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Assessing visual requirements for social context-dependent activation of the songbird song system

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Social context has been shown to have a profound influence on brain activation in a wide range of vertebrate species. Best studied in songbirds, when males sing undirected song, the level of neural activity and expression of immediate early genes (IEGs) in several song nuclei is dramatically higher or lower than when they sing directed song to other birds, particularly females. This differential social context-dependent activation is independent of auditory input and is not simply dependent on the motor act of singing. These findings suggested that the critical sensory modality driving social context-dependent differences in the brain could be visual cues. Here, we tested this hypothesis by examining IEG activation in song nuclei in hemispheres to which visual input was normal or blocked. We found that covering one eye blocked visually induced IEG expression throughout both contralateral visual pathways of the brain, and reduced activation of the contralateral ventral tegmental area, a non-visual midbrain motivation-related area affected by social context. However, blocking visual input had no effect on the social context-dependent activation of the contralateral song nuclei during female-directed singing. Our findings suggest that individual sensory modalities are not direct driving forces for the social context differences in song nuclei during singing. Rather, these social context differences in brain activation appear to depend more on the general sense that another individual is present.

Keywords: *egr-1*; *ZENK*; directed singing; social behaviour; vocal nuclei; courtship

Anatomical abbreviations: A, arcopallium; AH, anterior hyperpallium; AMD, anterior dorsal mesopallium; AMV, anterior ventral mesopallium; AN, anterior nidopallium; AreaX, a vocal nucleus; cVTA, caudal ventral tegmental area; DLM, dorsal lateral nucleus of the thalamus; DM, dorsal medial nucleus of the midbrain; E, entopallium; GLd, dorsolateral geniculate nucleus; H, hyperpallium; Hp, hippocampus; HVC, a vocal nucleus (no abbreviation); IH, interstitial lamina of the hyperpallium; M, mesopallium; LAreaX, lateral part of AreaX; LMAN, lateral magnocellular nucleus of the anterior nidopallium; MLd, dorsal part of the lateral mesencephalic nucleus; MD, dorsal mesopallium; MV, ventral mesopallium; MVe, ventral mesopallium near E; N, nidopallium; Ne, nidopallium adjacent to E; nXIIts, 12th nucleus tracheosyringeal part; P, pallidum; PH, posterior hyperpallium; PMD, posterior dorsal mesopallium; RA, robust nucleus of the arcopallium; Rt, nucleus rotundus; rVTA, rostral ventral tegmental area; SNc, substantia nigra pars compacta; St, striatum; Ste, striatum adjacent to E; TeO, optic tectum; VTA, ventral tegmental area

1. INTRODUCTION

Interactions with conspecifics, either positive or negative, are critical in many ways for the survival of individuals and species. Within these interactions, the precise social context in which animals interact can strongly modulate the level of activity in specific brain areas in a wide range of vertebrates, from fishes (Burmeister *et al.* 2005), frogs (Yang & Wilczynski 2007) and birds (Jarvis *et al.* 1998; Hessler & Doupe 1999; Vignal *et al.* 2005), to primates (Fujii *et al.* 2007), including humans (Sassa *et al.* 2007; Van den Bos *et al.* 2007). Social context-dependent brain modulation is especially strong in relation to communicative behaviour, where brain activity during the production of specific vocalizations is dependent on the presence of a communicative target individual. Such modulation has been well characterized in the songbird brain during singing (Jarvis *et al.* 1998; Hessler & Doupe 1999), and recently found in human language production and processing brain areas during speaking (Sassa *et al.* 2007). In both systems, the level of brain activation during vocalization differs by orders of magnitude depending on who is listening. When male zebra finches sing undirected song, the level of neural activity and expression of the immediate early gene (IEG) *egr-1* (also known as

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zif268, *NGF1-A*, *krox24* and avian *ZENK*) is high throughout the vocal motor pathway (figure 1*a*(i)(iii); black solid arrows, figure 1*b*) and the vocal pallial-basal-ganglia-thalamic loop (figure 1*a*(i)(iii)(v); white arrows, figure 1*b*). However, when they sing directed song, activity and/or *egr-1* levels are low in the robust nucleus of the arcopallium (RA) of the vocal motor pathway and in the lateral portion of the vocal pallial-basal-ganglia loop (figure 1*a*(ii)(iv)(vi)) (Jarvis *et al.* 1998; Hessler & Doupe 1999). The opposite result is found for the *FoxP2* gene, a gene necessary for normal speech production in humans and song learning in songbirds (Lai *et al.* 2001; Haesler *et al.* 2004, 2007), which shows decreased expression in AreaX of the vocal pallial-basal-ganglia-thalamic loop during undirected singing but no change during directed singing (Teramitsu & White 2006). Directed singing is used during courtship to females, and undirected singing appears to have other communicative functions (Dunn & Zann 1996), including for practice or vocal exploration (Jarvis *et al.* 1998; Olveczky *et al.* 2005; Kao & Brainard 2006). However, the songs produced are very similar, with only subtle differences in tempo (directed sung slightly faster) and variability (undirected more variable) (Sossinka & Bohner 1980; Kao & Brainard 2006).

This social context-dependent modulation of brain activation does not depend on the males' hearing their own voice during singing or hearing the calls of the females in response to their singing, as it still occurs in deaf birds when they sing (Jarvis *et al.* 1998; Hessler & Doupe 1999). In addition, directed singing is associated with increased neural activity levels and *egr-1* expression in brainstem motivation-related areas, namely the ventral tegmental area (VTA; Yanagihara & Hessler 2006; Hara *et al.* 2007). In mammals, activity of VTA neurons is driven by visual input and it receives visual input from the superior colliculus (Comoli *et al.* 2003; McHaffie *et al.* 2006), the homologue of the avian optic tectum. In both mammals and birds, the VTA has been implicated in reward and social context-dependent sexual behaviour (Maney *et al.* 2003; Riters *et al.* 2004; Young & Wang 2004; Aron *et al.* 2005; Esch & Stefano 2005; Heimovics & Riters 2005), and provides a strong dopaminergic input to the striatum, including to the lateral AreaX (LAreaX) of the vocal pallial-basal-ganglia loop (figure 1*b*; Lewis *et al.* 1981). The above findings led to the hypothesis that social context-dependent activation of vocal communication brain systems could depend on the visual stimulus reaching the VTA or some other brain areas and then those brain areas modulate the social context activation of the song system.

Here, we tested this hypothesis by blocking visual input into one hemisphere while male zebra finches sang directed song to females. There are two visual pathways in birds and mammals: the thalamofugal pathway (grey arrows, figure 1*c*) and the tectofugal pathway (black arrows, figure 1*c*; Karten 1991; Shimizu & Bowers 1999). In birds with laterally placed eyes, such as the zebra finch, the visual pathways are nearly completely crossed at the optic chiasm, such that visual input from one eye projects primarily to both visual pathways of the contralateral hemisphere (Weidner *et al.* 1985). We found that although visual input was necessary for the visually induced IEG activation of the visual pathways of the contralateral hemisphere and for the activation in part of the VTA, it was not apparently required for the social context-dependent activation of the song system. Our findings suggest that the social context-dependent activation of the brain is not driven by direct primary sensory or motor processes, but rather by an indirect perhaps association process indicating that another individual is present.

2. MATERIAL AND METHODS

(a) *Animals*

We used 19 adult male zebra finches (more than 90 days old) that were bred in our aviaries at RIKEN and at the Duke University Medical Center. All experiments were performed according to the RIKEN BSI guidelines and were approved by the RIKEN Animal Experiments Committee and the Duke University Animal Care and Use Committee.

(b) *Behaviour*

One eye of each bird was covered with several layers of black vinyl electrical tape; the innermost layer was placed so that the smooth surface covered the eye to prevent irritation. The tape was sealed at the edges with super glue to the surrounding skin and feathers, to prevent light leakage. We alternated covering of the preferred (right) and non-preferred (left) eye in different birds to prevent potential biases in the results; when allowed to use only one eye, zebra finch males tend to sing slightly more songs to females when viewing them with their right eye (George *et al.* 2006). Birds were then isolated at least overnight in individual cages that were in sound attenuation boxes. They were divided into three groups: female-directed singing ($n=8$; right eye covered $n=4$ and left eye covered $n=4$); silent alone ($n=5$; right eye covered $n=3$ and left eye covered $n=2$); and silent in the dark ($n=3$; right eye covered $n=1$ and left eye covered $n=2$). We also used another control group of female-directed singers with both eyes open ($n=3$). For the female-directed singing groups, a female was placed in the cage with the male in the dark on the night before the recording session, but separated by a cage wall barrier. The cage wall barrier was made from the same metal bar material as the rest of the cage. Thus, with the lights on, the male and female could interact visually, but not physically. From a slit outside the sound box, we placed a thick, opaque paper barrier attached to a string alongside the cage wall barrier. In the morning, the lights were turned on, and then for every 5–10 min the paper barrier was removed so that the male could see the female and become stimulated to sing to her. This was done for 45 min. The intermittent removal of the paper barrier and the presence of the cage barriers induced more singing behaviour than normally occurs with continuous sight of the female and with tactile interactions with the female. The behaviour was videotaped, and songs were recorded using an Avisoft Recorder (Avisoft Bioacoustics, Berlin, Germany). The silent alone birds remained alone with the lights on for 45 min and did not sing; the silent dark birds remained silent alone in the dark, during waking hours. After the sessions, birds were killed, their brains were removed and embedded in optimum cutting temperature compound (Sakura Fine Technical), frozen and stored at -80°C. For the female-directed group, we used birds that sang approximately 20 or more song motifs (range 17–88).

(c) *Gene expression analysis*

Serial coronal brain sections (12 μm) were cut throughout the telencephalon and brainstem, and mounted on silanated glass slides. Radioactive *in situ* hybridization was performed with

a zebra finch *egr-1* clone containing the full-length coding region (obtained from Dr Osceola Whitney, Duke University), using a previously described procedure (Wada *et al.* 2004). We performed two separate *in situ* experiments: one in which sections from an original five female-directed singers (right eye covered $n=3$ and left eye covered $n=2$) were hybridized together with all brain sections from all other animals, and another where we replicated the female-directed group with three additional animals to test for possible statistical differences in IEG activation depending on which eye was covered (right eye covered $n=4$ total and left eye covered $n=4$ total). Two pictures per brain region in each hemisphere from the same section were taken with a Leica DMXRA microscope under a 60× objective. We carefully chose the areas using lamina boundaries, Nissl stain and overall gene expression profiles, so that the same areas were measured from animal to animal. The density slice and analyse functions of Scion Image (NIH) were used to count silver grains, as previously described (Hara *et al.* 2007), in visual areas (PH, PMD, TeO, Ne, MVe, Ste and E), song nuclei (LAreaX, LMAN, HVC and RA), motivation-related (rostral VTA (rVTA) and caudal VTA (cVTA)), movement-associated (AMV and AN) and auditory midbrain (MLd) areas (see abbreviations list). To generate representative figures of gene expression profiles, darkfield pictures were taken with a Wild M420 microscope and processed in Adobe Photoshop (San Jose, CA, USA).

(d) *Anatomy*

The anatomical terminology used in this paper follows the new avian brain nomenclature (Reiner *et al.* 2004; Jarvis *et al.* 2005) with modifications (Feenders *et al.* 2008; see abbreviations list). For visually activated areas adjacent to and near the entopallium (E) of the tectofugal pathway that have been called lateral nidopallium and lateral ventral mesopallium, we called them nidopallium adjacent to the entopallium (Ne) and ventral mesopallium near the entopallium (MVe). For those of the thalamofugal pathway, we called them posterior hyperpallium (PH) and posterior dorsal mesopallium (PMD; figure 1*c*). For the connectivity of the vocal motor pathway, HVC (a letter-based name) projects to RA, and RA projects onto the midbrain dorsomedial nucleus (DM) and motor neurons of the 12th tracheosyringeal nucleus (nXIIts, black arrows, figure 1*b*). The vocal pallial-basal-ganglia loop consists of the lateral magnocellular nucleus of the nidopallium (LMAN), which projects to AreaX in the striatum, AreaX projects to the dorsolateral medial nucleus (DLM) in the dorsal thalamus, which in turn projects back to LMAN, forming a loop (white arrows, figure 1*b*). The movement-associated areas are regions in the anterior nidopallium (AN) and anterior dorsal mesopallium directly adjacent to the song nuclei LMAN and the mesopallium oval nucleus, respectively, and show movement-associated gene expression when birds hop (Feenders *et al.* 2008), which zebra finches normally do during directed singing.

(e) *Statistics*

The numbers of silver grains from two pictures per brain region per animal were averaged and used for statistical tests with SigmaStat v. 3.1 (Systat Software). We performed two types of statistical tests. (i) We compared the average number of silver grains (non-normalized values) between hemispheres *within a group* and in each hemisphere *across groups* by two-way repeated-measures ANOVA (factors: hemispheres and groups) with a Holm–Sidak *post hoc* test; statistical results within a group are in the figures and across groups are described in the main text. (ii) We compared ratios of silver grains on the side contralateral to the open eye relative to the side contralateral to the covered eye in each brain area across groups also by two-way repeated-measures ANOVA (factors: areas and groups). With the first test, the raw values, we can determine the direction of change for one group relative to another. With the second test, the ratios, we theoretically cannot determine the direction of change for either hemisphere, but we can use the ratios to internally normalize the data for variability between individual animals and for the amount of singing (Jarvis *et al.* 1998); therefore, the ratios should provide a more sensitive assay to detect small quantitative differences. The ratios also allow us to compare data from different experiments, as they can normalize out experimental variation. When performing ratio analyses between hemispheres or brain areas within the same animal as its own control, sample sizes of three to six per group have been sufficient to detect statistically significant small differences in singing-regulated gene expression in vocal nuclei among groups (Jarvis & Nottebohm 1997; Hara *et al.* 2007; Kubikova *et al.* 2007).

3. RESULTS

(a) *Visual areas*

Occlusion of one eye blocked induction of *egr-1* expression in the visual pathways of the contralateral brain hemisphere (figure 2). In the two groups exposed to light, silent alone and female directed, there was significantly less *egr-1* expression in both the thalamofugal (PH and PMD) and tectofugal (TeO, Ne, MVe and Ste) pathways contralateral to the covered eye relative to the open eye (figure 2*d*,*e*,*h*,*i*; quantification in figure 3*a*). Furthermore, although there remained a low level of expression in these areas in some animals on the side contralateral to the covered eye (figure 2*e*), these levels were not significantly different from that seen in the silent dark group (lower half of graph, figure 3*a*). In the silent dark group, the level of *egr-1* expression in visual areas was low and similar in both hemispheres (figures 2*a*,*b* and 3*a*). The first-order thalamic recipient neurons of the tectofugal pathway in the pallium, called the entopallium (E; figure 1*c*), normally do not express high levels of *egr-1* (Mello & Clayton 1995), but they had low *egr-1* levels that were significantly higher on the side contralateral to the open eye in the silent alone group (figure 3*a*; but see ratio analysis).

Next, we determined whether there were differences in brain areas among groups, using the ratio measures (contralateral to the open eye: contralateral to the covered eye). Except for the entopallium, all measured visual areas had significantly higher ratios of *egr-1* expression in the silent alone and female-directed groups relative to the silent dark group (figure 4*a*), supporting the non-normalized results. (The comparison for the entopallium approached significance, $p=0.022$, α-level$=0.017$; two-way repeated-measures ANOVA.) Furthermore, we observed a higher ratio of expression in the PH of the silent alone relative to the female-directed group, but higher expression in the Ne of the female-directed relative to the silent alone group (figure 4*a*). In general, high-induced contralateral *egr-1*