationship with male's selective aggression to unfamiliar females (Aragona et al. 2006). Because NAc is one of the major projection targets of VTA (Fallon et al. 1978), VTA may give a pair-bond go sign.

As for CG, Bharati et al (2006) reported that A11 DAergic neurons in CG are more strongly activated in male zebra finches who engaged in courtship behavior with females than males who did not try to interact with females. So the activation of A11 in CG may indicate a higher arousal during courtship compared to non-courting males.

If any of the DAergic system above is active during male-female interaction, its activity level may be diverse depending on the likelihood for the birds to become a couple (= valence score). And that could drive later long-lasting pair bonding in the zebra finch couples, or force them to stay as the strangers each other.

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Chapter 2:

2.1 Background previous works

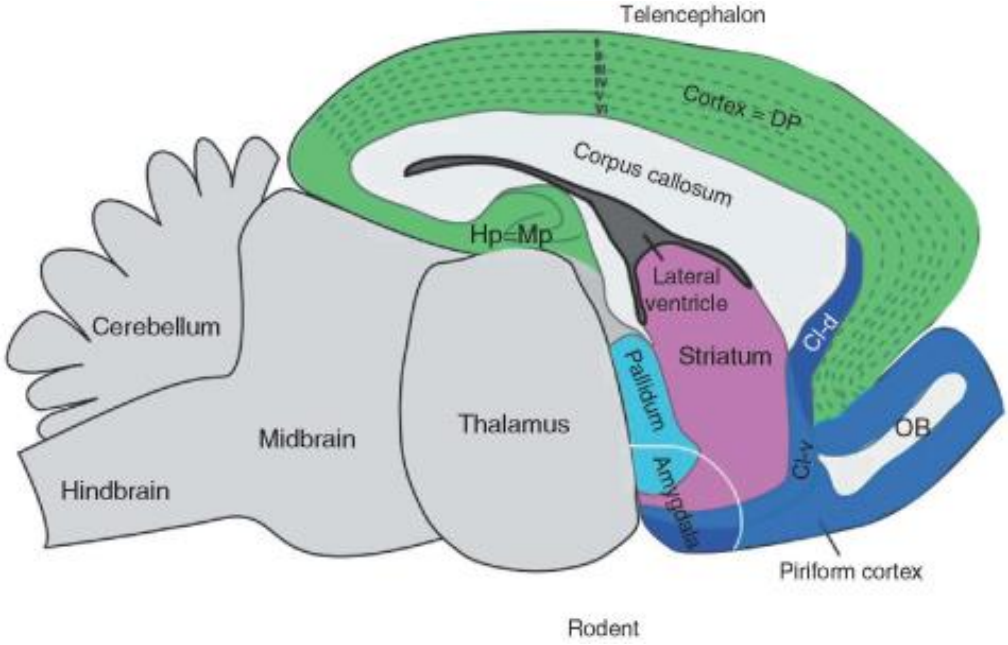
Before I move to my 1st journal paper "Singing-related activity in anterior forebrain of male zebra finches reflects courtship motivation for target females", here I will briefly explain the background previous works on "basal ganglia", because songbirds' Area X which I treated in my 1st journal paper corresponds to mammal basal ganglia. By understanding the homology between them, we may get some idea to associate general basal ganglia's motor modulation (not only oscine Area X) with the fluctuation in social motivation too.

Basal ganglia; Tuning of voluntary actions

The basal ganglia is a set of subcortical nuclei that control voluntary actions.

General Structure

The basal ganglia include two major structures: the striatum and the pallidum.



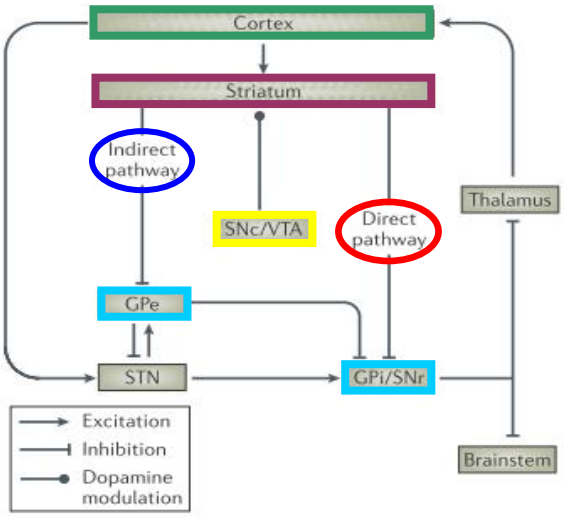
↑Fig.2.1-1 (Jarvis.2009)

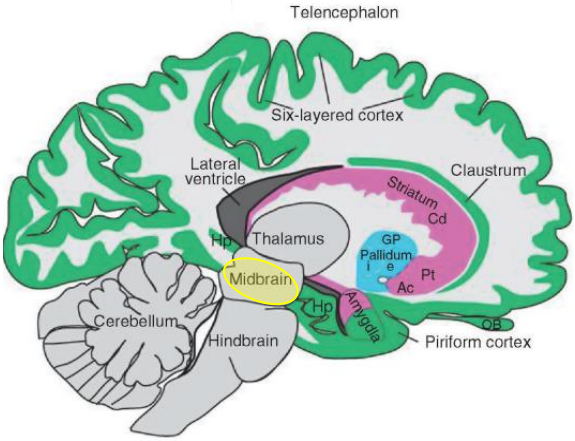
The striatum is the primary input structure of the basal ganglia, receiving glutamatergic input from most of the cortex (in mammal) or pallium (in avian) and from certain thalamic nuclei, as well as dense dopaminergic innervation from the midbrain.

The striatum does not project out of the basal ganglia; instead, its GABAergic (γ-aminobutyric acid) spiny projection neurons innervate the pallidum and substantia nigra pars reticulata (SNr).

The pallidum and SNr are collectively considered the output station of the basal ganglia, making GABAergic projections to a variety of targets outside of the basal ganglia, including the thalamus and brainstem. All of these basic features are shared among mammals and birds.

Direct and Indirect Pathway





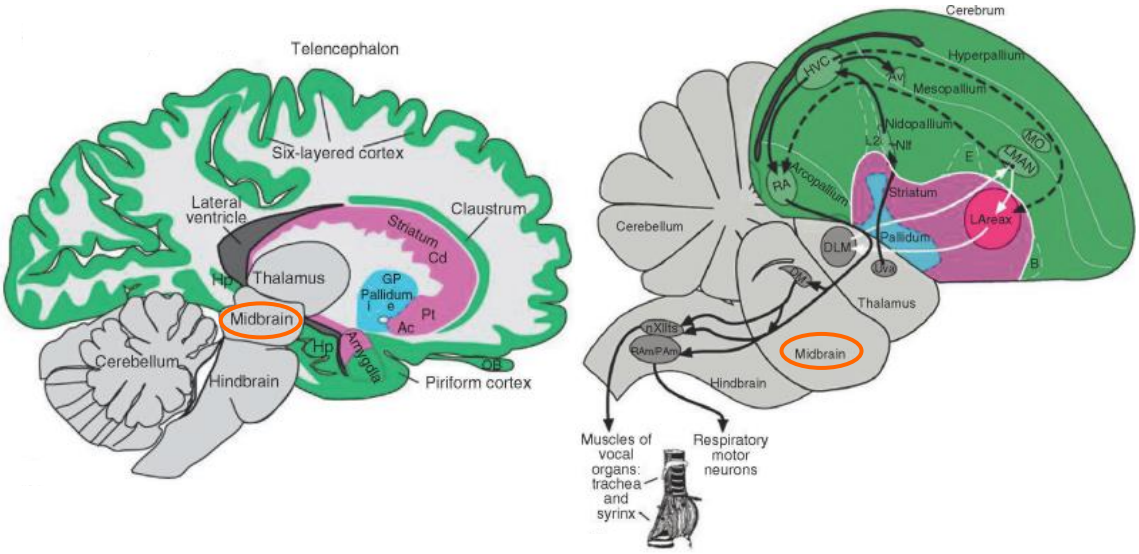
↑left, Fig.2.1-2 (Yin et al. 2006)
↑right, Fig.2.1-3 (Jarvis.2009)

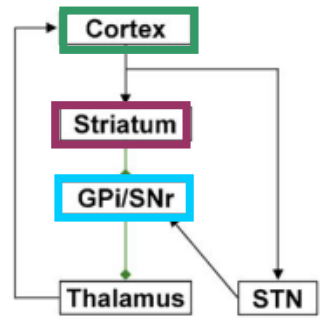
The GABAergic (inhibitory) projection neurons which project from the striatum are often quiescent owing to their intrinsic membrane properties (Wilson et al. 2004), and when they are activated by strong and coherent inputs from the cortex (and, to a lesser extent, the thalamus), they tend to suppress the tonically active pallidal neurons. The outcome of this disinhibitory pathway, the most basic pathway in the basal ganglia, is the facilitation of the targeted motor network (Deniau et al. 1985). It is called “direct pathway”.

However, a different pathway, traditionally known as the ‘indirect pathway’, appears to exert inhibitory control over downstream thalamocortical and brainstem networks (Albin et al. 1989).

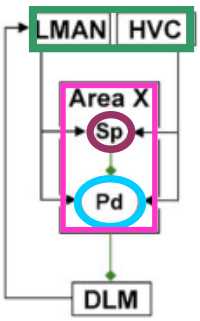
Direct- and indirect-pathway striatal neurons are co-activated during movement initiation, and are inactive when the animal is not moving. Neuronal activation in both pathways preceded movement initiation (with a latency appropriate for movement control) Co-activation of both direct and indirect pathways is important for action selection, with direct-pathway neurons promoting the wanted motor program and indirect-pathway neurons inhibiting competing motor programs (Yin et al. 2006).

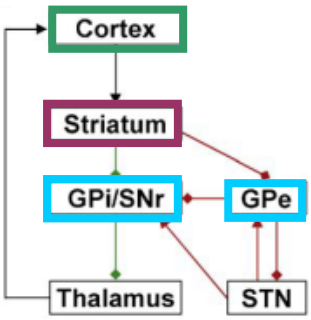
Comparison of basal ganglia between Oscine bird and Mammal



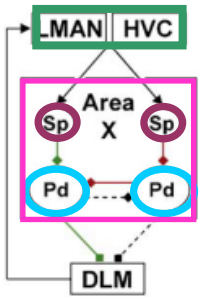


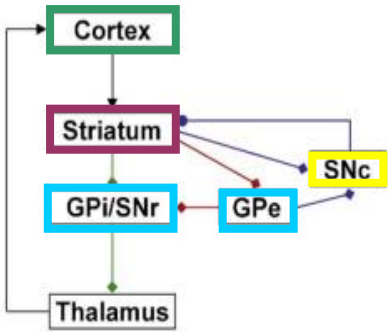
Direct pathway
(facilitation)



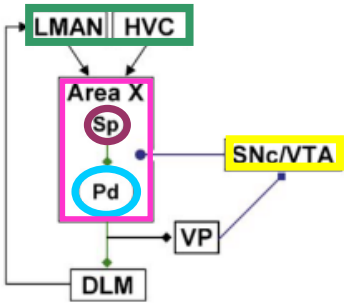


Indirect pathway
(inhibition)





DA Input to Striatum



↑Fig2.1-4 top (Jarvis.2009), bottom (Gale et al. 2010)

In mammals the globus pallidus(GP) is divided into two parts: an "external segment" (GPe) whose projections remain within the basal ganglia and an "internal segment" (GPi), which, like the SNr, projects out to the brainstem and thalamus (mammalian anatomy reviewed in Parent and Hazrati, 1995).

The GPe is reciprocally connected with the subthalamic nucleus(STN) (→indirect pathway), whereas the GPi receives subthalamic input but does not project there (→direct pathway).

The avian GP, by contrast, is not divided into distinct parts but rather exhibits the anatomical connections of both segments of the mammalian GP (Medina and Reiner, 1997). Area X is effectively a mixture of striatum and pallidum that retains much of the functional organization of the mammalian basal ganglia, even though it mingles cell types that are physically segregated in mammals (Jarvis, 2009).

2.2 Singing-related activity in anterior forebrain of male zebra finches reflects courtship motivation for target females

PLoS One. 2013 Nov 29;8(11)

Abstract

A critical function of singing by male songbirds is to attract a female mate. Previous studies have suggested that the anterior forebrain system is involved in this courtship behavior. Neural activity in this system, including the striatal Area X, is strikingly dependent on the function of male singing. When males sing to attract a female bird rather than while alone, less variable neural activity results in less variable song spectral features, which may be attractive to the female. These characteristics of neural activity and singing thus may reflect a male's motivation for courtship. Here, we compared the variability of neural activity and song features between courtship singing directed to a female with whom a male had previously formed a pair-bond or to other females. Surprisingly, across all units, there was no clear tendency for a difference in variability of neural activity or song features between courtship of paired females, nonpaired females, or dummy females. However, across the population of recordings, there was a significant relationship between the relative variability of syllable frequency and neural activity: when syllable frequency was less variable to paired than nonpaired females, neural activity was also less variable (and vice-versa). These results show that the lower variability of neural activity and syllable frequency during directed singing is not a binary distinction from undirected singing, but can vary in intensity, possibly related to the relative preference of a male for his singing target.

Introduction

For both males and females, interaction with individuals of the opposite sex is critical for successful reproduction. In addition, a subset of species further expands such interaction to prolonged pair bonding (Freeman et al. 2012, Stoesz et al. 2013). Such social monogamy is especially widespread among songbirds (~90% of species (Lack 1968). Within monogamous pairs, though, individuals occasionally choose to engage in extra-pair interactions (Griffith et al. 2002). Such decisions, which can influence an individual's reproductive success and maintenance of their pair-bond, are among the most important that females and males make.

In zebra finches, males court females by producing a stereotyped "directed" song, in response to which the female can choose to either continue the courtship interaction, or reject the male. In non-courtship contexts, males also produce a similar "undirected" song, apparently without a specific target (Zann 1996, Sakata et al. 2012). While both song types are very similar acoustically, directed songs can be distinguished by a lower level of variability of fine frequency control, and a slightly higher singing tempo (Sossinka et al. 1980, Kao et al. 2005). Importantly, female zebra finches can discriminate between these songs, and prefer to approach directed ones (Woolley et al. 2008).

Production of both directed and undirected songs is controlled by a specialized brain network known as the song system that is only present in songbirds (Nottebohm 2005). Activity in one subset of the song system, the anterior forebrain pathway (AFP), determines whether singing output is directed or undirected. The AFP network consists of the basal ganglia Area X, its efferent dorsal lateral nucleus of the medial thalamus (DLM), and the lateral magnocellular nucleus of the nidopallium (LMAN), which provides input to the singing premotor robust nucleus of the arcopallium (RA).[1]
This input (AFP to RA) controls a characteristic feature of directed singing, reduced variability of syllable fine frequency control. During undirected singing, LMAN projection neurons fire with a relatively variable pattern related to song elements, while during directed singing this neural activity is much more stereotyped. Critically, inactivation or lesions of LMAN reduce the variability of all singing, causing undirected songs to become similar to directed ones (Ölveczky et al. 2005, Kao et al. 2006).[2]

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[1] ►AFP Nuclei
HVC→AreaX→DLM→LMAN→RA

►**The HVc/LMAN → Area X → DLM → LMAN loop**
may be related to the **mammalian**
cortex → basal ganglia → thalamus → cortex loops
(Bottjer et al. 1995, Luo et al. 1999 J Comp Neurol, Luo et al. 1999 J Neurosci, Alexander et al. 1986, Parent et al. 1995, Veenman et al. 1997)

[2] Proven cases of "Variable activity in AFP causes variable song feature"

This input control of neural variability in AFP nuclei and of courtship-related features of singing appears regulated by dopaminergic input from the ventral tegmental area (Sasaki et al. 2006, Yanagihara et al. 2006, Hara et al. 2007, Huang et al. 2008, Leblois et al. 2010). Stronger activation of dopaminergic neurons during directed singing causes higher levels of dopamine release in AFP nuclei, and reduces the level of neural variability during courtship. Thus, in contrast to undirected singing, female-directed courtship singing is associated with strong activation of dopaminergic systems that are related in mammals with motivation and goal-directed behaviors (Kelley et al. 2002, Wise 2004). When males sing during courtship of females, but not when they sing while alone, the level of neural activity (Yanagihara et al. 2006) and of activity-dependent gene expression (Hara et al. 2007) in VTA is selectively modulated, and higher levels of dopamine are present in Area X (Sasaki et al. 2006). Further, block of dopamine receptors restricted to Area X causes directed singing to become more similar in variability to undirected singing (Leblois et al. 2010).

►Variable LMAN activity causes frequency CV in harmonic stacks both across (fundamental frequency) and within(spectral entropy) syllable renditions.

In the study which reversibly silenced LMAN in singing zebra finches by bilateral reverse microdialysis of the GABA A receptor agonist muscimol, LMAN inactivation acutely reduced undirected song variability, both across (freq xrCV) and even within syllable renditions, to the level of directed song variability (Stepanek et al. 2010).

Within-syllable variability which decreased under LMAN inactivation uses the features of spectral entropy as defined in (Sakata et al. 2006).

Spectral entropy is the entropy of the Fourier transform of the entire syllable collapsed across time, normalized such that sounds with qualities similar to pure tones have values approaching 0 and sounds similar to white noise have values approaching 1 (Stepanek et al. 2010).

► Variable AreaX activity causes fundamental frequency CV across renditions both in harmonic and other type of syllables.
D1R antagonist SCH23390 administration abolishes social context-dependent changes in the variability of the fundamental frequency of sub-syllablic elements not only displaying a clear harmonic structure, but also in other types of syllables (Leblois et al. 2010).

In zebra finch songs, harmonic stacks are typically present in only 10–50% of the syllables (Leblois et al. 2012; unpublished data).

Variability in non-harmonic syllable can be computed by pair-wise cross correlations between their spectrograms (Nelson et al.1994) after adjusting syllable length through time warping (Anderson et al. 1996).

The modulation of neural activity and song features present in directed singing, and absent in undirected singing, reflects in some way the male's female-directed courtship behavior. Here, we tested whether AFP activity and singing features were consistently less variable, characteristic of courtship singing, when males sang to females of a particular pairing status. For each male, a female was operationally defined as "paired" or "nonpaired" based on whether or not they had previously cohabitated with him for 1-2 weeks. We found that across all recordings, variability of both AFP activity and songs were not consistently dependent on female pairing status. However, within individual recordings, the variability of neural activity was closely related to syllable variability. These results highlight the complexity of courtship interactions for both males and females.

Materials and methods

Ethics Statement

All procedures were reviewed and approved by the RIKEN Animal Experiments Committee (Approval ID: H23-1-223).

Pairing procedure

Eight adult zebra finches (over 120 days old, 4 each male and female) were used in these experiments. For each opposite sex pair, birds were taken from a group housing cage in an open room containing other zebra finches, and moved into a cage (27 x 27 x 20 cm) in a sound attenuation chamber (60 x 50 x 50 cm). Males were moved into the cage one day before females. Following 8-13 days of housing together, all pairs had attained some level of pair-bonding, as defined by the occurrence of clumping behavior and allopreening (Zann 1996, Butterfield 1970, Silcox et al. 1982, Clayton 1990, Svec et al. 2009). Both birds were then transferred into separate small cages (20 x 20 x 20 cm) in sound attenuation chambers containing no other birds.[3]

Surgical procedure

1-4 days after separation from the female, males were implanted with a miniature movable microdrive, as described previously (Hessler et al. 1999, J Neurosci). In brief, under inhalation anesthesia (isoflurane), a movable microdrive containing two insulated tungsten electrodes (~3Mohm, Microprobe) was positioned with electrode tips 1.3 mm below the surface of the brain, and fixed to the skull with 5-minute epoxy (Devcon).

[3] ►Fewer than half of the zebra finch male and female form a pair relationship within 24 h, but 48 h results in over 75% of animals pairing (Pedersen et al. 2012).

Recording procedure

After 2-3 days of recovery, a flexible wire lead containing a headstage (Plexon, Dallas TX, USA) was connected to the microdrive, and signals from the electrode were passed through a rotating commutator (Dragonfly, Ridgeley WV, USA), amplified, and digitized at 40 kHz, along with the acoustic signal in the chamber (Plexon). During each recording session, electrodes were slowly lowered until apparently isolated single-unit activity was present. At intervals of about ten minutes, the door of the sound attenuation box was opened and a small cage containing either the female who had been paired with the male, a female who had not been paired with the male, or a painted dummy model of a female zebra finch or java sparrow was placed next to the male's cage.

For most recording sessions, either 2 or 3 nonpaired females were used, including females of similar and different color morphs (e.g., Fig. 2.2-1A,B), but in 4 sessions only a single nonpaired female was used (3 similar color, 1 different). Females that were operationally defined as "nonpaired" for an individual male were used as "paired" females for other experimental males, and thus had recent (within 3 weeks) experience of living with a male, and of being used as a singing stimulus. Subsequent behavior of both birds was monitored by video camera, and often included directed singing of the male towards the female. The caged female was removed after about 2-3 minutes, whether the male sang or not.

Recording sessions occurred over a period of 2-9 days, from 3-14 days after males were separated from paired females. Behavioral data were analyzed only from recording sessions in which neural activity was recorded.

Analysis

After recordings were completed, unit activity was sorted with Offline Sorter (Plexon). Unit isolation was confirmed as > 99% of ISI's were greater than 1 msec (Yanagihara et al. 2012). All subsequent analysis was performed with Matlab (Mathworks, Natick, MA, USA). For each recording session, productions of a typical song motif (containing 4-6 syllables, of duration 475 - 769 msec) were detected by matching a template of sound amplitude level to the acoustic signal recorded along with neural recordings.

[4]In order to account for some variability in motif duration, for each song motif the estimated deviation of motif initiation and termination time from the average motif

[4] Because area X is responsible for motor control of singing, its spike timing is likely to be coupled with the correspondent sound production. Then, to compare spike pattern across rendition, we compressed or expanded spike intervals depending on its correspondent song motifs' compression or expansion level, relative to the average motif duration.

initiation and termination time was determined by visual comparison of the smoothed rectified waveforms of both. Following this, unit firing times for each motif were shifted to account for these deviations. In brief, spikes occurring at motif initiation were shifted forward or backward in time according to the estimated deviance of a motif's onset from the average motif, and spikes occurring at the motif termination were shifted according to the estimated deviance of the motif termination time.

Spikes within motifs were shifted proportional to their position relative to motif initiation and termination. After shifting, the instantaneous spike rate for each song motif rendition was estimated by smoothing with a 10 msec Gaussian window. While this method assumes that motif temporal variability is uniform across all song elements, it could fail to fully characterize variability in timing of individual syllables in motifs. However, frequent vocalizations of females made accurate identification of all syllable boundaries difficult.

Two measures of variability of unit activity with repeated song motifs were calculated for songs produced to each female class (Hessler et al. 1999, J Neurosci).The cross-rendition coefficient of variance (C.V.) was calculated by dividing the standard deviation of the mean instantaneous spike rate by the mean spike rate, in 1 msec intervals. For each recording session, the mean C.V. of all song motifs was used for comparisons between female classes[5]. To quantify within-rendition variability, the cross-correlation (xcorr) between the instantaneous spike rate of each motif rendition and the average spike rate across all motifs was calculated.[6] As no clear differences were apparent in neural activity to conspecific or heterospecific model dummies (n=3: zebra finch / java sparrow ratio of mean C.V. = 1.03, mean xcorr = 1.04, both ns sign rank test), results from both were combined in analyses.

Variability in production of song motif syllables for each female class was determined by measuring either the fundamental frequency of a relatively constant frequency harmonic stack, or the frequency of a tonal element (Kao et al. 2005). For harmonic stacks, the fundamental frequency was estimated by finding the frequency of the first peak of the autocorrelation of the spectral density of a 10 msec window within the stack. For tonal

[5] The cross-rendition coefficient of variance (xrC.V.) was calculated in 1 msec intervals. Namely, for every 1 msec, the mean spike rate across rendition was divided by the standard deviation of instantaneous spike rates. The mean xrC.V. over the song motif duration was used for comparisons between different units. Further, xrC.V. for each female type were calculated, and their 20 consecutive time intervals were averaged and compared between female conditions using paired sign-rank test.

[6] We calculated the average spike pattern across all renditions per one unit. Then, we got the cross-correlation between the average spike and the spike during each rendition (xcorr). The xcorr values were averaged for each female type, and compared using Mann-Whitney *U* test.

elements, the frequency of the first peak in the autocorrelation of a 10 msec window was used.

Global features of singing related to timing of motif production were determined by first calculating the latency of each motif onset relative to the time at which the female cage was placed into the recording chamber. For each female presentation, the latency of the first motif produced, the number of motifs produced, the tempo of motif renditions (motif onset latency$_{x+1}$-motif onset latency$_x$) and the acceleration of motif renditions (tempo $_{x+1}$-tempo$_x$) were calculated. Examination of latencies of the first motif produced for all recordings revealed that only 3/113 were above 40 seconds, and all others below 20 seconds. Thus, only those less than 20 seconds were used for calculating mean initial latency values. Quantification of the remaining features was restricted to singing bouts beginning prior to 20 seconds, as well.

In order to focus on motifs produced within the initial continuous singing bout, we further examined the distribution of singing tempos. The majority of successive motifs were produced with intervals of 4.2 seconds or less, with progressively fewer of longer intervals (11/468 > 4.7 sec, 12 > 4.2 sec, 14 > 4 sec, 18 > 3.8 sec). Therefore, we limited quantification of motif number, singing tempo, and acceleration to motifs produced prior to the first motif interval greater than 4.2 seconds. Within this subset of acceleration values, the mean acceleration variability was quantified by calculating by the S.D. of acceleration values, and the mean level of acceleration alternation was quantified by the percentage of successive acceleration values that were of opposite sign (percentage of successive acceleration values that crossed the zero level).

For quantification of average motif duration, initiation and termination times were estimated by visual comparison of each rendition's sound amplitude waveform to the average motif rendition. Variability of motif durations calculated in this way were in the range of those reported in previous studies (motif duration C.V. = 0.007 - 0.013).

Because undirected singing was produced in only 3 recording sessions, and consisted of few motif renditions (5-9), we did not further analyze this in detail.

Statistical analysis

The majority of analyses tested whether characteristics of neural activity and singing features were different during singing directed to paired females than other female types. Thus, we performed statistical tests comparing neural activity and singing between paired females and nonpaired females, and between paired females and dummy females. Because data from paired females were used for 2 comparisons, we used a conservative significance level of $p = 0.01$ for all tests.

For tests comparing neural results between paired vs. other female classes, we used the average values of cross-rendition C.V. and within-rendition xcorr for each recorded unit. A

test of whether neural variability of the population of units was different between paired and other females was made with a paired statistical test (e.g., Fig. 2.2-2A).

For tests comparing singing features, we used the average values within a single day of recording for each bird, because multiple units were recorded on some days. Tests comparing singing features between paired and other females were made using paired statistics, as above (e.g., Fig. 2.2-3C).

The choice of parametric or non-parametric tests for each comparison was based on a Lilliefors test of normality. Those distributions consistent with normality, paired t-tests were used, and for others sign-rank tests were used.

In addition to these paired population tests comparing paired with other females, we compared measures of neural variability for each single unit, during singing to paired and other females (indicated by asterisks in Fig. 2.2-2). The comparisons of cross-rendition C.V. and within-rendition xcorr were made in different ways. Because calculation of the average cross-rendition C.V. results in a single time-series for each female type (e.g., Fig. 2.2-1C), for statistical comparison the average C.V. waveform was divided into 20 equal duration epochs (e.g., for a 1000 msec motif, 20 epochs of 50 msec duration). The average cross-rendition C.V. in each of these epochs was then used to make paired comparisons between female types, with choice of tests based on distribution normality as above.

In contrast, a single value of within-rendition xcorr is calculated for each song motif. Comparisons between female classes of the resulting independent samples of within-rendition xcorr were made with unpaired statistical tests within single days. Tests between groups with normal distributions were made with t-tests, and others were made with Mann-Whitney U tests. Unless otherwise noted, S.D. is used to indicate error values.

Results

A. Neural characteristics of directed singing compared between paired and other female classes.

An increased variability of AFP nuclei neural activity, which is reflected in song feature variability, occurs when a male sings alone compared to when singing to a female bird (Kao et al. 2005, Hessler et al. 1999 Nat Neurosci, Kao et al. 2008). Such reduction of neural and singing variability during directed singing is thus characteristic of the behavioral state of motivated courtship. Here, we tested whether such neural and song variability related to courtship is lower when males sing to females with whom they had previously been paired, compared to when males sing to females with whom they had not (classified as "paired" and "nonpaired" females, respectively).

Several days after males had been paired with a female zebra finch (for 1-2 weeks), single unit activity was recorded from the song system AFP nuclei Area X or LMAN (n=13 Area X, n=4 LMAN). As variability of activity in both nuclei is lower during directed compared to undirected singing (Hessler et al. 1999 Nat Neurosci), results were combined in analyses except as noted. During each recording, cages containing the female with whom the male had been paired, a female with whom they had not been paired, or a dummy model of a female finch were placed into the recording chamber at intervals of several minutes. Males quickly oriented toward and began directing song to all female types during most such presentations (singing % of all presentations, paired = 93, nonpaired = 86, dummy = 95).

Of the 13 units recorded from Area X, most were of the putative pallidal type, as indicated by their relatively high firing rate (> 58 Hz) and regular firing (median ISI C.V. = 0.42; n = 11) during non-singing periods, while few were of the putative striatal type (n = 2; mean s.r. < 30 Hz, mean ISI C.V = 1.02, (Goldberg et al. 2010)).[7] Consistent with this distinction, across all Area X units there was a strong negative correlation between firing rate and ISI C.V. during baseline non-singing periods (Fig. 2.2-6A, r = 0.81, p < 0.001, Pearson's correlation).[8] [9]

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[7] ►Some study reports the opposite, "The vast majority of neurons within AreaX are striatal (slow spiking) neurons".
The vast majority of neurons within Area X are striatal spiny neurons, which are homologous to mammalian medium spiny neurons (Farries et al. 2002, Reiner et al. 2004), but do not project outside of Area X. Spontaneous firing rate is lower than 25 spikes/sec (Leblois et al. 2009).

[8] Consistent with this distinction, across all Area X units there was a strong tendency that neurons whose spike faster have higher regularity of isi (low isi C.V.) at baseline non-singing

[9] I first applied Mann-Whitney *U* test to compare spike rate between base and during singing. Some neurons significantly increased its spike rate during singing compared to during base, some neurons decreased its spike rate significantly during singing compared to during base, and others were not judged that the spikerate changed depending on singing or silent status. Apparently, p_values from this mwwtest distribute along logarithmic scale.

Next, I tried to estimate spike reproducibility across song renditions. Because I already calculated xcov between average spike of paired, nonpaired, dummy, I calculated the average of those xcov. (mean xcov).If the neuron has high "mean xcov", that neuron is more likely to be involved in song production.

Then, I calculated correlation between "log10(p_mwwtest)" and "mean xcov". After I removed LMAN's data, its correlation improved a lot. In the linear approximate equation, R^2=0.4144 (n=15), after 1 outlier removed, R^2=0.6626 (n=14).

The average firing rate of putative pallidal units was not consistently modulated during singing: both increases and decreases of activity were observed (Fig. 2.2-6B; mean singing/non-singing spike rate = 1.08, n = 4 < 1, n = 7 > 1 (Hessler et al. 1999 Nat Neurosci). Instead, a more characteristic feature of pallidal unit activity during singing was an increase of phasic modulation from non-singing periods, including both decreases and increases of firing rate in single song renditions (resulting in a higher ISI C.V. during singing).

Across all Area X units, the level of such phasic modulation was strongly correlated with the non-singing spike rate (Fig. 2.2-6C), [10]with modulation of pallidal units significantly higher during singing than non-singing periods (mean ISI C.V. singing / non-singing = 1.44, p = 0.001, t-test). Such phasic decreases and increases of pallidal firing rate are critical for driving modulation of efferent thalamic neurons during singing (Person et al. 2005, Leblois et al. 2009). [11]In contrast, the average firing rate of all LMAN units was consistently increased during singing (mean singing/non-singing = 2.42 ± 0.42).[12]

Briefly, maybe we can conclude that in areaX, if the neuron's spike rate increased (or decreased) from base to singing a lot, that neuron always spikes in almost the same pattern along singing vocalization (So to say, Sensitive, or Responsible neuron). On the other hand, there is not so reactive neuron which tends to spike more randomly (So to say, Numb, not Responsible neuron).

In my analysis, neurons which have higher xcov during singing were actually the neurons which increased (or decreased) its firing rate more from base. But, neurons with lower xcov (their firing pattern don't strictly go along with singing) were actually the neurons whose spike rate were not significantly different from base's (Fig.2.2-7).

[10] Across all AreaX neurons, neurons with higher firing rate at non-singing tended to have "higher isi C.V. during singing" = "more irregular isi during singing compared to non-singing"

[11] ►At least two thirds of AreaX pallidal neurons appear to send an axon to DLM (Person et al. 2008).

►Each DLM neuron receives input from single or at most two large pallidal AreaX neurons (Luo et al. 1999, J Comp Neurol).
►The response of DLM neuron is mainly driven by changes in their GABAergic input from pallidal cells. (Leblois et al. 2009)

[12] This accords with the previous report. (Kao et al. 2008)

Typical features of Area X activity related to singing are evident in a representative recording, in which a male was presented with different female types (Fig. 2.2-1). From a relatively high and regular firing rate before singing, activity of this unit was mainly reduced during singing, with periods of phasic increases. Characteristic pauses of activity are seen in expanded periods of panel A containing final song motifs and non-singing periods (Fig. 2.2-1B, epoch indicated by color band in panel A). When aligned by a common song motif of this bird, characteristic patterns of inhibition and excitation are evident in spike timing during successive motif renditions (Fig. 2.2-1C) and in the average spike rate across all motifs (Fig. 2.2-1D, upper).[13]

During singing directed to females, activity of AFP units consistently is less variable across renditions compared to undirected singing with no particular target (Hessler et al. 1999 Nat Neurosci, Kao et al. 2008). This distinction appears to reflect a common phasic pattern of activity modulation present in both singing types driven by input from the premotor nucleus HVC, while higher levels of activity that are less related to this pattern also occur throughout the song motif during undirected singing.[14]

Thus, across multiple renditions of a song motif, the pattern of the average firing rate is similar for both directed and undirected singing.[15] The higher level of variability

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[13] ►Typically, each AreaX neuron generated a characteristic song-locked average firing pattern as were reported for LMAN (Kao et al. 2008).

[14] Despite of the distinction in variability across rendition, the average spike pattern across rendition is remarkably similar between directed and undirected singing. It appears that the premotor nucleus HVC drives a common phasic pattern in AreaX and LMAN both during directed and undirected singing, but only during directed singing, variability is added throughout the song motif.

[15] ►Previous recordings within HVC showed modulations in firing rate that were tied to individual syllables and repeated by motif (Yu et al. 1996).

HVC exhibits rhythmic, patterned activity (Hahnloser et al. 2002, Solis et al. 2005),

precisely time-locked to song in the awake bird (Long et al. 2008).
►LMAN projection neurons provide excitatory input to RA neurons and synapse on the same neurons as HVC projection neurons. (Mooney et al. 1991, Spiro et al. 1999, Stark et al. 1999)

►Spike timing is highly correlated across HVC, RA, and LMAN. (Kimpo et al. 2003)

characteristic of undirected singing can occur in addition to the basic singing-related pattern in two ways.

At a given location within the song motif, there can be variability in the firing rate across multiple song renditions (e.g., Fig. 2.2-1C raster plot around the end of third syllable). Such *cross-rendition variability* of the firing rate was quantified by the C.V., the standard deviation divided by the mean firing rate for each timepoint (Fig. 2.2-1D, see Materials and Methods for details).

In the converse dimension, within each motif rendition the phasic instantaneous firing rate can be more or less similar to the average firing rate pattern. Such *within-rendition variability* was quantified by calculating the cross-correlation of instantaneous firing rate for each motif rendition and the average firing rate across all renditions (defined as the mean xcorr, (Hessler et al. 1999 J Neurosci).

Across all recordings, neither of these measures of neural variability were significantly different between singing to paired compared to nonpaired females (Fig 2.2-2A, B; paired vs. nonpaired mean C.V. $p = 0.44$, mean xcorr $p = 0.92$). Within individual recording sessions, while the mean cross-rendition C.V. was significantly different in some experiments, though there was no clear pattern favoring one female class (C.V. significantly higher paired n = 5/17, nonpaired n = 2/17).

Previous behavioral experiments have suggested that model dummies of female birds are less potent at eliciting song than live females (Bischof et al. 1981, Garson et al. 1980) but see (Caryl 1981) for divergent results). Thus, presentations of paired females were interspersed with presentations of dummies, to test whether such a lower potency stimulus may be reflected in higher neural variability during directed singing. For the mean cross-rendition C.V., there was no significant trend among all units for a distinction between singing directed to paired or dummy females (Fig. 2.2-2C; paired vs. dummy $p = 0.50$).

The mean within-rendition xcorr had some tendency to be higher during singing to paired females compared to dummies (Fig. 2.2-2D, paired vs. dummy $p = 0.02$, mean ratio paired / dummy= 1.05), and was higher for paired than dummy females within all but two recordings (15/17, $p = 0.002$, chi-square test).

B. Comparison of song features typical of directed singing between different female classes.

Along with reduced variability of neural activity during directed compared to undirected singing, previous work has characterized lower variability of syllable spectral structure and an increase in singing tempo during directed singing (Sossinka et al. 1980, Kao et al. 2005, Ölveczky et al. 2005, Leblois et al. 2010, Leblois et al. 2012).[16]

[16] Support Info for an increase in singing tempo (Cooper et al. 2006, Kao et al. 2006)

We thus compared these features, as well as several other global singing parameters that have been previously shown to be related to singing motivation, between singing directed to different female classes (Sossinka et al. 1980, George et al. 2006). For each singing parameter, data from single days of recording for individual birds were combined for group analyses.

Variability of spectral structure was quantified by measuring the frequency of a motif syllable component containing a sustained stable frequency (e.g., Fig. 2-1C, syllable element about 150 msec from onset). The cross-rendition variability of frequency was then compared between target female classes for each day. Over all experiments, there was no tendency for frequency variability to differ during singing to paired vs. nonpaired or dummy females (Fig. 2.2-3B; $p = 0.82$ and 0.75).[17]

Among global features of singing, the latency to sing, number of motifs produced, and the tempo and acceleration with which sucfcessive motifs were produced were quantified for each female presentation in which a male sang (e.g., Fig. 2.2-3A). Across all recording sessions, the strongest trend was for a higher singing tempo to paired than nonpaired or dummy females (Fig. 2.2-3C, mean ratio of tempo paired / nonpaired = 1.12, paired / dummy = 1.06).

►Shorter song motif doesn't appear soon after the female presentation, it needs 2min.
In all birds, HVC temperature increased in response to the presentation, reaching an average value of 0.94 ± 0.19 Celsius degree above baseline after 2 min (Aronov et al. 2012).

Changes in song tempo, quantified by measuring the duration of the song motif (Glaze et al. 2006), did not occur immediately upon the presentation of the female, but followed a slow time course taking close to two minutes to reach equilibrium.

Most of the context-dependent difference in tempo could indeed be explained by a linear dependence on temperature (Aronov et al. 2012).

[17]►Across-rendition variability in fundamental frequency of harmonic syllables is higher during undirected singing associated with LMAN variability (Kao et al. 2005, Kao et al. 2008).

►Within-rendition syllable entropy is higher during undirected singing associated with LMAN variability (Stepanek et al. 2010).

►Across-rendition variability in fundamental frequency of harmonic & non-harmonic syllables associated with AreaX variability (Leblois et al. 2010).

Although these tendencies were not significant across all recordings, ($p = 0.26$ and 0.28 respectively), this reflected some variability in two components of singing rendition tempo - the duration of each song motif and the duration of inter-motif intervals (pauses). For one bird, the duration of pauses was close to significantly shorter when singing to paired than dummy females ($p = 0.014$, mean pause durations paired and dummy: 552 and 780 msec).[18]

For two others, the duration of motifs sang to paired females were shorter than those sang to nonpaired females (bird A; paired / nonpaired=476 / 480 msec, $p = 0.00038$; bird B; paired / nonpaired = 597 / 605 msec, $p = 0.0011$; Mann-Whitney *U* test), and also shorter than motifs sang to dummy females for one of these (bird B; paired vs. dummy $p < 0.0001$, paired / dummy = 597 / 608 msec).[19]

While there was a clear group of recordings in which males began singing more quickly to paired females than others (Fig. 2.2-3D, note paired latencies clustered at about one second), the high variability across recordings resulted in no significant difference between female classes ($p > 0.9$, median ratio of latency paired / nonpaired, paired / dummy = 0.86, 0.82 respectively).[20] The average number of motifs per singing bout was also not significantly

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[18] ►In undirected song, inter-motif gaps (=pauses) and the beginning syllables of motifs (=just after the pauses) are especially responsible for song elasticity (Glaze et al. 2006).

[19] Related Info "Directed (more motivated) singing has shorter motif duration"
►Three in four birds, there was a significant decrease in the motif duration when the bird sang in the presence of a female relative to when he was singing alone ($P < 0.001$ in all three birds). It was not affected by D1R blockade in Area X (Leblois et al. 2012).

[20] Physiological change before song onsets
►Pre-song heart rate is higher for directed than undirected.
Mean of both "pre-" and "during-" song heart rate are significantly higher for directed singing than it was undirected singing. Further, both "pre-" and "during-" song heart rate are negatively correlated with the duration of the first motif (Cooper et al. 2006)

►For undirected singing, AreaX firing increases before song onsets measured by multiunit. But for directed is not known.
►For undirected singing, LMAN (18 of 19) firing increases before (in a 500ms interval from 600 to 100ms before) song onsets, but not for directed (Hessler et al. 1999, J Neurosci).

►Some HVC neurons exhibit a gradual increase in firing rate as much as several seconds before song onset, perhaps reflecting a role in motor preparation or initiation (McCasland et al. 1987, Dave et al. 1998).

different between groups (not shown, paired vs. nonpaired, dummy p = 0.36, 0.42, mean ratio motif number paired / nonpaired, paired / dummy = 1.24, 1.12 respectively).[21]

As briefly noted previously (Chi et al. 2001, Glaze et al. 2006, Cooper et al. 2006), zebra finches have some tendency to reduce their singing tempo within a song bout. We suspected that such deceleration may reflect in part a habituation of singing males to their female target, and thus could be a sensitive indicator of male courtship motivation within single bouts.[22] In order to quantify reduction of singing tempo during successive motif renditions, we calculated the rate of motif acceleration (derivative of tempo). The average level of acceleration over all song motifs was consistently negative (mean = -0.13 motifs/sec^2, $p < 0.001$ less than zero, t-test), with the average acceleration for paired, nonpaired and dummy females -0.13, -0.19, and -0.09 respectively.

Further, the average acceleration level in the majority of singing bouts was negative (paired, nonpaired, dummy = 0.89, 0.94, 0.82 respectively). Average acceleration levels within each recording day were not significantly different between female classes (Fig. 2.2-3E, paired vs. nonpaired, dummy p = 0.45, 0.38, mean acceleration paired, nonpaired, dummy = -0.15, -0.21, -0.11 respectively). A closer examination of instantaneous acceleration levels within individual singing bouts revealed an unexpected pattern (Fig. 2.2-4A).

While the simplest pattern of singing deceleration would be a monotonic slowing of successive motifs, it was more common that both decreases and increases of singing tempo occurred - thus acceleration was both negative and positive within singing bouts. For all recording sessions, about 1/3 of song motifs were produced with a faster tempo than immediately preceding ones (29 ± 3 %). Further, deceleration and acceleration of singing tempo often appeared oscillatory, such that successive motifs were produced with opposite signs of acceleration (e.g., Fig. 2.2-4A lower panel, dummy female).

As two ways of quantifying the strength of such variable and oscillating accelerations, we measured the standard deviation of acceleration values within song bouts (*acceleration*

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[21] ►The number of motifs per bout was significantly increased when the bird sang in the presence of a female and Area X blockade of D1 receptors had no effect on it (Leblois et al. 2012).

[22] For undirected song, negative and positive acceleration is briefly reported in previous study.
►In 9 of the 10 birds having consistent motifs, the second motif was longer by 5.9 ±4.4 ms (range of 0.04–12.7ms) or 0.82 ±0.49% (range of 0.00–1.49%) than the first motif.
In the five birds with at least three motifs, the third motif was longer than the second (range of 1.2–3.7 ms).
In all five of these birds, the gap between motifs two and three was longer than the gap between motifs one and two (Glaze et al. 2006).

variability) and the fraction of successive motifs that were alternately positive and negative, as above (*acceleration alternation*). Across the group of recording days, acceleration variability within singing bouts[23] was nearly significantly lower for singing to paired than to nonpaired or dummy females (Fig. 2.2-4B, paired vs. nonpaired, dummy p = 0.027, 0.029; median accel. S.D. paired / nonpaired = 0.34, mean accel. S.D. paired / dummy = 0.61).

While a similar comparison of acceleration alternation level across recording days did not reveal clear differences between paired and other female classes (Fig. 2.2-4C; paired vs. nonpaired, dummy p = 0.16, 0.57 sign-rank test; median ratio paired vs. nonpaired, dummy = 0.76, 0.58), such alternating accelerations were less common when singing to paired females. While among all singing bouts, 53% of successive acceleration values crossed the zero level (alternated from acceleration to deceleration or vice versa), only 43% crossed when singing to paired females, and over half crossed when singing to other female classes (nonpaired = 56%, dummy=57%).

C. Relationship of singing and neural correlates of directed singing within single recordings.

We next tested whether neural correlates of directed singing were related to singing features within single recording sessions. In each recording session, the ratio of syllable frequency C.V. between singing directed to paired and other female classes was used to compare song variability between them (e.g., syllable frequency C.V. for paired / nonpaired females). Similarly, in each recording session, the ratio of neural cross-rendition C.V. was used to compare neural variability during singing directed to different female classes. Among all recordings, the ratio of syllable frequency C.V. was significantly related to the ratio of cross-rendition C.V. of neural activity between paired and nonpaired singing (Fig. 2.2-5A, p = 0.002, r = 0.79 Pearson's correlation).

During recordings in which singing was more "directed" for paired females (i.e., lower syllable frequency C.V. for paired than nonpaired), neural activity was also more "directed" (i.e., lower cross-rendition neural C.V. for paired than nonpaired). Thus, across the population of recordings, fine control of syllable frequency structure was closely linked to regularity of neural activity. The same comparison for paired vs. dummy singing conditions was similar, but not significant (p = 0.1, r = 0.49). A relationship between the ratio of frequency C.V. with the ratio of mean within-rendition xcorr was not clear for either paired vs. nonpaired or paired vs. dummy comparisons (p = 0.3, 0.58).[24]

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[23] SD comparison was done for all bouts within the day.

[24] Variability in neural activity seems to be shared among whole neurons in Area X or LMAN.

►In both the striatopallidal region AreaX and the AFP output nucleus LMAN (lateral magnocellular nucleus of the anterior nidopallium), the immediate early gene (IEG) egr-1 is

Among global singing features, the strongest trend was a positive one between the ratio of tempo and the ratio of cross-rendition C.V. comparing paired and nonpaired singing (Fig. 2.2-5B, $p = 0.018$, $r = 0.58$).[25] No other comparisons between singing features and neural variability, both cross-rendition C.V. and within-rendition xcorr, were significant.

Discussion

The main result of this study is that within individual recordings the variability of neural activity was closely related to syllable variability, but across all recordings variability of both were not consistently dependent on female pairing status.[26]

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induced in more neurons when males sing alone ("undirected") than when they sing to females ("directed") (Jarvis et al. 1998).

Activation of IEGs is highly sensitive to the pattern of stimulation (Sheng et al. 1993, Worley et al. 1993), and bursts can be more effective at inducing IEGs than tonic activity. During undirected singing, more LMAN neurons may appear to be active because marked bursting drives greater IEG induction (Kao et al. 2008).

[25] This indicates that when males sang with higher tempo to one female class, their neural activity was less "directed"-like, in contrast to the opposite relationship of frequency C.V. to neural C.V. noted above (Fig.2.2-5B). This relationship was not present comparing paired vs. dummy singing ($p = 0.22$).

Song Tempo has a circadian fluctuation reaching the fastest around 2~4hrs after lights on.

►Songs speed up in the first 4 h after lights on and slow down between hours 5and 11. Acoustic activity (singing time per an hour) tends to decline steadily over the day beginning at hour 5 (Glaze et al. 2006).

►There was a peak in brain temperature roughly 2 h after lights-on, followed by a gradual decline throughout the rest of the day (-0.032±0.007 C/h during hours 2–10).
Motif duration showed the opposite pattern, exhibiting a minimum at roughly 2 h after lights on, followed by a slow increase during hours 2–10 (Aronov et al. 2012).

[26] The main result of this study is that within individual recordings (within one male bird, one day, and one neuron) the variability (cross-rendition C.V.) of neural activity was closely related to syllable variability (cross-rendition C.V.), while there was no clear tendency for the collection of these variabilities to depend on female category (pairing status).

Based on previous results that such neural variability directly controls song variability, and that low levels of both are characteristic of courtship as opposed to non-courtship singing, these results suggest that male courtship motivation may vary depending on the target female, and can be evident in singing-related AFP activity.

Within single recordings from individual males, the variability of AFP activity and song syllable structure were consistently related: when activity was less variable to one female class, syllable frequency was also less variable to that female class. Previous studies had shown that both neural and song variability are consistently lower during directed than during undirected singing (Kao et al. 2005, Kao et al. 2006, Leblois et al. 2010, Hessler et al. 1999 Nat Neurosci, Kao et al. 2008, Sakata et al. 2008, Hampton et al. 2009, Stepanek et al. 2010).[27]

The current results extend this, by showing that within the behavior of directed singing, there can be distinct levels of neural and song structure variability. Directed songs with relatively low neural and song variability could reflect a male's high level of courtship motivation toward a target female.

The correlation of syllable and AFP neural variability dependent on female identity could be causally related. Based on a variety of approaches, the level of neural variability in the AFP appears closely related to the strength of dopaminergic input, including that from VTA (Sasaki et al. 2006, Yanagihara et al. 2006, Hara et al. 2007, Huang et al. 2008, Leblois et al. 2010). Thus, the levels of VTA activity and dopamine in Area X could signal the relative preference of a male for individual female, and subtly modulate the level of syllable frequency variability (Leblois et al. 2012).

Although there was no overall trend for males to court paired or nonpaired females with higher motivational level (as defined by low neural and singing variability), among recordings such motivation appeared higher for sometimes for paired and sometimes nonpaired for females. Such labile courtship preferences may reflect individual males' personal history and current motivational state. Related to this, here and in most other

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[27] ►For LMAN, demonstrated that LMAN inactivation (muscimol, GABA-A receptor agonist) deprived only frequency variability during undirected, but didn't affect on tempo (Stepanek et al. 2010).

►For AreaX, Leblois demonstrated that Dopamine-1 type receptor antagonist deprived only frequency variability during directed, but didn't affect on tempo (Leblois et al. 2012).
►For tempo modulation, temperature change specific to HVC (but not RA) affect on song elasticity, so HVC is likely to play a key role (Long et al. 2008).

studies examining physiological correlates of pair bonding, individuals were classified either as paired or nonpaired.

However, it is likely that operationally paired males and females could have been relatively satisfied or unsatisfied with their randomly selected partners, which may influence their relative motivation for courtship with paired or nonpaired individuals. Female zebra finches who form a pair with a relatively preferred or nonpreferred male will reject and accept extra-pair courtship, respectively, even though they have similar number of offspring with either male type (Houtman. 1992). Further work to rigorously quantify pair-bond strength after extended separation will be required to address this question.[28]

In sum, this study indicates that directed singing is not a uniform behavior, but can vary in intensity. This variation could reflect both preferences of a singing male, and the relative preference of the target female for the proposing male.

[28] ►In zebra finch, extra-pair courtship have been observed in the wild (approx. 0.5 courtships an hour per male; Birkhead et al. 1988)
and in captivity (0.46 courtships an hour per male; Burley et al. 1994)
and which led some successful extra-pair copulation (2.4% in the wild and 3.3% in captvity).
Extra-pair paternity have been reported from the wild (2.4% of 82 offspring; Birkhead et al. 1990) and captivity (28% of 278 aviary-bred offspring; Burley et al. 1996).

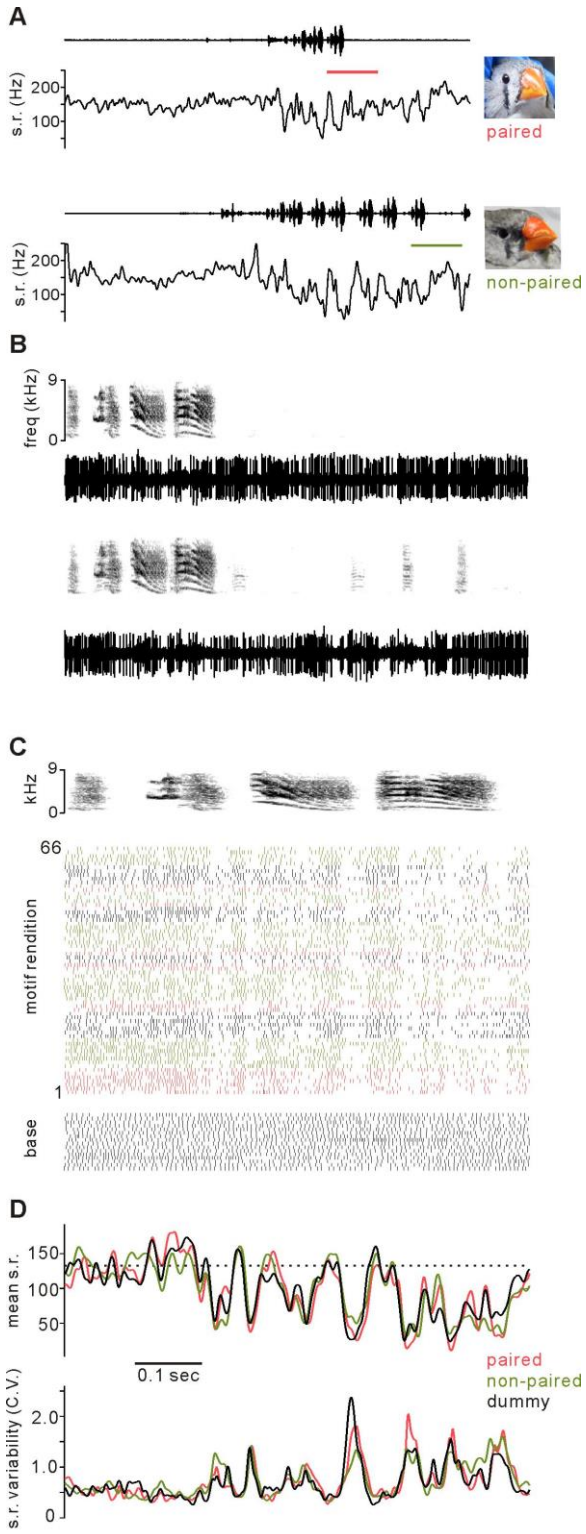

Figure legends

Figure 2.2-1. Example unit activity before and during singing directed to paired, nonpaired, and dummy females.

A, Concurrently recorded sound oscillogram (upper traces) and instantaneous firing rate of representative Area X unit taken from epochs including non-singing and singing to either a paired female (upper panels) or a nonpaired female (lower panels).

B, Expanded views of sound (upper panels, spectrogram) and neural activity (lower panels, activity waveform) in periods of panel **A** indicated by orange (paired) and green (nonpaired) color bars, which contain similar singing and non-singing periods for both females.

C, Activity of unit during multiple renditions of the characteristic song motif directed to different female classes. Upper spectrogram is a representative song motif. For each successive motif rendition, unit firing time is indicated by vertical tick. Raster of activity during equal duration epochs when male did not sing is indicated by base.

D, Average firing rate for each msec of analysis epoch (upper) and variability of firing rate across all renditions to each female class (C.V. = S.D. / mean).

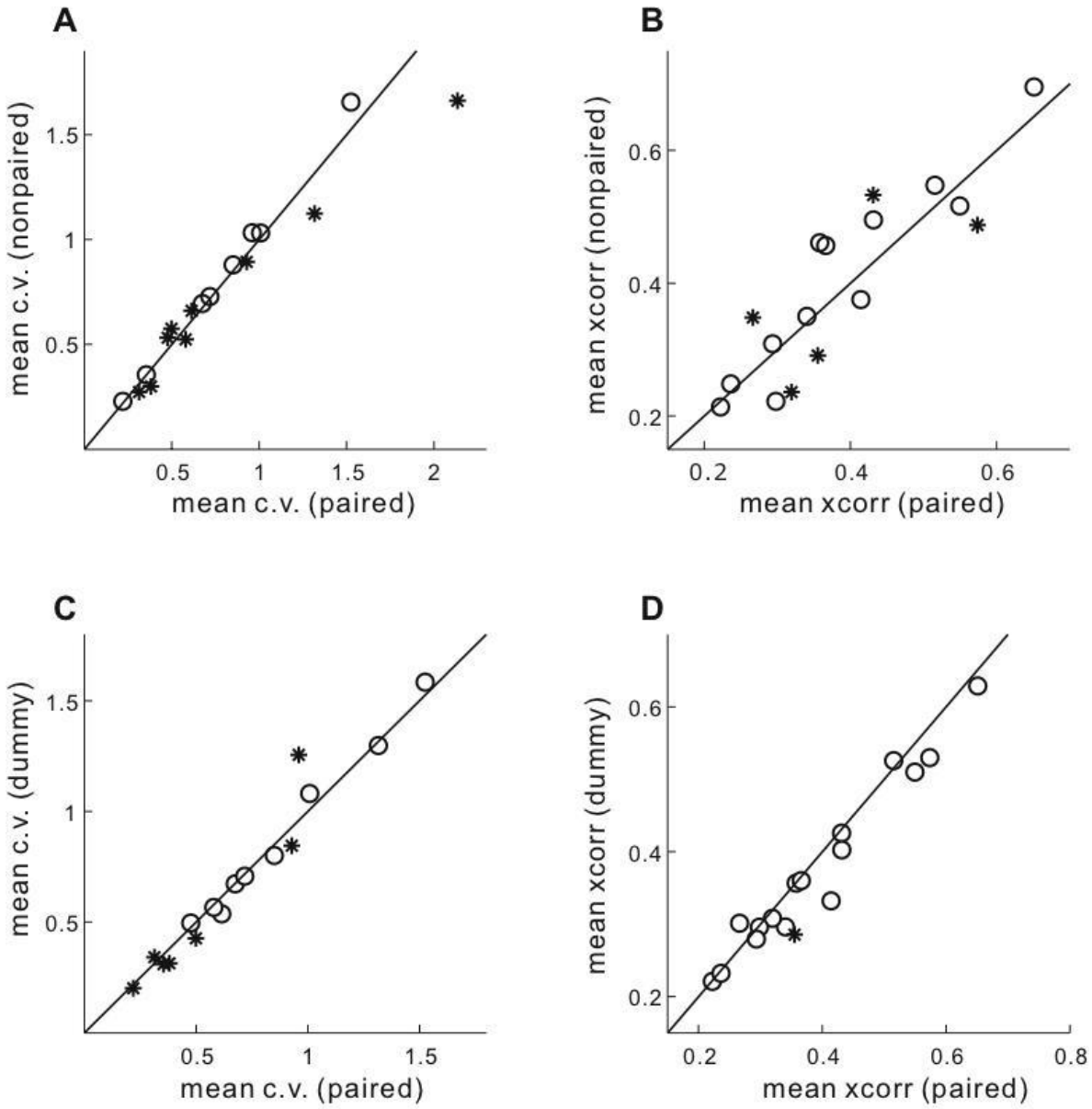
A
mean c.v. (nonpaired)
mean c.v. (paired)
0.5
1
1.5
2
B
mean xcorr (nonpaired)
mean xcorr (paired)
0.2
0.4
0.6
C
mean c.v. (dummy)
mean c.v. (paired)
D
mean xcorr (dummy)
mean xcorr (paired)
0.8

Figure 2.2-2. Variability of unit activity during song motifs directed to different female classes. ;

A - B, Comparison between paired and nonpaired females. Mean cross-rendition variability (C.V., left panel) and mean within-rendition variability (xcorr, right panel). Across the population of recordings, variability during motifs directed to paired and nonpaired females was similar. Individual recording sessions in which mean C.V. or mean xcorr were significantly different indicated by *.

C - D, Comparison between paired and dummy females, as in panels **A - B**.

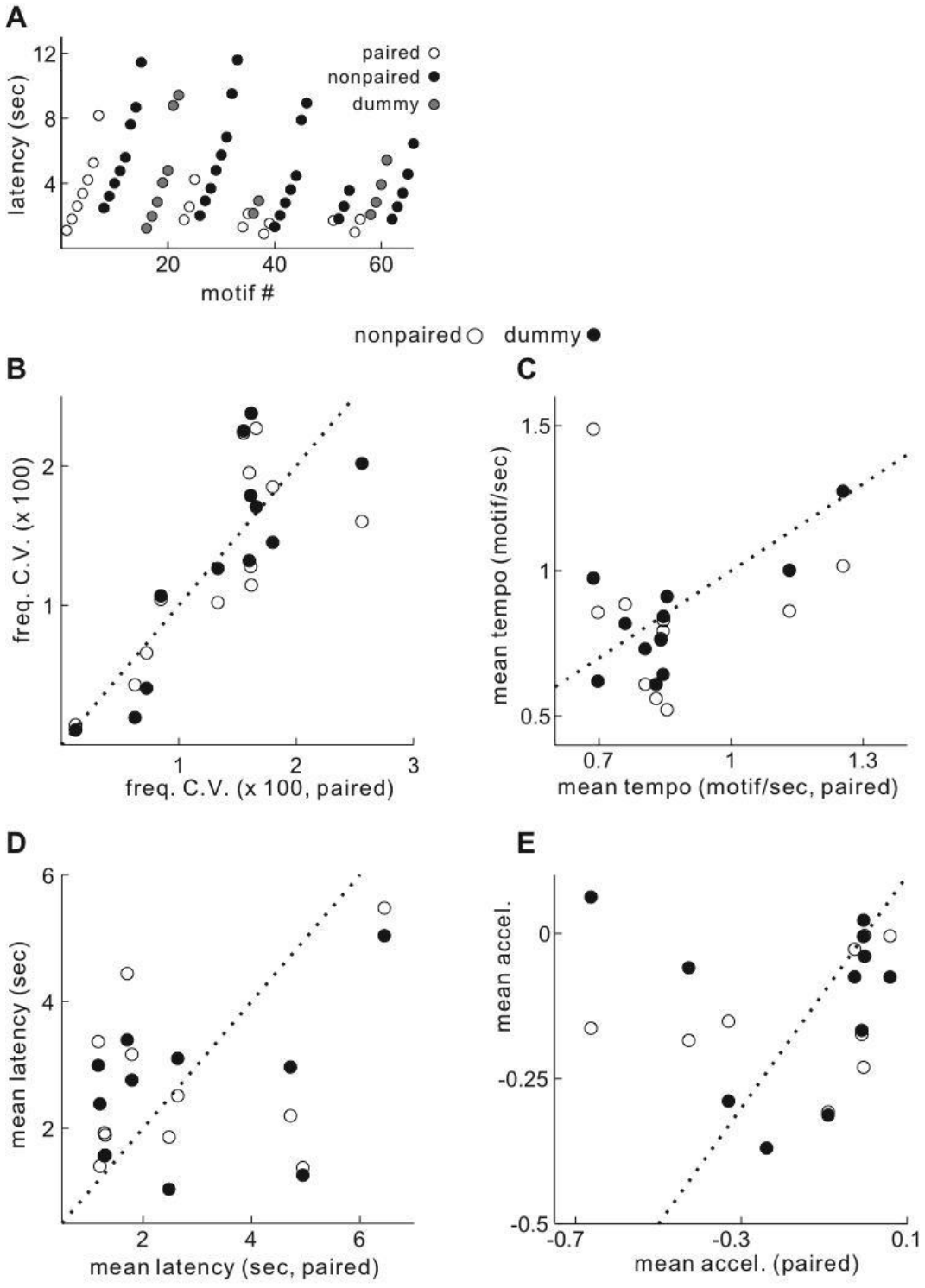
A
latency (sec)
motif #
paired
nonpaired
dummy
nonpaired
dummy
B
freq. C.V. (x 100)
freq. C.V. (x 100, paired)
C
mean tempo (motif/sec)
mean tempo (motif/sec, paired)
D
mean latency (sec)
mean latency (sec, paired)
E
mean accel.
mean accel. (paired)

Figure 2.2-3. Spectral and global features of singing directed to different female classes.

A, Timing of song motif production in a representative recording session used for example Figure 2. Latency of each motif initiation during song bouts directed to paired (open circles), nonpaired (black circles), and dummy females (gray circles).

B-E, Mean of song features within each recording session for paired female plotted versus both nonpaired (open circles) and dummy (black) females. For each panel, one point summarizes results from singing of one bird in one recording day.

B, Mean variability of syllable fundamental frequency.

C, Mean tempo of motif production over all singing bouts.

D, Mean latency of first motif produced.

E, Mean acceleration of motif production.

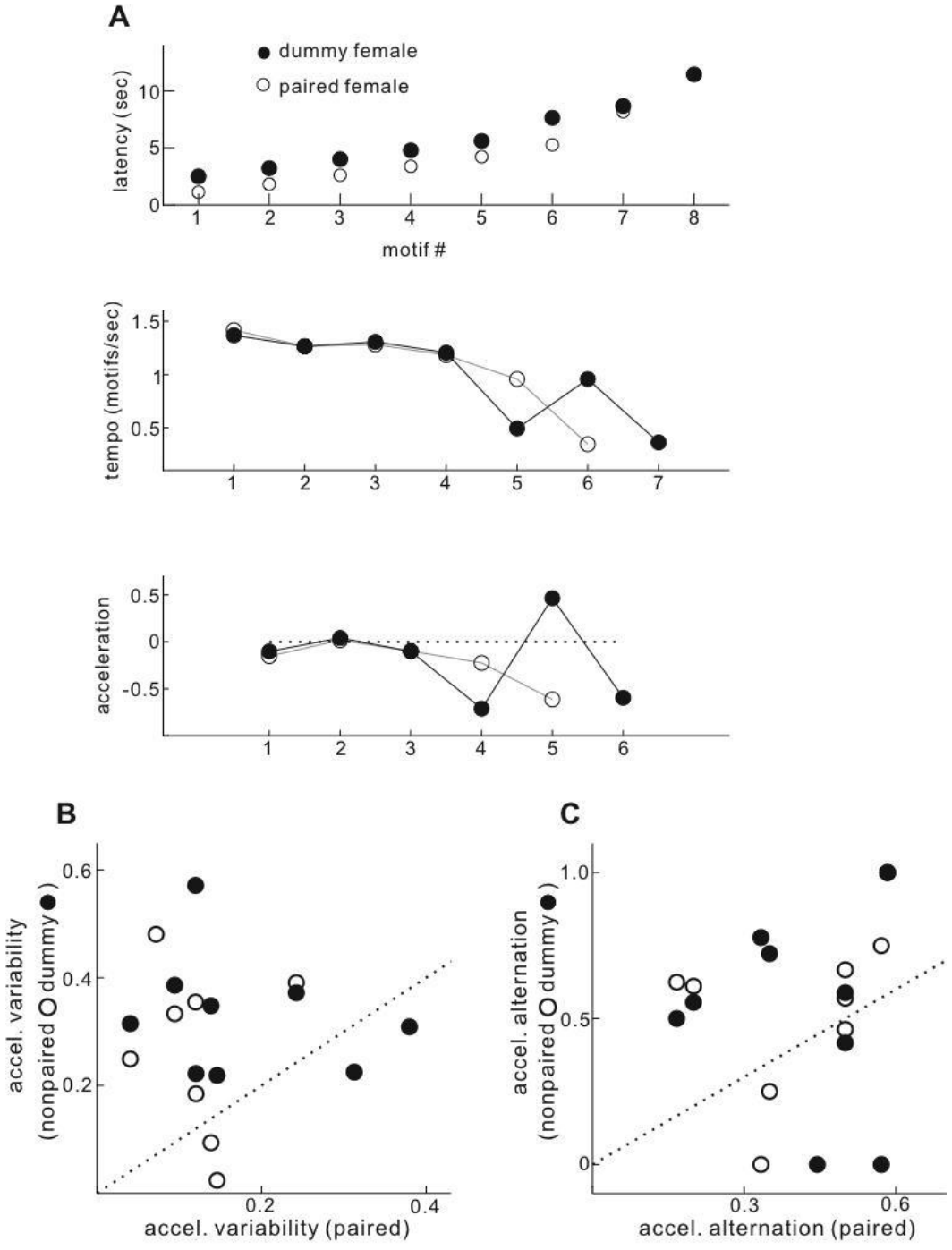
A
dummy female
paired female
latency (sec)
motif #
tempo (motifs/sec)
acceleration
B
accel. variability (nonpaired ○ dummy ●)
accel. variability (paired)
C
accel. alternation (nonpaired ○ dummy ●)
accel. alternation (paired)

Figure 2.2-4. Variability of acceleration in successive song motifs is lower during singing to paired females.

A, Upper, Latency of motif initiations (as in Fig.2.2-3A) during single bouts of singing to paired and dummy females. Middle, Instantaneous tempo of motif production (1/(motif onset latency$_{x+1}$-motif onset latency$_x$). Lower, Instantaneous acceleration of motif production (tempo $_{x+1}$-tempo$_x$).

B, Mean variability (S.D.) of motif acceleration within singing bouts.

C, Mean level of acceleration alternation within singing bouts (fraction of successive acceleration levels that change sign: negative to positive, or reverse).

For panels **B** and **C**, one point summarizes results from singing of one bird in one recording day. Quantification of acceleration features was not possible in 2 recordings for nonpaired and dummy conditions, due to the sparsity of sustained singing.

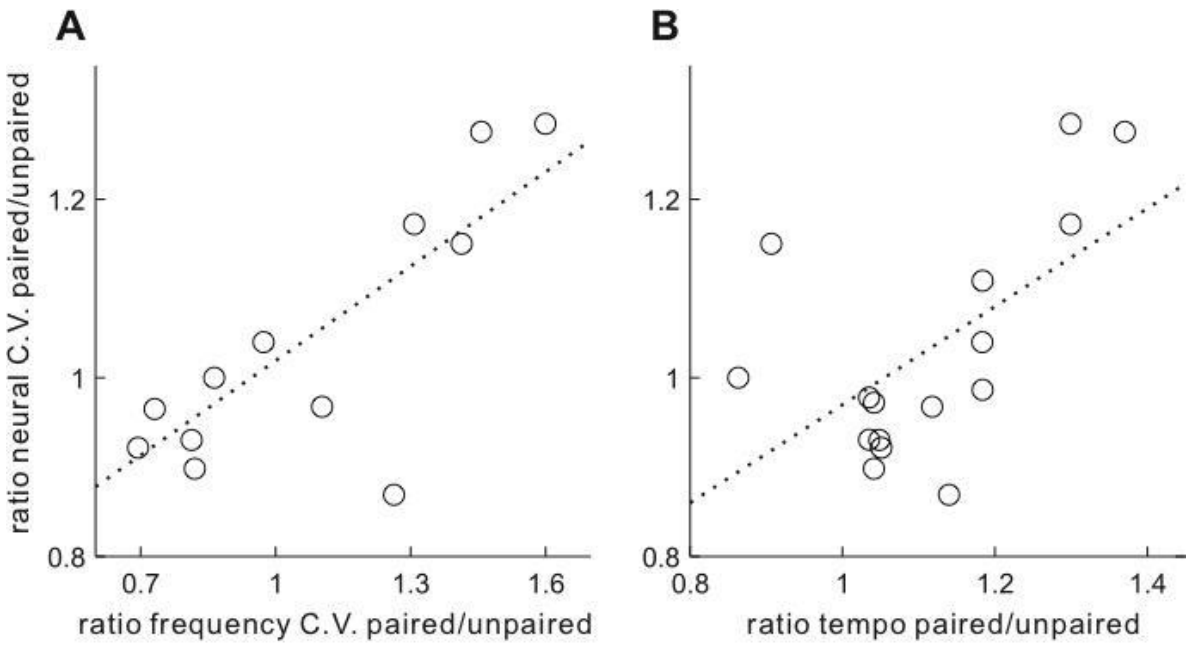

Figure 2.2-5. Singing features related to variability of neural activity across all recordings.

A, The ratio of fundamental frequency C.V. during singing to paired relative to nonpaired females is plotted versus the ratio of mean cross-rendition C.V. of neural activity level during paired relative to nonpaired females. Values of ratio frequency C.V. greater than 1 indicate recordings in which syllable frequencies were more variable during singing to paired than nonpaired females, while values of ratio neural C.V. greater than 1 indicate recordings in which neural activity was more variable during singing to paired than nonpaired females. One recording was excluded due to lack of a constant frequency syllable element.

B, The ratio of mean tempo during singing to paired relative to nonpaired females is plotted versus neural variability ratio as in panel **A**. For both panels, each point indicates singing and neural data from a single recording session. For sessions in which 2 units were recorded, the results from both units were combined.

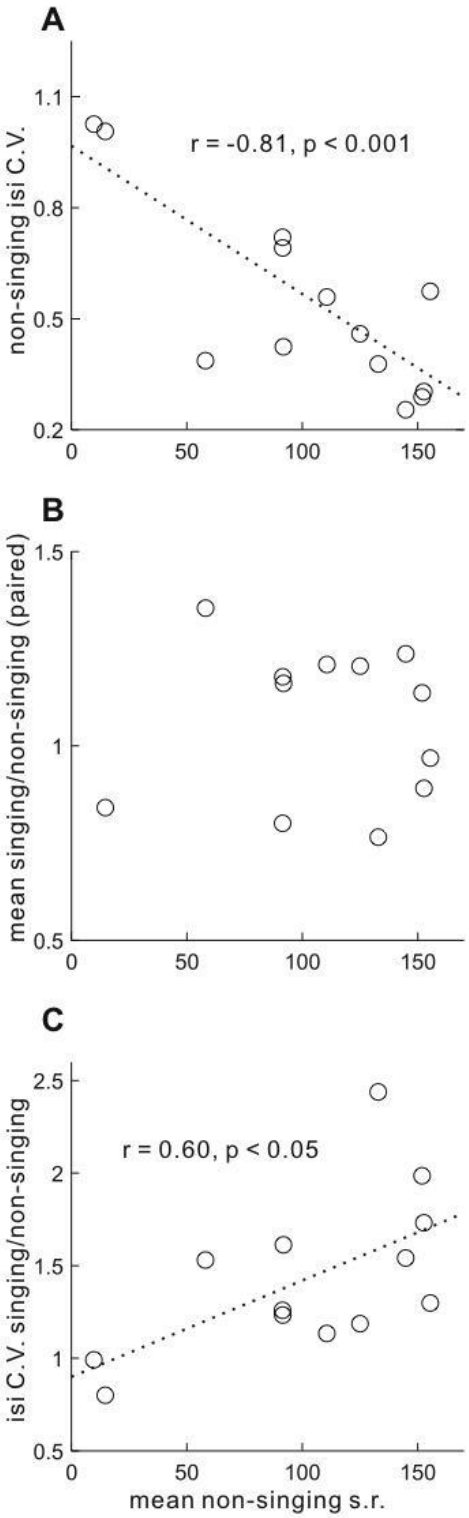

Figure 2.2-6. Summary of Area X unit activity modulation during singing.

A, Mean unit firing rate during quiet non-singing periods (mean non-singing s.r.) is plotted versus variability of instantaneous firing rate in non-singing periods.

B, Modulation of average firing rate during singing compared to non-singing periods.

C, Relative variability of instantaneous spike rate during singing compared to non-singing periods. Data from one putative striatal unit (non-singing s.r. = 9.8 Hz, singing/non-singing mean = 4.3) is not visible at the scale used in panel B.

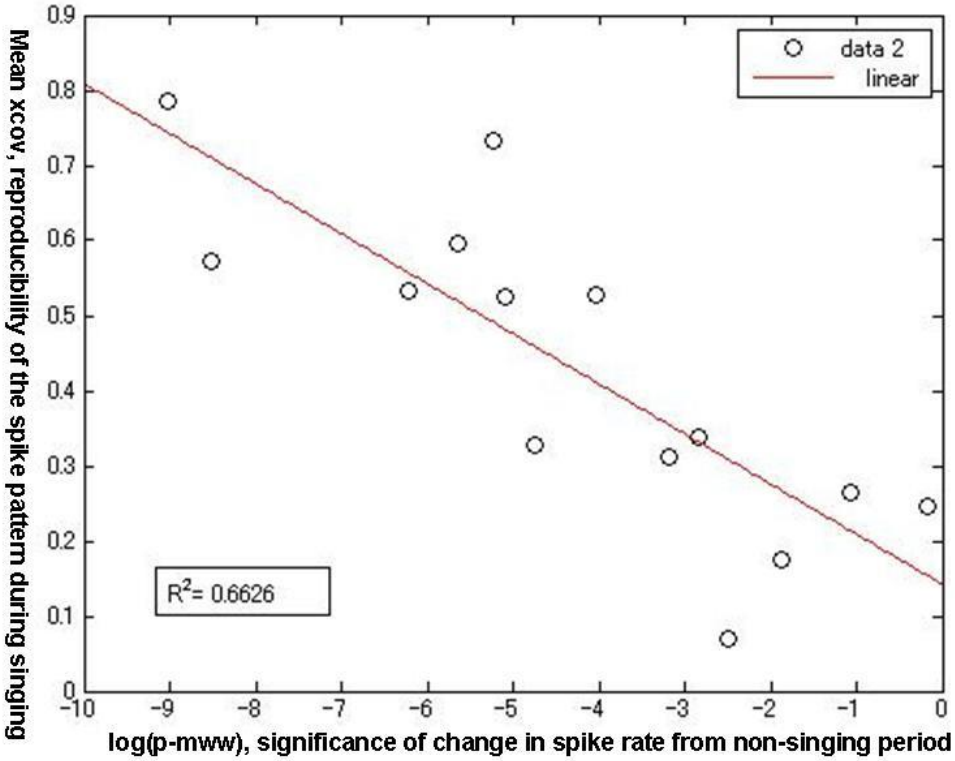

Figure 2.2-7.
For the figure, I used log(p-mww) for x-axis and meanxcov for y-axis. On this plot, neurons located in left, upside are more likely to be involved rhythm control of singing. On the other hand, neurons in right downside are less strictly firing along with singing.

p-mww; p-value of Mann-Whitney *U* test which compared spike rate between base and during singing

meanxcov; For each single singing period, xcov was calculated between the spike pattern during the rendition and the average spike pattern of all renditions. Then, those xcov were averaged within the single neuron.

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Chapter 3:
Sexually dimorphic activation of dopaminergic areas depends on affinity during courtship and pair formation

Front Behav Neurosci. 2014 Jun 11;8:210

Summary

For many species, dyadic interaction during courtship and pair bonding engage intense emotional states that control approach or avoidance behavior. Previous studies have shown that one component of a common social brain network (SBN), dopaminergic areas, are highly engaged during male songbird courtship of females. We tested whether the level of activity in dopaminergic systems of both females and males during courtship is related to their level of affiliation. In order to objectively quantify affiliative behaviors, we developed a system for tracking the position of both birds during free interaction sessions. During a third successive daily interaction session, there was a range of levels of affiliation strength among bird pairs, as quantified by several position and movement parameters. Because both positive and negative social interactions were present, we chose to characterize affiliation strength by pair valence. As a potential neural system involved in regulating pair valence, the level of activity of the dopaminergic group A11 (within the central gray)[29] was selectively reduced in females of positive valence pairs. Further, activation of non-dopaminergic neurons in VTA was negatively related to valence, with this relationship strongest in ventral VTA of females. Together, these results suggest that inhibition of fear or avoidance networks may be associated with development of close affiliation, and highlight the importance of negative as well as positive emotional states in the process of courtship, and in development of long-lasting social bonds.

Keywords

social behavior, courtship, video tracking, dopamine

[29] A11 in CG*…It means “dopaminergic neural population called A11 in CG (central gray) in avian, which is homologous to mammal PAG (periaqueductal gray). Cathecolaminergic neural population are classified into group A1~A12 in brainstem reticular formation. A11 is known as dopaminergic, and Goodson says that it is dopaminergic in birds. (Goodson et al. 2009)

Serotonergic ones are group B1~B9.
Mammal PAG is "folded" CG. PAG surrounds aqueduct of the midbrain. That is why it is called "peri-" aqueductal gray. On the other hand, avian CG structure is "opened" PAG. Dorsal PAG corresponds to latreral CG, and ventral PAG is equivalent to medial CG. (Kingsbury et al. 2011)

INTRODUCTION

Successful dyadic interactions with individuals of the opposite sex are critical for reproductive success. In many species, both short and long-term processes of courtship and pair bonding engage intense emotional states which control approach or avoidance behavior. Among the best characterized forms of courtship is that of male songbirds singing to attract a preferred female.[30] Courtship in some species, such as zebra finches, is a dyadic interaction in close proximity. While a male sings, the target female listens and decides whether to continue courtship interaction based on her judgment of the song and other factors. As a final step, the female may produce a copulation solicitation display (CSD), involving a horizontal posture and tail quivering, which the male can view (Morris, 1954).[31] Thus,[32] during the courtship interaction, both individuals make ongoing judgments regarding the other, and produce behavioral signals reflecting these decisions.[33] In addition, it is likely that brain activity reflecting the choice of each individual can be influenced by responses of the other. For example, the relative activation of brain reward networks in a male attracted to a female bird should depend on whether she makes a positive or negative response to his singing.

Courtship behavior of vertebrates is controlled by largely evolutionarily common areas known as the social brain network (SBN: Newman, 1999; Goodson, 2005). As emphasized in a recent review, reward and motivation related dopaminergic areas are an essential component of the SBN (O' Connell and Hofmann, 2011). These areas are involved in motivational processes for affiliative behavior and other natural rewards (Kelley and Berridge, 2002; Stuber et al. 2008). Further, a critical role of dopaminergic systems in courtship and pair formation has been demonstrated in mammals (Aragona et al. 2006) and suggested in birds (Goodson et al. 2009; Alger et al. 2011; Pawlisch et al. 2012; Banerjee et al. 2013; Lyilikci et al. 2014). In songbirds, both dopaminergic and non-dopaminergic neurons in VTA are selectively active when male zebra finches sing to court females but not when they sing in a non-courtship context (Yanagihara and Hessler, 2006; Hara et al. 2007; Huang and Hessler, 2008). Singing of males may be interpreted as a sign of attraction to the female, and the level of VTA modulation is high when males sing to females but lower when males only see the female but do not sing (Yanagihara and Hessler, 2006). Thus, activity in VTA may reflect the degree of motivation of a male

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[30] In the wild and captivity, single male songbird will approach any single female to initiate a sexual encounter by his singing and dancing.

[31] However, even if both male and female are receptive, courtship does not proceed very far.

[32] gradually over the course of days

[33] If each decides to accept the other, they spend more time together, and courtship becomes more frequent, although it does not lead complete copulation until they build a nest. (Zann, 1996)

to mate with a female, based on sensory characteristics of the female and a male's current internal state.

Previous studies in songbirds have focused almost exclusively on males, because of their prominent courtship display. However, as noted above, courtship requires a critical decision by females - whether to accept the singing male's proposal. Further, most studies have used reduced experimental paradigms, in which females and males can't physically interact. For example, males and females are often kept in separate cages during courtship experiences, so that both birds are aware at some level that there was no access to the other (Sasaki et al. 2006; Yanagihara and Hessler, 2006; Hara et al. 2007; Huang and Hessler, 2008). It seems likely that brain networks involved in affiliative decision making may be fully engaged only when normal outcomes of interaction are possible.[34] Therefore, in order to investigate brain activation related to courtship and pairing, we wished to examine behavioral interactions in a more natural setting, so that both birds have the opportunity to complete behavioral approach or withdrawal behaviors. Also, as the majority of previous studies examined brain function in only male birds, we examined both sexes. We tested whether activation of previously characterized areas related to male courtship, as well as other dopaminergic areas associated with the SBN, is characteristic for pairs that have behaviorally defined negative or positive experience related to courtship and pairing.[35]

In order to quantify behavioral features of interaction, we developed a system for automatically tracking the position and movement of both birds.[36] This allowed us to systematically quantify specific measures related to affiliative behavior, such as interbird distances. During a third daily interaction session, there was a range of affiliation level among pairs of birds. As expected, among these randomly selected pairs of birds some displayed mainly positive social behaviors and some mainly negative. Thus, we chose to

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[34] In addition, while courtship is aimed to copulation, not only such sexually intense interaction but also apparently non-sexual interaction is critical to form long term pair bond. The first sign that a bond has formed is when the pair sit in contact (clumping) and allopreen and this criterion chosen in most field studies. Complete copulation tends to occur sometime after this when the pair starts nest-building.

[35] As a general pair bond indicator, Caryl and Silicox and Evans focused on the changes in behavior that occur over the course of pair formation, and found that the difference in clumping between pairing and non-pairing individuals reached significance until day2 to day5. On the other hand, 30% of male and female birds fail to establish bonds within 10 days of contact even when the sex ratio is 50%. (Caryl 1976, Silcox et al. 1982, Zann 1996)

That is why we chose day3 for the sacrifice day because when some birds start shortening their distance, a responsible brain circuit must be cueing this behavioral switch while other birds still remain in taking long distance.

[36] It was observed during a third daily interaction, by which a whirlwind of courtship already calmed down

characterize relative affiliation of pairs by a valence score ranging from -1 to +1.The valence during the third session was strongly predicted by a specific female-male interaction during their initial meeting session: pairs in which aggressive behavior occurred during male courtship singing developed negative valence, while those lacking aggression had positive or neutral valence.

The level of activation of the immediate early gene (IEG) protein c-Fos (product of c-fos) following this final interaction session was quantified in several dopaminergic areas included in the SBN. As a potential neural system involved in distinguishing relative affinity level, the level of c-Fos in putative dopaminergic neurons in A11/PAG was selectively reduced in positive valence pairs for females, but not males. Further, activation of non-dopaminergic neurons in VTA was negatively related to pair valence, with this relationship strongest in ventral compared to dorsal VTA of females. Together, these results suggest that inhibition of fear or avoidance networks may be associated with development of a female's close affiliation with a male. The lack of such reduction in males could indicate a slower development of affiliation, or a slower recognition of ongoing affiliative behavior of the female partner. These results highlight the importance of both positive and negative emotional states in the process of courtship, and in development of long-lasting social bonds.

MATERIALS AND METHODS

(a) Animals and behavior protocol

Adult zebra finches (19 each female and male) bred in our laboratory facility were used in this study. All birds lived in a cage with parents and siblings until 70-100 days old, and thereafter in a communal cage with others of the same sex. Birds were adapted to living in a small cage (27 x 27 x 20 cm) containing two perches inside a sound attenuation box for several days to weeks before experimental observations began. To eliminate disruptive effects of social isolation on dopaminergic systems (Jones et al. 1990; Huang and Hessler, 2008; Feder et al. 2009) birds were housed during this period with a zebra finch of the same sex.

Following this, one female and one male from each cage were placed inside the same cage for 30 minutes, over three successive days. Each day, this interaction began three hours after the light period began, to minimize nonspecific arousal related activation of dopaminergic neurons (Lu et al. 2006; Takahashi et al. 2010), as well as interference with social motivation by strong thirst and hunger upon waking. Both birds were introduced into a neutral cage for interaction sessions, to reduce potential dominance effects caused by cage residence. In one pair, an unfamiliar female was placed in the cage during the third session with a male who had been paired with another female during the two previous sessions. Behavioral and neural results from this third session only were combined with other results. All procedures were reviewed and approved by the RIKEN Animal Experiments Committee (Approval ID: H23-1-223).

(b) Anatomical analysis

One hour after the third interaction session began (both birds remained in the cage for the thirty minutes after interaction session), both birds were quickly anesthetized (Sodium pentobarbitol, IM) and perfused with 0.1M PBS / 0.4% heparin followed by 4% paraformaldehyde to fix brain tissue. Brains were removed from the skull, postfixed for two days, soaked in 0.1M PB / 30% sucrose and embedded in gelatin. Frozen brains were cut in 40μm sections with a cryostat, collected in cryoprotectant, and stored at -30˚ C. The immunohistochemical procedure began with inactivation of endogenous peroxidase by 2 min in 0.3% H_2O_2, 30 minutes in blocking solution (1% tritonX + 5% normal goat serum in PBS), and overnight incubation in rabbit anti-c-Fos antibody (Santa Cruz Biotechnology, Santa Cruz, California, USA) diluted 1:5000 in 0.5% tritonX + 2.5% normal goat serum / PBS. Sections were incubated for one hour in anti-rabbit IgG (H+L) biotinylated goat antibody (Vector Laboratories, Burlingame California, USA) diluted 1:500 in 0.5% tritonX + 10% normal goat serum / PBS, 30 minutes in VECTASTAIN Elite ABC Standard Kit (Vector Laboratories, Burlingame California, USA), and antibody was visualized by incubation in DAB substrate kit for peroxidase (Vector Laboratories, Burlingame California, USA). TH was labeled by overnight incubation in mouse monoclonal anti-rat tyrosine hydroxylase antibody (Acris Antibodies, Herford, Germany) diluted 1:1000 in 0.5% tritonX + 2.5% normal goat serum / PBS, 30 minutes in VECTASTAIN Elite ABC Standard Kit, 13-min reaction of TMB substrate kit for peroxidase (Vector Laboratories, Santa Cruz California, USA), and decoloration of TMB until DAB stain became visible. Sections were rinsed three times in 0.1 M PBS between each step.

The number of neurons containing label for both TH (blue, cytosol) and c-Fos (brown, nucleus), and for TH only were counted by direct observation with a 20x objective (OLYMPUS UPlanFl 20x/0.50, ∞/0.17) in dopaminergic groups (from rostral to caudal) A14, A15, A11 in stratum cellulare internum (SCI), A10 in caudal VTA, and A11 in CG (Fig. 3-3). In VTA, the number of neurons containing label for c-Fos only were counted using a grid to divide the region 300-550 μm from midline into dorsal and ventral subregions.

(c) Video tracking

Paired videos (30 fps, VGA resolution) were recorded by two Logitech web cameras (Logitech International S.A., Switzerland) positioned at perpendicular points outside of the cage (front and side, Fig. 3-1). Videos were processed using Matlab (MathWorks, Natick, Massachusetts, USA) to detect the outline of both birds, and these boundaries were combined mathematically to identify for each video frame the three-dimensional location of the female and male within the cage. This procedure required: 1. determination of the background image of cage apparatus not including birds, 2. subtraction of each frame of video from background to detect birds, 3. identification of female and male birds based on characteristic feather colors, 4. combination of views from both cameras to calculate three-dimensional position of both birds.

As most behavior relevant for this study occurs during perching, an example of the system's performance during a perch event shows a critical advantage allowed by stereo tracking (Fig. 3-1). Positions of both a female (gray) and a male (orange) calculated using both views is distinct from the information extracted from the side-view camera alone. The system was able to accurately identify both birds in almost all video frames (median = 94% of frames)**,** but sometimes could not separately distinguish the female and male when they were in contact with each other (median = 1% of identified frames), or when characteristic features became obscured (remainder of missing frames).

Here, we intended to compare neural activation in pairs who experienced relatively positive or negative courtship interaction. When unfamiliar birds first meet, their interaction likely entails a high level of general arousal. In an attempt to increase the relative importance of specific judgment regarding the other bird, we assessed their behavior during a third successive interaction session, when they have had some experience with each other. Based on previous behavioral studies, we expected that some pairs would at this point have began initial stages of pair bonding, and some would have not (Caryl, 1976; Silcox & Evans, 1982).

Position information was used to generate an estimate of the strength of affiliation for each pair. We chose relatively simple static and dynamic features that included information about inter-bird interactions, and could be reliably calculated (were not sensitive to small changes in measurement windows). Three stationary position measures used were: % of total time both birds spent sitting on the same perch (X2), % of total time both birds spent sitting on opposite perches (X5), and average distance between birds when they both sat on the same perch for longer than 100 video frames (3.33 secs, X3). Two movement related measures used were: the number of times either bird left a perch that both were sitting on for over 100 frames (X1), and the average maximum distance between both birds during these movements from the perch, within 3.33 secs (X4). [37] This measure quantifies how far the actively "withdrawing" bird moves from the other.

37

X1	% time spent jumping between perches (number of times either bird left a perch that both were sitting on for over 100 frames)
X2	% of total time both birds spent sitting on the same perch
X3	average distance between birds when both on same perch
X4	maximum distance between two birds which was made by one jump during jumping between perches. The maximum distance for every 100 frames' of jumping period were then averaged. The movement can be anywhere in the cage, not just between the perches (although that's where most of the movement occurred).
X5	% of total time both birds spent sitting on opposite perches

PC1=-0.0532*x1 + 0.6085*x2 -0.4196*x3 -0.2382*x4 -0.6278*x5
PC2= 0.0230*x1 + 0.7470*x2 + 0.0617*x3 + 0.0568*x4 + 0.6592*x5
Affinity Score= PC1*0.25 + PC2

In order to relate these measures to pair affiliation status, a principal component analysis was performed on them (princomp, Matlab). Based on the two major components, a single scalar score was calculated as PC1*0.25 + PC2. As we expected and observed both positive and negative social interactions that varied between pairs, we normalized this scalar score over the range of -1.0 to +1.0, as a measure of pair valence[38].

Singing periods were detected based on the acoustic power of the video audio channel (Matlab). All singing was confirmed by visual inspection to be of the "directed" type used during courtship, with males oriented toward females while performing stereotyped "dance" movements. Singing bouts, used for examining female and male behavior associated with courtship, were defined as successive songs that had less than three second long pauses between them.

During such bouts of male courtship singing, copulation and aggressive behavior were detected by scanning for video frames where both birds were adjacent or body centers were within 7 cm. Successful copulations were counted as those in which the male remained above the female for longer than 1 second, while both birds remained relatively stationary. Aggressive behaviors during courtship were detected by scanning video for rapid beak pecking of the female or male targeting the other bird. For each such aggressive event, the pecked bird responded by either retreating (fleeing) or not retreating (remaining in the same position). From these data, we obtained for each interaction session the number of aggressive behaviors by either bird toward the other, and the response of the target bird to each aggression.

Comparisons between groups were made with parametric or nonparametric statistical tests based on results of Lilliefors goodness of fit tests for normality.

RESULTS

(a) Video tracking and valence quantification

The positions of female and male birds interacting in the same cage were automatically tracked by a simple video system. By combining orthogonal views (Fig. 3-1, front and side), this system calculated the three-dimensional position of both birds in each frame of a video (female, gray box; male, orange box). The use of such a system was critical for our experiments, as normal bird behavior utilizes three-dimensional space, and thus occasionally a view of one camera could be obscured (Fig. 3-1 lower right).

Results of tracking were used to quantify several measures related to both static and dynamic positions of individuals, as well as behavioral interactions. The distance in space between two individuals is negatively related to their affiliative activity. In the cages we used, one feature relevant to this is the absolute inter-bird distance during epochs when both birds were sitting on the same perch (X3). Because the cage contained two perches,

[38] -1.0(worst pair) to +1.0(best pair)

the relative amount of time both birds spent on the same perch also seemed related to affiliation (X2). Besides these features which provide information of positive interaction within pairs within pairs, several features containing information about negative or neutral behaviors were: time spent sitting on opposite perches (X5), the relative level of jumping between perches (X1=time), and the maximum distance between birds during such perch jumping episodes (X4, see Materials and Methods for details of quantification).

To systematically characterize properties of these features across all pairs without knowledge of their relative importance, we performed a principal component analysis (PCA). With the first two components,[39] that included 89 % of the variance, all pairs were well separated (Fig. 3-2A). From this plot, it is clear that the two position features most strongly distinguishing between pairs are the amount of time spent on the same (X2) and opposite (X5) perches. Further, the distance between birds when on the same perch influences PC1 but not PC2 (Fig. 3-2A,B). The PC1 component thus is positively related to the amount of time birds spent on the same perch, and negatively related to the amount of time on opposite perches, as well as the inter-bird distance while on a perch. This component could discriminate between pairs with varying 10 strengths of affiliation.

In contrast, the PC2 component is mainly related to the amount of time birds spent on any perch, whether together or separately. The significance of this component in potentially discriminating pairs is somewhat subtle. In our system, typical behavior of birds when apparently content (based on posture, and other observations) consisted mainly of sitting on perches. Thus, PC2 may be somewhat independent of birds' ongoing affiliative behaviors, but correlated with an affective state such as low arousal/reactivity or lack of stress. We suspected that this component could influence affiliation status, by distinguishing between pairs based on their potential for successful participation in social interactions[40].

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[39] PC1 and PC2

[40] X2 may be high in pairs with high affinity because both clumping and preening require birds' staying on the same perch.

On PC2 dimension (pc2=0.0230*x1+0.7470*x2+0.0617*x3+0.0568*x4+0.6592*x5), both X2(% time both birds on same perch) and X5(% time both birds on opposite perches) raise PC2 value. Bad pairs have low PC2 value because "% time both birds on same or opposite perches" is low. Because those pairs are bad, one bird might try to take more distance then eventually sat on the floor for long time.

On PC1 dimension (pc1=-0.0532*x1 + 0.6085*x2 -0.4196*x3 -0.2382*x4 -0.6278*x5), good pairs take high value in X2(right side on pc1 axis), intermediate pairs took high value in X5(left side on pc1 axis). And bad pairs take low value both in X2 and X5(middle of pc1 axis, near zero), because X2 has positive sign, and X5 has negative sign, then they cancel each other out. Or, as is shown on the axis PC2, basically in those pairs, two birds hardly stayed on either perch at the same time.

In order to generate a scalar estimate of pair valence (relative affiliation status, ranging from -1 to +1) we wished to combine these two PC values together. When pairs were separated by combining the values of PC1 and PC2 directly (i.e., for each pair the PC1 and PC2 values from Fig. 3-2A were added), the distribution of valence estimates was highly skewed, with almost half of the values from -0.8 to -1. Most pairs within the negative cluster did not display aggression during this session, and several occasionally displayed positive social behavior such as preening. The inaccuracy of this estimate of pair valence appeared to result from the high variability of PC1 values compared to PC2, so that information contained in the PC2 component was obscured. We thus tested whether more equal weighting of the contributions of PC1 and PC2 could yield a better correspondence with affiliation status. Based on the 4-fold higher variation within the PC1 distribution, we combined the values of PC1*0.25 + PC2. The resulting valence estimates did correspond well with independently observed social behaviors - pairs with the lowest valences exhibited aggression, and pairs with the highest valences preening.

As an example of characteristic movement and position features of positive and negative valence pairs, perching behavior is shown in Fig. 3-2C. For the upper positive valence pair, the high density of magenta indicates frequent sitting on the same perch. For the negative valence pair, such perch sharing was rare, with more common opposite perch sitting and high rates of moving to and from perches.

In many songbirds, including zebra finches, one male cue on which females could base their judgment is singing frequency or duration (Houtman, 1992; Collins et al. 1994). However, there was no clear relationship between the total duration of singing on day 1 and valence score on day 3 ($r = -0.33$; $p = 0.18$), and the amount of singing on day 3 was negatively correlated with valence score (Fig. 3-2D, $r = -0.49$, $p = 0.04$), which could reflect a reduced need for active courtship in positive valence pairs. Further, most males sang less on day 3 than day 1, consistent with reduced courtship motivation due to beginning of successful or unsuccessful pair bonding (Fig. 3-2D; paired t-test, $p < 0.001$).

Because male singing is critical as the initial step in courtship, we further quantified pair behavior during singing periods within the first day's interaction session. During a typical courtship sequence, the male sings to a female, and she either accepts by adopting a copulation solicitation display (CSD) posture, or rejects by not. If the male chooses to attempt copulation based on this signal, the female can then continue with copulation or reject him. Surprisingly, there was no clear relationship between the amount of male copulation attempts or successful copulations during singing on day 1, and later valence of pairs. Pairs in which males did or did not attempt copulation during day 1 courtship had similar valence scores on day 3 ($p = 0.08$, t-test; copulation attempts valence = 0.18, no attempt valence = -0.20). Further, the success rate (% of completed copulations)of males that attempted copulation was not related to later valence score ($r = 0.43$, $p = 0.24$).

Thus, we closely examined behavior of both females and males during bouts of male singing, to identify interaction features related to later valence. The clearest distinction between pairs of birds that had relatively high and low valence during day 3 interactions was aggressive behavior during courtship in the first interaction session. During singing

bouts, aggression consisted of beak pecking by either bird, which was responded to with either beak pecking or withdrawal. Pairs that fought during male singing on day 1 had significantly lower valence on day 3 than pairs that did not fight (Fig. 3-2E, $p = 0.025$, t-test; aggressive mean = - 0.2, $n = 10$; nonaggressive mean = 0.24, $n = 8$). Such fighting was specifically related to courtship singing of males, as it occurred about 50 times more frequently during singing than non-singing periods (across all pairs, the rate of fights / second during singing compared to non-singing median = 54, range 23-162, $p < 0.01$, sign test)[41].

Among the pairs that fought during male singing, we examined whether later valence level was associated with particular interaction features. Pairs that developed the lowest valence tended to have frequent retreats by the female when males attacked during courtship (> 50% female retreats, Fig. 3-2D, black). Pairs in which females mainly attacked the male when he sang were associated with somewhat higher valence (< 50% female attacks; Fig. 3-2D, medium gray), while those in which females did not retreat from male attacks were associated with neutral valence levels (> 50% male attack, < 50% female retreat; Fig. 3-2D, light gray).

(b) Anatomical analysis

Following the third interaction session, which was the basis for calculating valence scores of each pair, the level of neural activation was quantified by immunostaining for the IEG[42] c-Fos protein. Based on results of previous studies (Charlier et al. 2005; Bharati and Goodson, 2006), we quantified expression of c-Fos in putative DA neurons (as defined by expression of Tyrosine Hydroxylase) in five dopaminergic cell groups of the diencephalon and midbrain. In VTA, the one area with boundaries that could be reliably distinguished[43], expression of c-Fos in non-DA neurons was also quantified.

The level of c-Fos expression was clearly related to pair valence scores in putative DA neurons of A11 of CG and in non-DA neurons of VTA. For A11 (CG), the level of c-Fos expression in dopaminergic neurons was much lower in females with positive valence (Fig. 3-4A , $r = -0.6861$, $p = 0.041$, $n = 9$). In contrast, expression in males was similar for all valence levels ($p = 0.77$, $n = 9$). In DA neurons of other areas, there was no clear relationship between the level of c-Fos expression and valence scores (Fig. 3-5).

In VTA, the level of c-Fos expression in non-DA neurons was also related to pair valence, and dependent on the sex of birds. As recent studies in mammals indicate that dorsal and ventral regions of VTA can have distinct functions (Lammel et al. 2008; Brischoux et al. 2009), we separately quantified the level of c-Fos expression in these regions. In both

[41] ((fights/second) during singing)/((fights/second) during non-singing) median = 54, range 23-162, $p < .01$, sign test

[42] immediate early gene

[43] It was defined by a grid (300-550um from midline) from dorsal to ventral region

females and males, about double the number of non-DA neurons expressed c-Fos in the dorsal compared to the ventral VTA. In females, the level of expression in ventral VTA was negatively correlated with valence scores (Fig.3-4B; $r = -0.62$, $p = 0.04$), while a similar trend in dorsal VTA was not significant ($r = -0.44$, $p = 0.18$). In males there was a similar tendency of negative relationship of c-Fos expression to valence score in both dorsal and ventral VTA (ventral: $r = -0.68$, $p = 0.045$; dorsal: $r = -0.66$, $p = 0.05$).

DISCUSSION

With a simple video tracking system, we could automatically quantify affiliative behavior of pairs of female and male finches. Across all pairs, there was a range of affiliation, from strong (positive valence) to weak (negative valence). In two dopaminergic areas, the level of neural activation was related to pair valence. In A11 (in CG) of females, but not males, expression of the IEG product c-Fos in putative DA neurons was selectively reduced in females within positive valence pairs, while in VTA there was a negative correlation between pair valence and activation of non-DA neurons, which was stronger in dorsal than ventral regions for females. These results suggest that the intense social interactions of courtship and pair formation involve regulation of negative emotional systems in the brain, rather than only positive ones.

(a) Anatomical results

In both A11 in CG and non-DA neurons in VTA, the level of expression of c-Fos had a specific relationship to the valence of pairs, and was dependent on sex.[44] These two dopaminergic areas adjoin each other in birds, with A11 located within the central gray (CG, overlapping with periaqueductal gray (Appeltants et al. 2000; Kingsbury et. al. 2011; also referred to as A10dc, Zahm et al. 2011). Song system nuclei of males receive input from dopaminergic neurons in both areas, with the striatopallidal Area X only from A10 of VTA (Lewis et al. 1981) and the motor control nuclei HVC and RA from both A11 of CG and A10 of VTA (Appeltants et al. 2000, 2002). It is likely that more general interaction of these areas with motivational and affective systems plays an important role in both sexes. In birds, dopaminergic neurons in both A11 of CG and A10 of VTA receive input from the medial preoptic nucleus of the hypothalamus (mPOA), which regulates sexual motivation (Berk and Butler, 1981; Balthazart and Absil, 1997; Riters and Alger, 2004). Beyond these specific afferent and efferent connections, quite extensive projections of VTA to avian forebrain areas have been demonstrated (Bottjer, 1993). In mammals, the dopaminergic A11 group (in PAG, homologous to avian CG) has many similar projection targets as A10 of VTA, but some unique ones such as the central

[44] In rats, non-dopaminergic neurons occupy more than 40% of VTA neurons. (Margolis et al. 2006, Swanson 1982). A large population of non-dopaminergic neurons in VTA can be cytochemically identified as GABAergic neurons. (Carr et al. 2000, Margolis et al. 2006, Van et al. 1995). Some evidence also indicates that some VTA neurons release glutamate. (Chuhma et al. 2004)

nucleus of the amygdala and bed nucleus of the stria terminalis (Hasue and Shammah-Lagnado, 2002).[45]

Recent studies highlight some important role of the dopaminergic A11 group in social behavior of birds. These neurons are more strongly activated in males that engaged in either courtship or sexual behavior with females than in non-interacting males (Charlier et al. 2005; Bharati and Goodson, 2006). While this distinction may indicate a specific function in these intense social behaviors, it could also reflect a higher arousal of courting compared to non-social males. Studies that reported a correlation of singing activity with A11 dopaminergic activation (Lynch et al. 2008; Goodson et al. 2009) may also reflect variability in baseline arousal level of males. Further, the level of c-Fos expression in a previous study (~50%) related to singing or sexual behavior was similar to that of all males in this study, and of females in negative valence pairs. In contrast, females in positive valence pairs had similar levels of expression in A11 of CG neurons as non-courting males of earlier studies[46] (Maney and Ball, 2003; Goodson et al. 2009). Thus, this suppression of A11 of CG function could reflect a reduction of females' arousal related to acceptance of a male's presence[47].

Both dopaminergic and non-dopaminergic neurons in VTA have been shown by a variety of methods to be involved in songbird courtship (Yanagihara and Hessler, 2006; Hara et al. 2007; Huang and Hessler, 2008; Lynch et al. 2008; Goodson et al. 2009). During courtship singing compared to non-courtship singing, the firing rate of presumed DA neurons was higher, while that of presumed non-DA neurons could either increase or decrease (Yanagihara and Hessler, 2006). Further, the strength of synapses onto DA but not non-DA neurons in VTA was increased following courtship singing but not singing while alone (Huang and Hessler, 2008). In mammals, a similar activation of DA neurons is associated with artificial and naturally occurring rewards such as drugs and food (Stuber et al. 2008). In studies such as this one examining IEG expression in VTA, non-DA neurons are typically more strongly activated during courtship interactions than DA neurons (Hara et al. 2007; Lynch et al. 2008; Goodson et al. 2009) [48]. However, this

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[45] A11 in PAG projects to either dopaminergic, GABAergic, or other type of neurons in VTA (Omelchenko et al. 2010) and GABAergic neurons in VTA project to PAG (Kirouac et al. 2010)..

[46] about 20%

[47] In our study, c-Fos&TH double-positive ratio in A11 CG was more **universal across males** (around 50%), and this tendency was also seen in the study of Sunayana et al (around 40%, after 2-4 days of living together).
As for females, double-positive ratio in A11 CG in Sunayana's study took wider range (around 10-30%) which resulted in no difference from the same sex interaction. In our study also, female c-Fos level in A11 took wider range than that of male, and which had negative correlation with PCA score (pair affinity level) (Sunayana et al. 2013).

[48] single unit recording (Yanagihara et al. 2006), slice recording (Huang et al. 2008),

distinction may be due to a relatively low level of IEG expression in DA neurons. In parallel with these studies characterizing VTA activity, a variety of experiments have demonstrated that directed courtship singing is associated with increased dopamine release in a major target of VTA, Area X (Sasaki, et al., 2006; Leblois, et al., 2010) [49]. Based on these previous studies, the activity level of both non-DA and DA neurons in VTA appear similarly dependent on intense social behaviors like courtship. While this may appear contradictory, given the inhibition of DA by non-DA neurons, a consideration of phasic activation patterns of both could suggest a mechanism. Recent studies suggest that phasic burst firing of DA neurons is critically dependent on phasic

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immunohistochemistry targeted to immediate early gene (IEG) product (Goodsonet al. 2009, Lynch et al. 2008), and in situ hybridization against mRNA of IEG (Hara et al 2007).

[49] Our TH/c-Fos double positive A10 neurons' % range in cVTA (around 0-25%, median=around 6%) is close to previous study; Around 0-10%, median=around3%, positive correlation with male courtship behavior (Goodson et al. 2009). Up to 5% of TH-ir neurons in the VTA expressed c-Fos after copulation. (Bharati et al. 2006, Balfour et al. 2004). These may reflect a low level of activity-dependent c-Fos expression in these neurons.

On the other hand, Sunayana et al. found that 2-4days whole-day hetero-sex interaction (which resulted in the increase of time spent in the same nest box) led c-Fos in 12% of DAergic neurons in VTA of both male and female zebra finches while same-sex interaction induced less than 5%. So, probably, c-Fos induction triggered by electrophisiologically measurable DAergic activation in VTA could be enhanced later than the period we investigated. (Sunayana et al. 2013)

As for non-dopaminergic neurons (non-DA), c-Fos positive non-DA in dorsal cVTA was about double of that in ventral cVTA. Judging from previous study, majority of non-DA in VTA are GABAergic interneurons, but it is controversial how GABAergic interneuron affects on dopaminergic neurons in VTA.

For example, single unit recording study reported that while putative DAergic neuron in cVTA was bursting during male's singing female-directed song, putative GABAergic neuron was silent (Yanagihara et al. 2006) which agrees with "GABAergic disinhibition onto DAergic burst" hypothesis (Lobb et al. 2010)

However, study using in situ hybridization against IEG egr1 revealed that number of egr1-positive(activated) non-DA in cVTA was positively correlated to the number of male's female-directed singing bouts (Hara et al. 2007)

removal of inhibitory input, including that from local non-DA interneurons (Lobb et al. 2010, 2011).

Based on this model, there was a negative correlation between the level of activation of non-DA and DA neurons in ventral VTA of females and pair valence score.[50] Females with strong neural activation in ventral VTA tended to have low valence scores. Although similar studies have not yet been done in birds, in mammals there are clear distinctions between dopaminergic neurons in ventral and dorsal VTA in anatomical projection targets, physiological properties, and responses to rewarding vs. aversive stimuli (Lammel et al. 2008; Brischoux et al. 2009). While activity of DA neurons in dorsal VTA is associated with positive events, activity of those in ventral VTA was associated with negative events such as electric shocks (Brischoux et al. 2009; Matsumoto and Hikosaka 2009; Lammel et al. 2011). Thus, stronger neural activation in ventral VTA of females in low valence pairs could reflect the negative social experience of interacting with a non-preferred male. The similar relationship of valence to neural activation of dorsal and ventral VTA in males may reflect a reduction of both withdrawal and approach motivation during an early stage of pair formation. While there was no clear relationship of valence to dopaminergic activation in VTA, unlike in previous study examining various social behaviors (Goodson et al. 2009), this may reflect a low level of activity-dependent c-Fos expression in this area.

(b) Behavioral Results

In complex interactions such as courtship, it is likely that full and normal activation of critical neural systems will only occur when both individuals can freely choose how to interact with the other. Here, we attempted to provide such opportunities by allowing females and males free movement within a cage. In pairs that had begun to initiate pair bond formation, as shown by the presence of close physical contact, movement was generally less agitated than in lower valence pairs (e.g., Fig.3-2C). This behavioral characteristic was associated in females, but not males, with reduced activation of A11 of CG neurons, as discussed above. Such a distinction in neural activation between females and males may reflect a higher level of judgment by females, in contrast to indiscriminate mating motivation of males. Further quantification of each individual's relative affiliation status, rather than the average within pairs, would be useful in cases of divergent motivation.

It has been proposed that during courtship, both birds must balance opposing tendencies to approach and flee from the other bird (Hinde, 1953). The negative affective tendency may reflect some general fear of close physical contact, as this often occurs during negative experiences such as aggression or predation (Porges, 2003). Thus, development of behavioral increase in affinity may be associated with or require suppression of

[50] It is new finding that number of active non-DA in dorsal cVTA is twice as big as that in ventral.

negative emotional affect and responsiveness, rather than only an increase of positive affect.[51]

[51] While a controlled laboratory environment is necessarily restricted compared to courtship in a natural setting, it seems likely that the ability of both birds to physically interact during courtship should be necessary for full activation of motivation dependent processes, as only during such interaction can the full process of decision making and judgment based on affective processes occur. Consistent with this, recent behavioral studies have begun focusing on similar arrangements, comparing female and male courtship interaction and judgements in "no-choice" chambers (like that used here) rather than those in "two-way" chambers in which females can choose to approach a male, but not have access (Rustein et al. 2007)

In addition, typical "choice chamber" study make female to choose preferable male from two males. However, inter-individual attachment develops through physically communicative period, not through a moment of female choice between two males. We discriminated general features of couple birds' interaction, which would predict a degree of female acceptance level toward male more accurately.

Svec et al also made PCA score to characterize zebra finches' pair affinity level, based on the manual observation. In their study, strong components of PC1 were "female approaching male", "frequency and duration of female-initiated clumping or in proximity of male", and "female preening female.

Their PC1 significantly discriminated the paired female attitudes toward her partner male from her attitudes toward new male, that was, she showed more proximity toward her partner male.

On the other hand, their PC2 characterizes more of birds' sexual courtship aspects , by the range of components like "frequency and duration of directed song", "beak wipe", "male approaching female", and "attempted mount". However, their PC2 could not discriminate "partner-specific" behavior of paired female toward her partner male from new male.

That study agrees with our way of birds' pair affinity measurement which is independent from any copulation-oriented acute rituals (Svec et al. 2009).

Here we examined directly how behavioral interactions between female and male during the male's initial courtship bout influenced eventual pair valence, or level of affiliation. Male courtship in later low valence pairs was often associated with aggression. While such aggression by the female bird toward the male when he sings seems to indicate a strong negative response to his courtship, it was somewhat surprising that males also sometimes attacked the female while they sang. However, in territorial songbirds, singing is used in both a "sexual" context to attract a female bird and an "aggressive" context to repel competitor males. Further, domesticated male zebra finches occasionally sing to other males in an apparently aggressive context (Hessler and Doupe, 1999). In general, previous experimental and observational studies suggest that highly aroused sexual and aggressive behavior may have some common neural mechanisms (Veening et al. 2005).

Such occurrence of aggressive behavior during courtship interactions may indicate that females' judgment of males is based on more than just song features. Specifically related to courtship singing bouts, a male's responses to feedback given by the female can influence her decision to mate or not. Male cowbirds and bowerbirds modulate the intensity of their courtship to avoid startling their target female (Patricelli et al. 2002; O'Loghlen and Rothstein 2012), and male cowbirds can modify their singing output to feature elements to which females respond positively (West and King, 1988).

Recent advances in computational efficiency that allow automated monitoring of animal behavior allow more efficient and comprehensive analysis critical for ethological studies, and especially useful for characterizing interaction of multiple individuals (Dankert et al. 2009; Branson et al. 2009). Further development of our video tracking system will aim to identify specific common behaviors, such as eating and grooming. Of more general utility, this system can be used for tracking other small animals moving in three-dimensional space, such as fish, and its implementation in the commonly available program Matlab allows easy customization by users.

This study, by assaying IEG expression, could quantify the level of neural activation within an entire interaction session. It will also be interesting to test whether neural activity in A11 of CG and VTA (both A10 dopaminergic and non-dopaminergic neurons) is acutely related to ongoing positive or negative interactions. As in previous studies focusing only on males (Yanagihara and Hessler, 2006), this could be done with acute single-unit recordings in semi-restricted birds, though it will be especially useful to record simultaneously from both birds in a setting where they can freely choose whether to interact or not. Clearly, the complex dyadic interaction during courtship involves additional neural systems besides the dopaminergic one examined here. Further studies using similar behavioral tests should also characterize peptidergic systems such as vasopressin/oxytocin that control the development of close social bonds in birds and mammals (Goodson and Thompson, 2010; Insel, 2010; McCall and Singer, 2012).

Acknowledgements

This work was supported by funding from the Brain Science Institute, RIKEN. We thank Yumi Ozawa and Keiko Asai for assistance with animal care and Tsutomu Hashikawa, Takumi Akagi, Ayako Ajima-Tanaka and Erina Hara for assistance with anatomical processing.

Figure

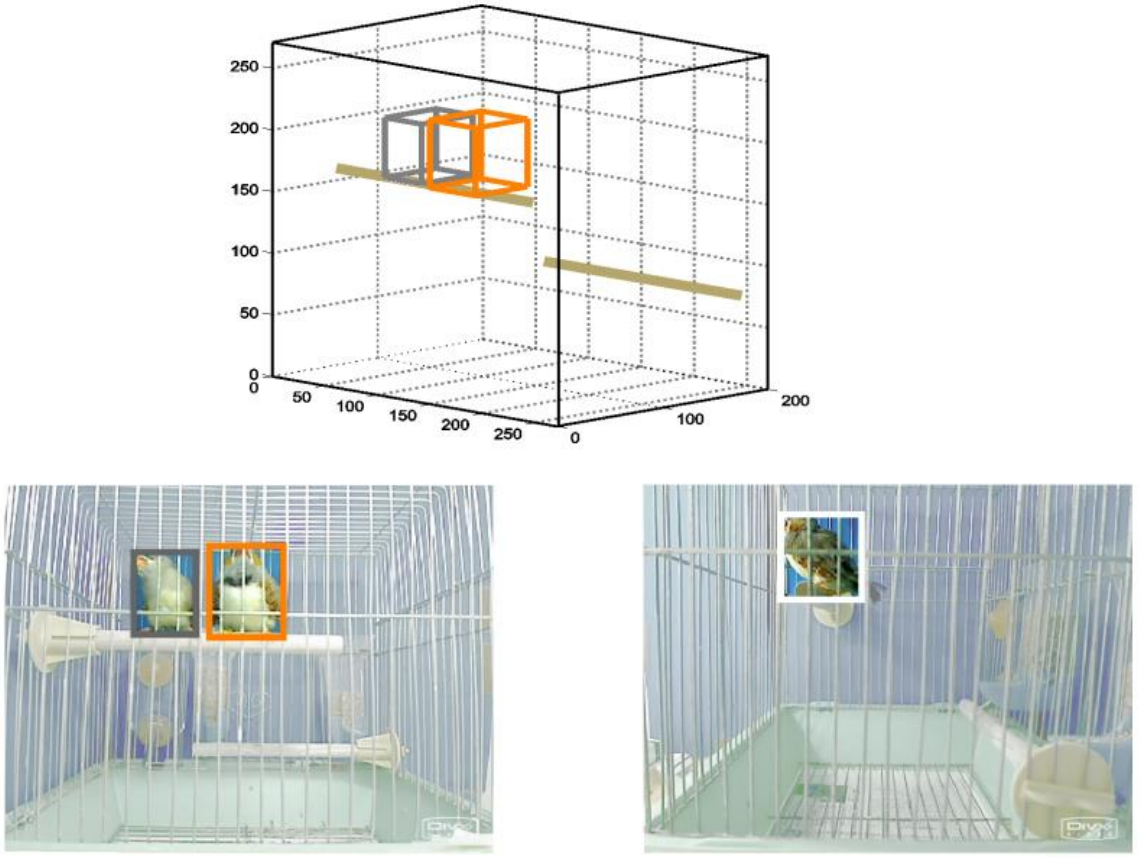
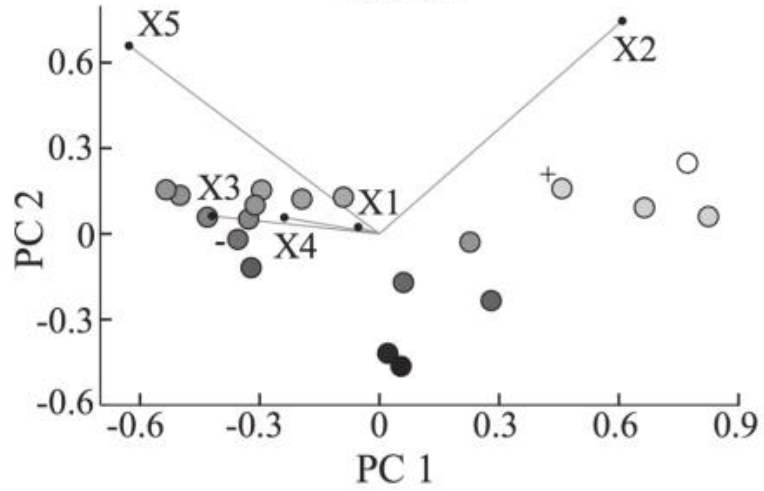


Figure 3-1. Schematic of system for automatic tracking of individual birds during interaction sessions.

Lower left and right panels are images obtained by two webcams positioned outside the front and side of a cage containing a female and a male zebra finch. Software could identify and discriminate between female and male birds (outlined in gray and orange respectively) based on color differences. In the side view, detection of female bird is prevented by the obscuring male. Positions detected by both cameras were combined to obtain three-dimensional coordinates of both birds in the cage (upper panel, axis labels indicate dimensions in mm).

A

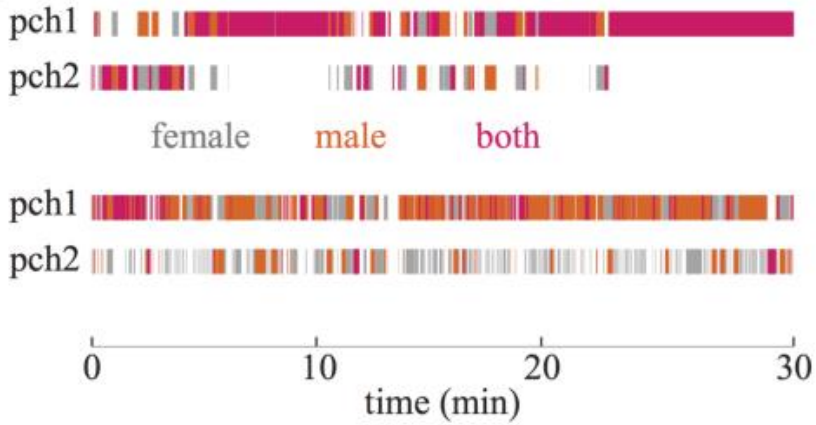
Figure 2.TIFF



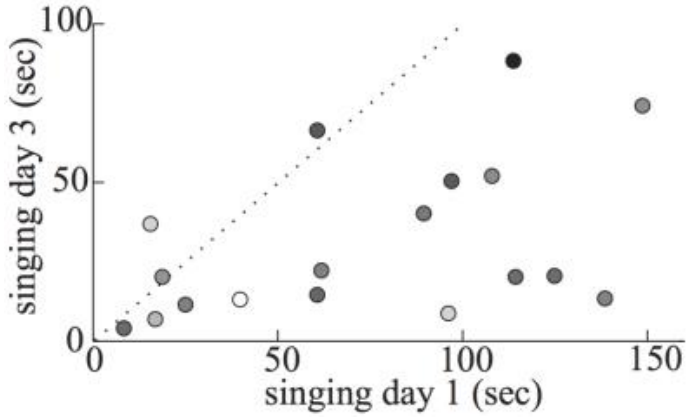
B

	X1	X2	X3	X4	X5
PC1	-0.05	0.61	-0.42	-0.24	-0.63
PC2	0.02	0.75	0.06	0.06	0.66

C



D



E

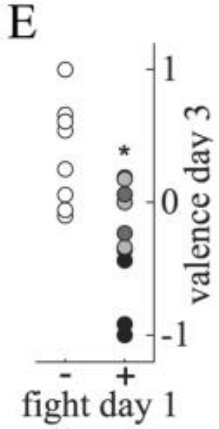


Figure 3-2. Information from tracking system used to obtain pair valence, and relation of courtship behaviors to valence.

A. A Principal Component Analysis (PCA) was performed on five behavioral features of interactions of female and male birds. Results from the first two principal components, which accounted for 88 % of the variance, are plotted for each pair. Mappings of each behavioral factor are overlaid projected within this plane, with the length of the line equal to the PC 1 and PC2 components. The five behavioral features are: X1 - % time spent jumping between perches, X2 - % time both birds on same perch, X3 - average distance between birds when both on same perch, X4 - distance moved when jumping between perches, X5 - % time both birds on opposite perches. Symbols for pairs which had positive and negative valences, illustrated in panel B, are labeled with "+" and "-" respectively. The final calculated valence of each pair is coded with a grayscale map, from black = -1(worst possible pair), to white = +1(best possible pair).

B. Relationship of PC1 and PC2 to the five behavioral features used.
C. Presence of female and male birds on either perch in the cage for each video frame. Video frames when only the female or male was on perch 1 (pch1) or perch 2 (pch2) are indicated with gray and orange respectively. Frames when both female and male were on the same perch are indicated by magenta. Upper and lower perch position plots represent data from day 3 recordings of positive valence (0.38, indicated by '+' to left of symbol in panel A) and negative valence (-0.24, indicated by '-' to left of symbol in panel A) pairs.

D. Total time singing by each male during first and third day interaction sessions. Dotted line indicates equal duration of singing on both days. Most males sang more during first day of interaction with a female. The valence of each male during the day 3 session is indicated by a grayscale map, with -1 to 1 indicated by black to white. The amount of singing in the third session was negatively related to valence.

E. Fighting during male singing bouts in the first session was associated with low valence during the third session. Typical aggression features associated with low, medium, and higher valence indicated by black, medium gray, and light gray were female retreat, female attack, and male attack/female nonretreat respectively. Valence scores were significantly lower during day 3 in pairs that fought in day 1 (*).

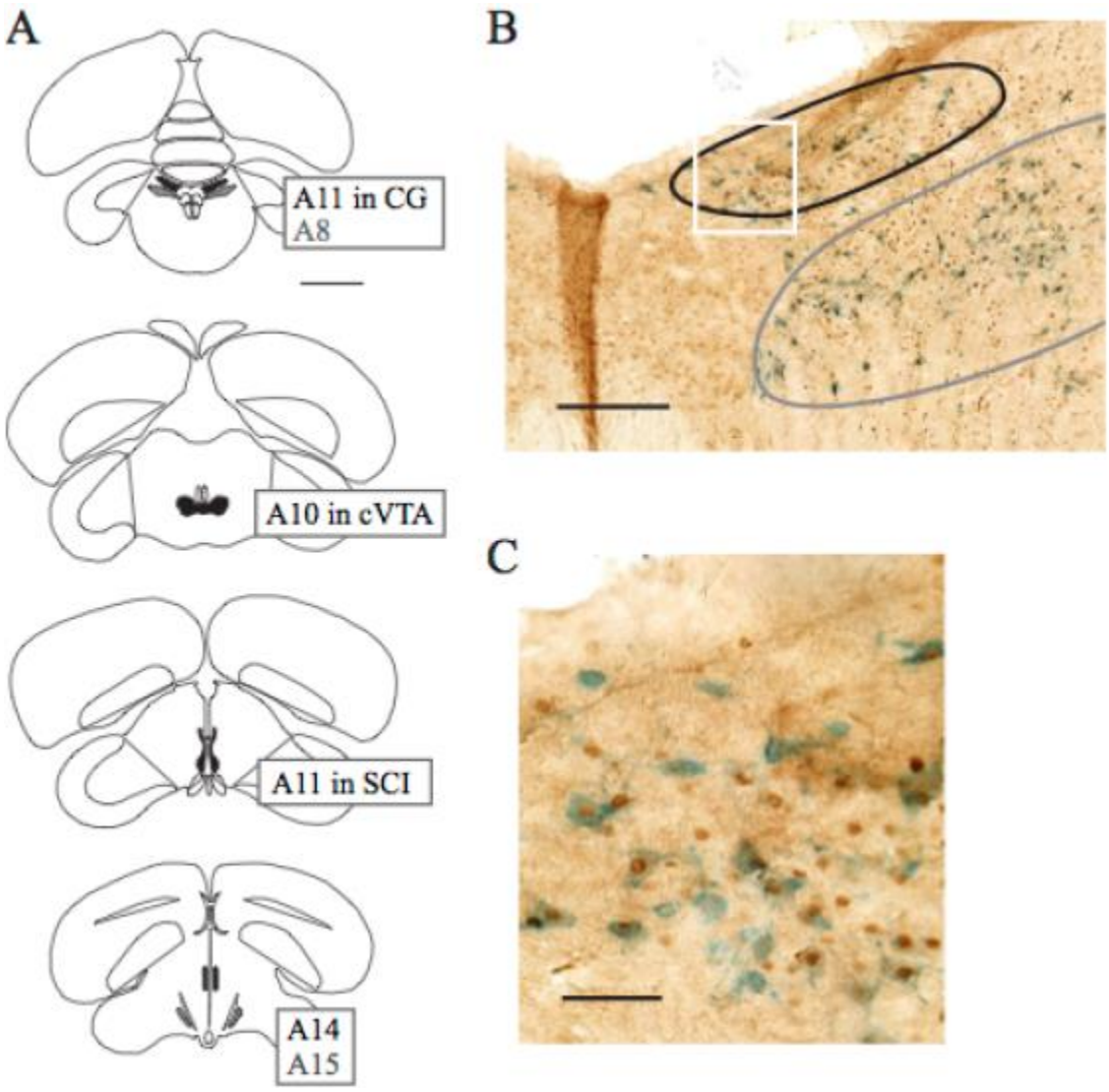


Figure 3-3.
Schematic diagram of dopaminergic areas in which expression of c-Fos was quantified, and example of colocalization.

A. From top (caudal) to bottom (rostral), sections included A11 in CG (black) and A8 (gray), A10 in VTA, A11 in SCI, and A14 (black) and A15 (gray). Expression in A8 was not quantified, but is included for reference to panel B.

B. Image of coronal section containing A11 in CG (black) and A8 (gray) Expression in A8 was not quantified due to difficulty in consistently defining its border.

C. Region outlined in white box in panel B is expanded to illustrate labeling of dopaminergic neurons with tyrosine hydroxylase (blue) and nuclear expression of c-Fos protein (brown). Scale bars in panels A, B, and C indicate 2mm, 250 μm, and 50 μm respectively.

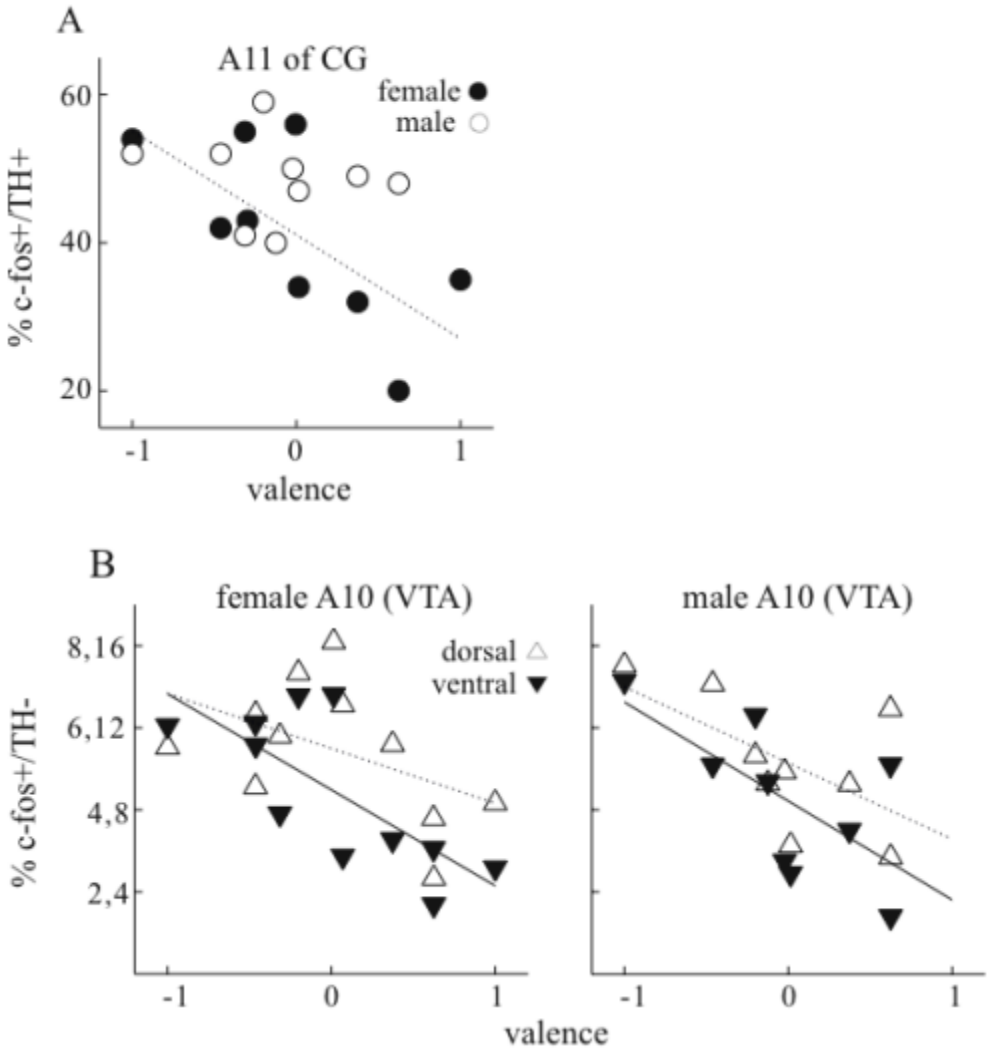


Figure 3-4.
Relationship of pair valence to expression of c-Fos in dopaminergic and non-dopaminergic neurons.

A. In dopaminergic neurons in central gray (CG/PAG), the level of expression of c-Fos was negatively correlated with valence in females (filled symbols) but not males (empty symbols). Dotted lines represent linear fit to female data.

B. In non-dopaminergic neurons in VTA, for females there was a stronger negative relationship of valence to c-Fos expression in ventral than dorsal VTA, and for males this negative relationship was similar in both regions. Because the level of expression in dorsal VTA was about double that in ventral VTA, the scale of y-axis indicates separate percentages for both ventral (lower values) and dorsal (higher values) regions.

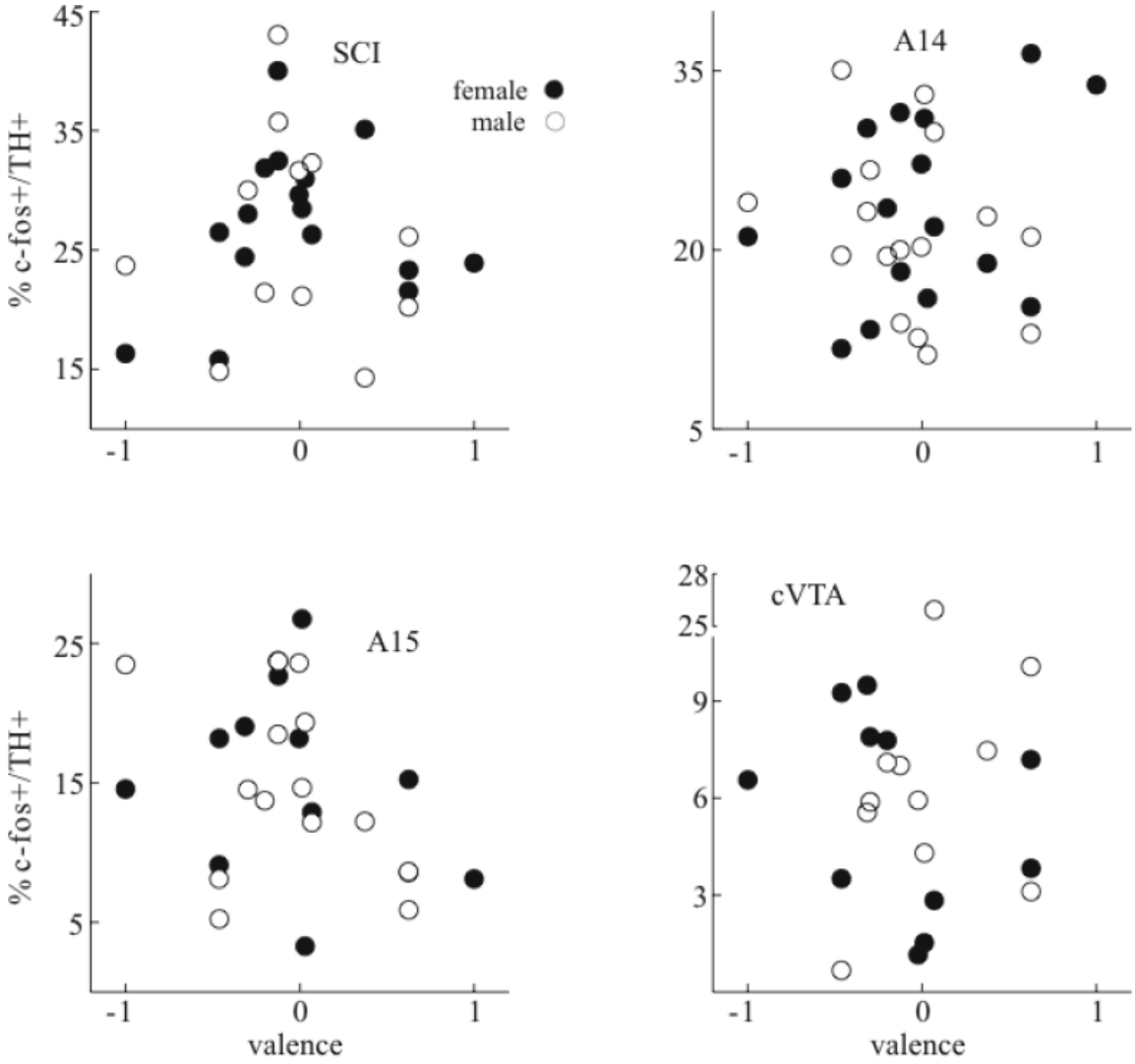


Figure 3-5.
Lack of relationship between pair valence and c-Fos expression in additional dopaminergic areas.

In A11 of SCI, A14, A15, and A10 of caudal VTA (cVTA), the percentage of dopaminergic neurons expressing c-Fos was not significantly related to valence of females (filled symbols) or males (empty symbols).

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Chapter4: General discussion and Conclusion

Male-female interaction of zebra finch accompanies the midbrain DAergic activation before they form life-long pair bond (Huang et al. 2008). Because DAergic neurons in the midbrain are known to differentially reinforce or inhibit diverse voluntary behaviors (Yin et al. 2006), the likelihood of successful sexual pair bond formation may be reflected in the DAergic activity, which may in turn, result in the adjustment of their motor output (=behavior) for the ongoing social interaction. In this thesis, I studied two topics related to this;

The first study elucidated that, in songbirds, male vocal motor neurons are controlled in real time possibly depending on the male's motivation to communicate with a female counterpart. This songbird model sets up how animals adjust their motor neural activity every moment, reacting to their peers. Because vocalization is the motor output made by muscles which is clearly intended to communicate with other individuals, monitoring vocalization gives useful indication of how animals voluntary control inter-individual interactions. Ultimately, each single behavioral interaction composes the animal collective actions, or further, their social dynamics.

The second study demonstrated that, long-lasting social distance which is measured by physical distance between two animals might be controlled by "fear" networks. So far, the dynamic change of inter-individual distance within an animal (or human) group has been tracked by ethologists (Partridge et al. 1981) or information technology engineers (Yano et al. 2012). And "fear" has been studied by neuroscientists, using foot shock (Johansen et al. 2010), recorded predator's calling (Mongeau et al. 2003), and others. Apparently, conspecific animal's approach is nothing to do with fear, however, keeping a distance from specific individuals despite of their ardent approach, seems to be driven by strong inclination of avoidance. We combined quantitative tracking of two individuals and monitoring their neurons which belong to fear networks, and then revealed the possibility that inter-individual distance might be shortened by inhibition of fear networks.

For the second study, I will further discuss the topic which we could not cover in the journal paper.

4.1 Interpretation of c-Fos signal within the TH-ir & TH-negative neurons in VTA

Previous electrophysiological study suggested that A10 DAergic neurons in VTA were active when male zebra finches sang to court females or just saw females without singing, but were not when they sang alone (Yanagihara et al. 2006). In that study, 60% of all recorded neurons (including non-singing-reactive neurons) in VTA showed the feature of DAergic firing, and this ratio accords with the population of DAergic neurons in mammal VTA (55-65%). It suggested that most DAergic neurons in VTA were active during the courtship context. In addition, 26% of all recoreded neurons showed the feature of GABAergic neuron which accords with mammal GABAergic population in VTA (~30%) and its activity was suppressed during female-directed singing.

Contrary to that result, previous studies which examined IEG expression in VTA found that IEG (egr-1) labeled DAergic neurons after female-directed singing was less than 5% of all the DAergic neurons while around 50% of all the GABAergic neurons were labeled with IEG (Hara et al. 2007). Clayton et al.(2000) advocated that IEG expression is not a surrogate for electrophysiology because its induction dissociates from depolarization, arousal, and novelty as some researchers have believed. Lobb et al. (2010) suggested that phasic burst firing of DAergic neurons is dependent on phasic removal of local GABAergic inhibitory inputs, so in case of VTA, "disinhibition" might induce IEG expression within GABAergic neurons.

In our study, c-Fos expression level in A10 in VTA was around 6% (median) and there was no clear relationship between the level of c-Fos expression and valence scores. Although birds in our experiment were not involved in the intense copulation-leading sexual courtship on day 3, DAergic activity in VTA shown by IEG expression took similar level to the previous study where male sung female-directed song. But we cannot eliminate the possibility that DAergic neurons which were not labeled with c-Fos were actually active in the electrophysiological aspect.

Next, we tried to examine if there is correlation between the valence scores and c-Fos induction level in non-DAergic neurons (putatively GABAergic neurons) in VTA. Then we found that in both males and females, about double the number of non-DAergic neurons expressed c-Fos in the dorsal compared to the ventral VTA. In mammals, activity of DAergic neurons in dorsal VTA is associated with rewarding events while activity of those in ventral VTA is associated with aversive events such as electric shocks (Brischoux et al. 2009; Matsumoto and Hikosaka 2009; Lammel et al. 2011).

Therefore, we separately quantified c-Fos positive non-DAergic neurons in dorsal and ventral VTA. In females, the number of c-Fos positive non-DAergic neurons in ventral VTA was negatively correlated with her pair valence, while a similar trend in dorsal VTA was not significant. This may suggest the history of GABAergic disinhibition in ventral VTA in female which was triggered by aversive experience as is shown by low valence toward the male bird, but further study is needed to interpret our result.

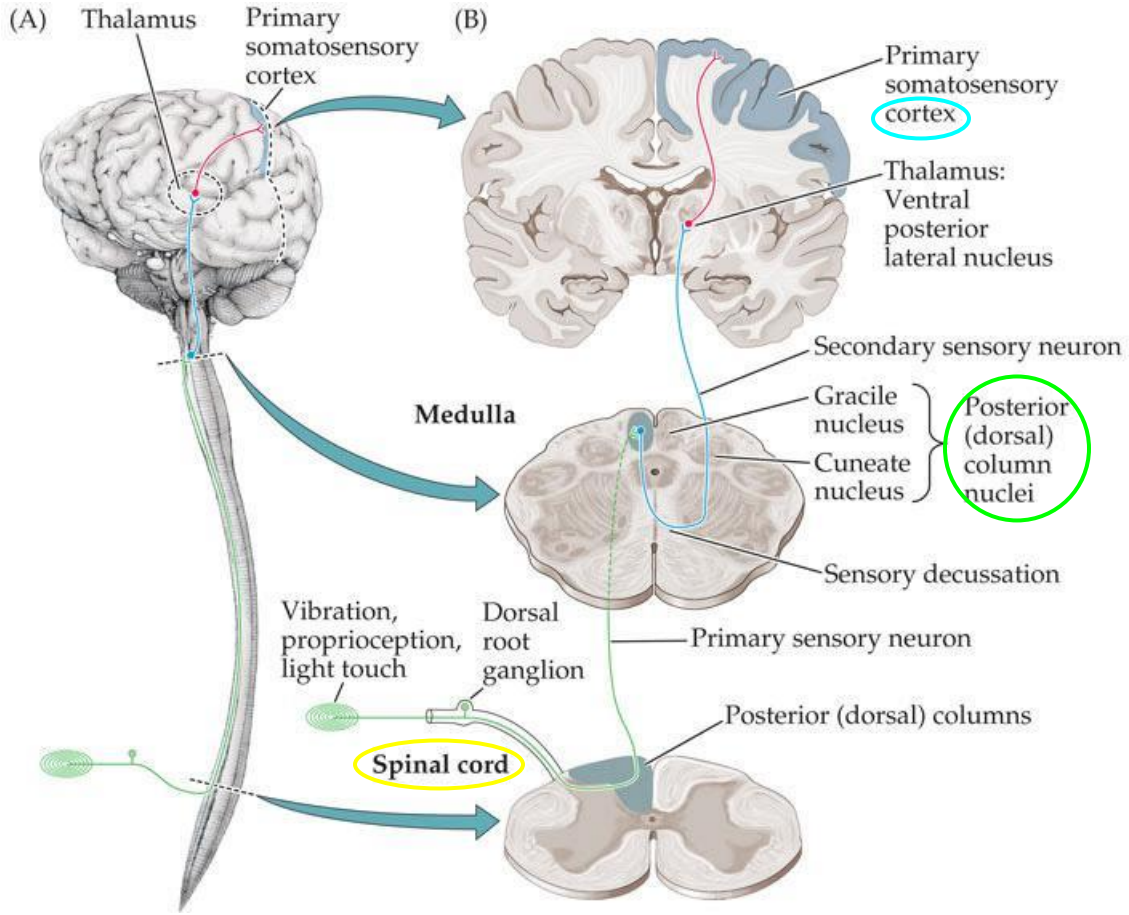
4.2 Interpretation of the result on TH-ir neurons in CG

4.2.1 Comparison of avian CG and mammal PAG

In my study, I observed TH-ir neurons in the central gray (CG) of zebra finches. However, there are too few papers today which treat avian CG to interpret the biological meanings of my results. Therefore, I had to refer to previous studies on mammal periaqueductal gray (PAG) which is proved to be homologous to avian CG.

Histochemical comparisons (Kingsbury et al. 2011) indicate a fairly specific correspondence between columns of the mammalian PAG (periaqueductal gray) and avian CG (central gray)/ICo(nucleus intercollicularis). Mammal ventreal lateral PAG (PAGvl) corresponds to medial avian medial CG (mCG), and mammal dorsal PAG corresponds to avian lateral CG and ICo (intercollicular nucleus).

Connectional studies also demonstrate that features of the avian ICo are consistent with those of the dorsal PAG. In mammals, overlapping projections from the spinal cord, dorsal column nuclei and sensorimotor cortex define an intercollicular (ICo) region that lies substantially within the lateral portion of the dorsal PAG, medial to the inferior colliculus (Hazlett et al. 1972)

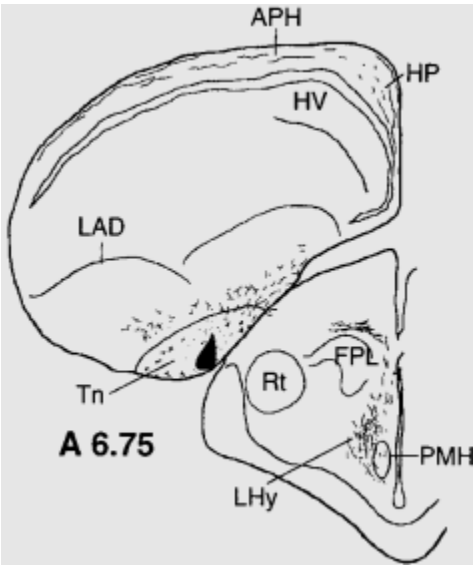


↑Fig.4.2.1-1 (Afton, 2012)

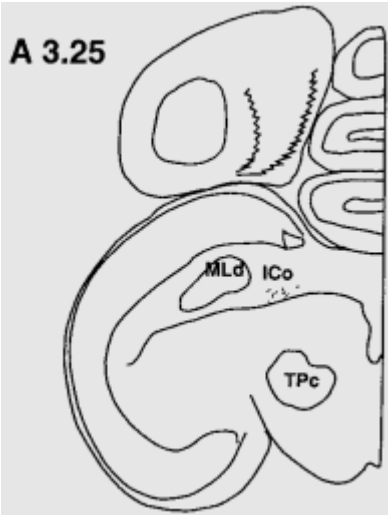
4.2.2 Avian "nucleus taenia→CG" projection corresponds to mammal "amygdala→PAG"

In mammal, projections from amygdala to PAG play an important role in fear conditioning (Johansen et al. 2010). Does bird have the homologous projection?

Nucleus taenia (Tn) is thought to be an avian homolog of the amygdala in mammals. Cheng et al. (1999) characterized Tn in terms of its connections and function using anterograde tracers Phaseolus vulgaris leucoagglutinin (PHAL) to map the efferent projections of Tn in ring doves.



←Fig.4.2.2-1 Solid black indicates the site of PHAL application to the mid Tn. Dots indicate the extent of its spread.



←Fig.4.2.2-2 In the midbrain, the presence of labeled, long fibers in nucleus intercollicularis (ICo) and its neighboring region of the central gray (CG).

Both fig.4.2.2-1 and fig.4.2.2-2 are cited from Cheng et al. (1999).

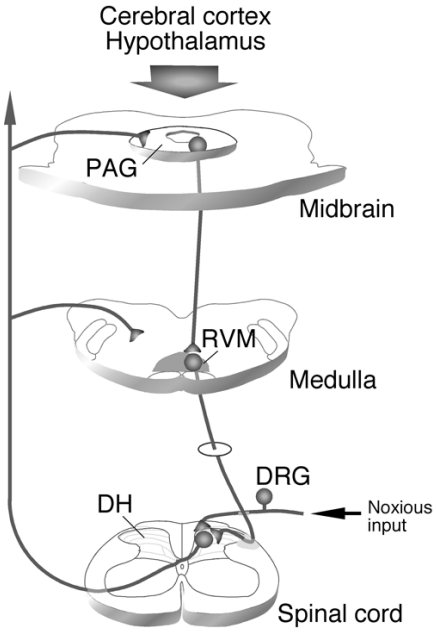
It will worth confirming to what extent avian "nucleus taenia→CG" projection has homologous function to mammal "amygdala→PAG".

4.2.3 Projection of catecholaminergic neuron in PAG, in mammal

Next, I will focus on the connection of TH-ir (catecholaminergic) neurons in PAG which are exclusively located within the ventral lateral PAG (PAGvl). In mammal, PAG is known to play a key role in anti-nociception, and catecholaminergic neurons in PAG have been studied in relation to anti-nociception (Hasue et al. 2002).

PAGvl appears to have the most catecholaminergic neurons projecting to the extended amygdala (central nucleus of the amygdala, bed nucleus of the stria terminalis, sublenticular extended amygdala) (Hasue et al. 2002). Those catecholaminergic neurons also project to locally within the PAG, substantia innominata (Dong et al. 2006), and tectum, thalamus, septal area (Ottersen et al. 1981).

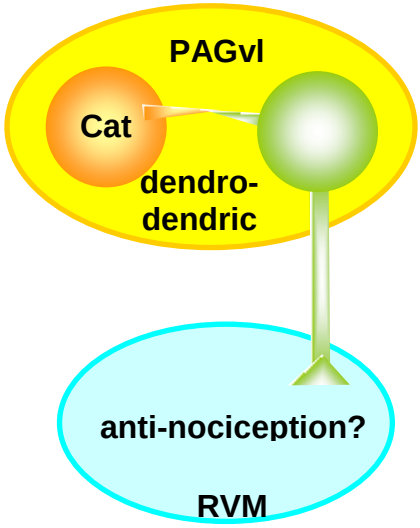
Many TH+ fibers were observed within the PAG. Although catecholaminergic neurons in PAGvl are located in close proximity to neurons that send projects to efferent targets of PAGvl, such as RVM (rostral ventromedial medulla), SN, and VTA, those projection neurons DO NOT contain catecholaminergic markers.

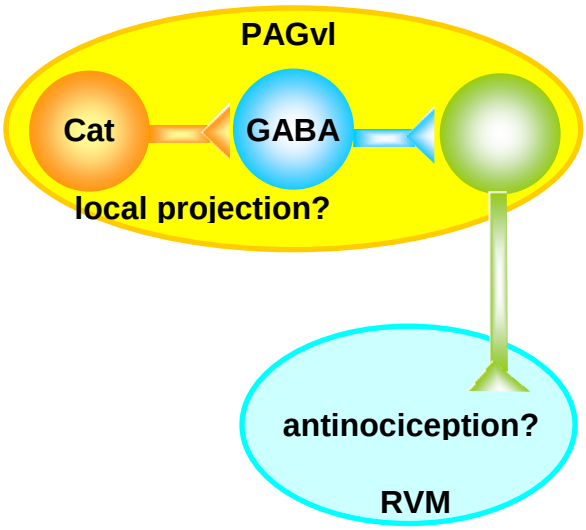


←Fig.4.2.3-1 (Guo et al. 2006)

But, those catecholaminergic neurons form dendro-dendritic contacts with neurons that project to the RVM, as well as other neurons in the vlPAG, suggesting a nonsynaptic mechanism for modulation of PAG activity.

Fig.4.2.3-2→



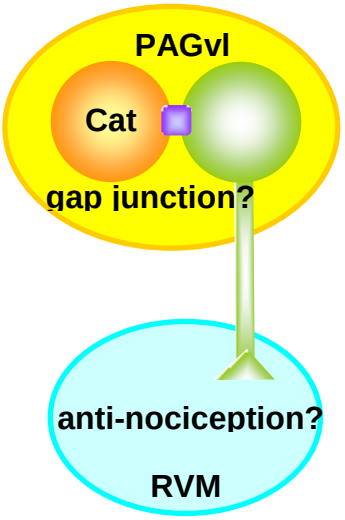


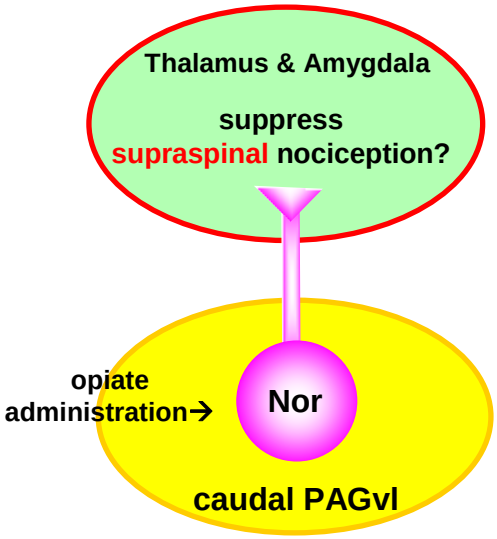
It is possible that these catecholaminergic neurons send local projections that synapse on other neurons in PAG such as inhibitory GABAergic neurons (Shelby et al.2013)

←Fig.4.2.3-3

Another possibility is that these catecholaminergic neurons and other cells in the PAG form gap junctions (structure of an electrical synapse) (Buma et al. 1992).

Fig.4.2.3-4→





↓ Fig.4.2.3-5

In caudal regions of the vlPAG, neurons contained both TH and DBH (Dopamine beta hydroxylase; catalyses the transform of dopamine to nor epinephrine) suggesting these neurons are noradrenergic (Herbert et al. 1992). Its size is relatively large (15–25 μm, Herbert et al. 1992; 30-40 μm, Flores et al. 2004) and have a multipolar morphology.

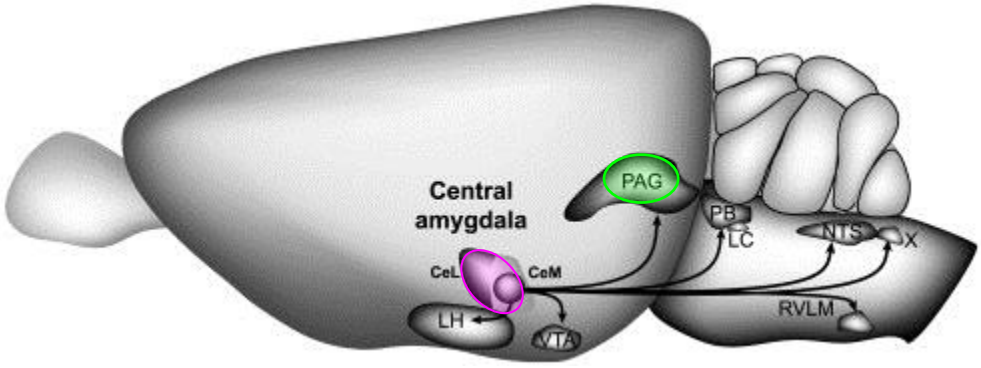
It is plausible that large, noradrenergic projections from the PAG onto diencephalic structures such as thalamus and amygdala would be related to control of supraspinal pain responses, because these centers are involved in supraspinal nociceptive control and affective aspects of pain (Hasue et al. 2002).

More rostral vlPAG regions contained TH-ir neurons but no DBH-ir was present suggesting these neurons are dopaminergic. Its size is relatively small (5–15 μm, Shelby et al. 2013; 10-15 μm, Flores et al. 2004) and the morphology is round (Shelby et al. 2013).

4.2.4 PAG and social fear processing

How can we apply the accumulated knowledge of anti-nociception for the physical pain to the inhibition of social anxiety? Actually, some researchers have already attempted to approach this topic. The establishment of social bonds is mediated by close proximity, and the proximity is caused by the ability to navigate themselves across physical distance via voluntary behavior. To interact socially in close proximity, we need to modulate and inhibit certain defensive behaviours (Zeki, 2007).

Neuroanatomical and neurophysiological research with animals reported inhibition of defensive behaviors via well-defined connections between the amygdala and the periaqueductal gray (PAG).



↑Fig.4.2.4-1

Amygdala activation acts as a warning signal of potential threat, posed by sensory stimuli. It is mediated via excitatory pathways connecting the central amygdala nucleus to the midbrain, and thence to the autonomic nervous system. Excessive amygdala activation during social encounters raises anxiety, create a sense of suspicion and foster social avoidance of others with whom we are not familiar (Stein et al. 2002).

On the other hand, PAG are organized to regulate flight, fight, or freeze behaviors and the autonomic states that support these behaviors. PAG stimulation rostrally within the lateral and dorsolateral PAG produces "fight". On the other hand, PAG stimulation caudally within lateral and dorsolateral PAG produces "flight" (Keay et al. 2001). If the other individual are perceived as trustworthy, the projection from the temporal cortex[52] to the

[52] The temporal lobes are involved in the retention of visual memories, processing sensory input, comprehending language, storing new memories, emotion, and deriving meaning (Smith et al. 2007)

central nucleus of amygdala inhibits the limbic structures[53] that control fight, flight, or freeze behaviors via PAG.

Defensive behavior such like fight or flight response accompanies accelerating heart rate, constriction of blood vessels, increasing blood pressure and so many other involuntary functions in the body. Bodily nervous system for fight or flight response is mobilized by the sympathetic nervous system. The myelinated vagus fosters calm behavioral states by inhibiting the sympathetic nerve influence, then that allows the animal to communicate socially.

In addition, strong social bonds require immobilization without fear as well. The immobilization system is dependent on the unmyelinated or dorsal vagal complex. PAG stimulation ventrolateral to the aqueduct evokes defensive immobilization (freeze). Immobilization without fear is achieved by the autonomic balance change toward sympathetic dominant (Heinrichs et al.2003, Kirsch et al. 2005).which results in cardiac slowing (Carter 1998). This autonomic balance change is mediated by pathways that lead from the central amygdala nucleus to the periaqueductal grey matter, the reticular formation and the nucleus accumbens (Huber et al. 2005).

4.2.5 Interpretation of my result based on mammal PAG study

In my study, female birds having high affinity with their male partners showed low activity of TH-ir neurons in CG (mammal PAG). Possibly, for those females, their male partners might be already trustworthy enough to allow amygdala and CG to keep low level of activity, accompanied with low sympathetic nervous activity. On the other hand, high activity of TH-ir neurons in female birds having low affinity score suggests that those female might need to actively suppress their fear and anxiety toward the co-housed males, utilizing PAG catecholaminergic antinociception (supraspinal).

Further, previous study showed that birds'nucleus taenia (Tn, Tn is homologous to mammal amygdala) sends projection to CG (Cheng et al. 1999) (CG corresponds to mammal periaqueductal gray, PAG) as mammal amygdala sends projection to PAG (Gavan et al. 2011). Therefore, birds' Tn-CG projection may have a similar function to the one which mammal Amygdala-PAG has; fear reaction.

Mammal PAG seems to relay social fear information from amygdala to medulla (Kirsch et al. 2005), probably resulting in autonomic balance shift to the sympathetic dominant (Heinrichs et al. 2003). Sympathetic nervous system makes whole animal body to be ready for fight or flight (Cannon 1929) via myelinated vagus (Porges 2007). Therefore, as

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[53] The limbic system is a complex set of structures that lies on both sides of the thalamus, just under the cerebrum. It includes the hypothalamus, the hippocampus, the amygdala, and several other nearby areas. It appears to be primarily responsible for our emotional life, and has a lot to do with the formation of memories.

was found in mammal, an intriguing possibility is that the female CG contributed to raise sympathetic activity by relaying social fear from amygdala to medulla, and that resulted in female birds' maintaining a certain distance from males.

If inhibition of fear networks is associated with development of close social affiliation, this neural phenomenon seems to accord with our finding in behavioral analysis; the occurrence of aggressive behavior on day 1 strongly predicted the low valence on day 3. To elucidate causal relationship between A11 activity in CG and behaviors with low valence, further study using free moving electrophysiology or optogenetical method would be useful.

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Chapter5: Future work

5.1 Future development of my study

In the first study, we examined if operationally "paired" or "unpaired" female evokes difference in song's acoustic feature or neural activity of Area X as is seen between female-directed singing and undirected singing. However, neither of them was significantly different between the female categories. One possible interpretation of it is that "operationally" paired and "unpaired" females might fail to differentially motivate male's singing. For example, ethological study reported that complete copulation occurs only after their nest building (Zann 1996). So male's preference for a specific female may become more evident after raising their own chicks. Further work to rigorously quantify pair-bond strength after all the recording had finished will be required. For this pair affinity measurement, video tracking targeted to an aviary full of developed couples may be one option.

We also found that within single unit, syllable frequency's across-rendition CV and Area X spike rate's across-rendition CV were linked. In case of the distinction between female-directed and undirected songs, stronger activation of DAergic neurons in VTA during directed singing causes denser dopamine release in Area X and it makes Area X's neural variability lesser (Leblois et al. 2012). Therefore, the higher variability of the Area X activity we found in this study might be also caused by lesser VTA activation. For the future study, it may be nice to record both from VTA and Area X to elucidate the relationship between them.

Further, across the group of recording days, the *acceleration variability* (the standard deviation of acceleration values within song bouts) was nearly significantly lower for singing to paired than to nonpaired females. According to the previous study, presentation of females causes elevation of HVC temperature possibly as a by-product of elevation of body temperature, resulting in higher singing tempo (Appeltants et al. 2001). So it might be interesting to investigate if HVC is responsible for the stability of song acceleration. Because HVC receives DAergic input mainly from A11 of CG (Dahlstöm et al 1964), it would be intriguing to examine if A11 dopaminergic input makes HVC's song timing control finer as A10 dopaminergic input from VTA does so for Area X.

In the second study, because we wished to assess male-female pair affinity (valence) based on the birds' natural approach or withdrawal from the counterpart during their interactive behaviors, we applied principal component analysis (PCA) to the five behavioral parameters acquired from the video tracking system. However, the cage size we used here was 27x27x20cm. Considering that the zebra finch couples in the wild generally stay within 2 meters in diameter (Zann 1996), we might not provide enough space for birds to keep a comfortable distance when they desired. The usage of larger aviary around 4 cubic meters seems more ideal for the future study.

For the histological analysis, in females, the number of c-Fos positive non-DAergic neurons in ventral VTA was negatively correlated with her pair valence. However, we

could not interpret this result properly because previous study in mammal used electrophysiological method to approach VTA reaction related to rewarding (Schultz et al. 1997) and aversive (Guarraci et al. 1999) stimuli. It may be nice to record DAergic activity electrophisiologically during the development of durable physical proximity between a male and a female, which is characteristic of pair bonding. Because mammal study suggested that the increase of D1R binding level within NAc caused male's selective aggression to unfamiliar females (Aragona et al. 2009), NAc projecting DAergic neurons in VTA seems intriguing target to monitor.

For A11 in CG, c-Fos expression level in DAergic neurons was much lower in females with higher valence. Previous study suggested that mammal PAG (=avian CG) seems to relay social fear information from amygdala (=avian Tn) to medulla (Kirsch et al. 2005) resulting in autonomic balance shift to the sympathetic dominant (Heinrichs et al. 2003). Sympathetic nervous system makes whole animal body to be ready for fight or flight (Cannon 1929) via myelinated vagus (Porges 2007). Anatomically, birds have the homologous projection to mammal amygdala to PAG projection; Tn to CG (Cheng et al. 1999). Therefore, an intriguing possibility is that the female CG contributed to raise sympathetic activity by relaying social fear from amygdala to medulla, and that resulted in female birds' maintaining a certain distance from males.

However, the involvement of A11 catecholaminergic neurons in PAG to the fear processing remains to be clarified even in mammal. In mammal, A11 in PAG has been studied related to anti-nociception (Hasue et al. 2002). Some A11 projects within PAG and other projects toward amygdala, or thalamus. Among them, amygdala or thalamus – projecting A11 population seems critical for supraspinal anti-nociception. So this projection is one candidate route for A11 in CG to be involved in social fear processing.

As well as VTA, free moving electrophysiological recording from CG (mammal PAG) and Nucleus Taenia (mammal amygdala) seems useful to examine if they are co-activated during social interaction with low valence, as was observed in human reaction to social fear stimuli. Related to CG activity, if we additionally measure the real-time alteration of autonomic balance between sympathetic and parasympathetic during social interaction, we may be able to investigate more about CG function as an output (or input) gate of the central nerve system toward whole body behavior.

5.2 Contribution to human society

I hope that this study on birds' communication takes a small step toward the breakthrough to approach difficulties in our human relations as our predecessors have achieved the contribution to medical treatments by studying model animals.

Human personality is not always consistent but usually flexible because the social context demands him/her to adjust his/her behavior to be more suitable for the situation. Someone who maintains wholesome ties between husband and wife or among friends can bully his subordinates if his company has a culture which encourages superior workers to behave bossy toward their subordinates. Sometimes we even hear that a ringleader of genocide returned to be a gentle father when he is at home.

Those distinctions of his changing attitude depending on the counterpart seem to reflect his differential labeling onto each counterpart, such as "in-group people to whom he should cooperate" or "out-group people to whom he should take hostile attitude". In fact, intergroup conflict is one of the most pervasive problems facing human society (Bowles, 2009). In fact, prejudice, terrorism, ethnic cleansing, and interstate war have persisted throughout trans-generations (Fiske 2002). Governmental genocidal policies killed more than 210 million people during the 20th century (Cohen et al. 2008).

So far, people who were handling those problems were the military top brass, government at the international issue level, and counselors, psychiatrists, lawyers at the personal trouble level, and journalists, social scientists, psychologists at the generalization level. However, the approach to the problems of human relationship from biological points of view has been relatively few even though we humans are also one of the animals which form swarm communities.

Today, due to the development of fast enough computer processing, researchers in information technology (Yano et al. 2012), zoology, and biology (Partridge, 1981) have gradually succeeded in capturing the animal (including human) communication quantitatively. Among them, we biologists are likely to have ability to bridge the gap between "inter individual communication" and "within individual neural network" called brain.

In the future, I want to investigate how in-group camaraderie and inter-group hostility differentiate in the reward circuit, or in the fear circuit in a person, and how this personal discrimination between friends and enemies eventually propagates throughout the whole in-group members. Those studies may give us a fundamental treatment for vicious circle of exchanges of revenge which is rampant in today's world.

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Acknowledgements

This work was supported by funding from the Brain Science Institute, RIKEN.

I owe my deepest gratitude to Prof. Neal A. Hessler in RIKEN BSI for his invaluable guidance, Dr. Thomas M. Poulsen in RIKEN BSI for his enormous amount of labor for the video-tracking development, and Prof. Kotaro Oka in Keio University for his constructive suggestions.

I received generous technical advice from Dr. Erina Hara in Duke University, Dr. Tsutomu Hashikawa , Dr. Takumi Akagi, Dr. Ayako Ajima-Tanaka in RIKEN BSI, Prof. Kazuhiro Wada in Hokkaido University, and Prof. James L. Goodson in Indiana University.

Prof. Melissa J. Coleman in Keck Science Department (for Chapter 2), Prof. Valérie Doyère in Centre de Neurosciences Paris-Sud (for Chapter 3), Prof. Paul M. Nealen in Indiana University of Pennsylvania (for Chapter 3), Prof. Juan M. Dominguez in University of Texas at Austin (for Chapter 3) gave insightful comments.

I appreciate the meticulous feedback offered by reviewers of this thesis, Prof. Akira Funahashi, Prof. Eiichi Izawa, and Prof. Junichi Ushiba.

I thank Dr. Yutaka Shindo for serving a good model for the composition of the doctoral thesis.

Without many people's encouragement, this dissertation would not have materialized. Once again, let me thank everyone who has offered warm support for my work!

Mai Iwasaki