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Neural Responses to Aggressive Challenge Correlate with Behavior in Non-breeding Sparrows

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Abstract

The present study was conducted on captive male song sparrows (*Melospiza melodia*) during the nonbreeding season in order to 1) examine Fos and Zenk responses of basal forebrain sites to simulated territorial intrusion (STI), and 2) determine how those responses relate to aggression. Numerous forebrain areas showed significant Fos and Zenk responses to STI, and in several areas of the hypothalamus and lateral septum, these responses were negatively correlated with aggressive behavior. Homologous areas in mammals show greater responses in subordinate subjects than in dominant subjects. Thus these brain areas may be responsive to social stressors across a wide range of vertebrates.

Keywords

amygdala; bed nucleus of the stria terminalis; dehydroepiandrosterone; DHEA; egr-1; estrogen; hypothalamus; lateral septum; songbird; testosterone

Introduction

In birds, immediate early gene (IEG) data relevant to agonistic behaviors are limited to studies of male song sparrows (*Melospiza melodia*), which have been examined only during the breeding season (spring-summer). Simulated territorial intrusion (STI; presentation of song playback and a caged male decoy) elicits significant Zenk (egr-1) responses within the medial bed nucleus of the stria terminalis (BSTm), multiple zones of the lateral septum (LS), lateral subdivision of the ventromedial hypothalamus (VMH), midbrain central gray, and areas related to song production and perception [1-3]. Fos is also a useful marker of neural response, and it yields results that are complementary (and only partially redundant) to Zenk [4,5]. Thus, one of the goals for the present experiment was to examine both Fos and Zenk responses of male song sparrows to STI.

Importantly, we collected the present data during the non-breeding season (autumn), thereby allowing comparison with our previous data from the breeding season. Although song sparrows are territorial year-round, the hormonal regulation of territorial behavior varies across seasons, shifting from a dependence upon sex steroids produced in the gonads during the breeding season, to dependence upon non-gonadal (perhaps neural) sources of sex steroids during the non-breeding season [6-11]. The present study examined whether hormone-sensitive brain

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regions show different responses in the breeding and non-breeding seasons. We also determined whether responses to STI are regulated by dehydroepiandrosterone (DHEA), a steroid implicated in non-breeding territoriality [10,11].

Materials and Methods

Animals and housing

Nonbreeding male song sparrows ($n = 21$) were captured by mistnet and song playback in San Diego, CA during late September and early October 2003. Birds were housed individually in cages (61 cm W $\times$ 46 cm H $\times$ 46 cm D) placed outdoors at the University of California's Elliot Field Station, San Diego. Finch seed mix and water were provided *ad libitum*. Mealworms were provided 3 times per week.

One week prior to STI, we distributed the subjects' cages throughout a large housing complex comprised of 11 buildings, each containing an average of six large, open-air housing units ~ 3 m $\times$ 4 m. These housing units were separated by concrete block walls but had chain-link walls to the front and rear and had a partial roof. Subjects' cages were placed individually into these units, onto elevated platforms 1.5 m high. In order to conduct STIs without disturbing other subjects that were being tested on the same day, we conducted STIs in multiple cohorts (i.e., groups of birds to be tested in a single day) containing no more than six birds each. Members of a cohort were distributed widely across the housing complex.

Steroid manipulations and radioimmunoassay

Based on previous findings that DHEA increases territorial singing in non-breeding sparrows [11] and is metabolized to sex steroids within the brain [12], we examined whether DHEA manipulations affect other agonistic behaviors and IEG responses to STI. Osmotic minipumps containing 50% DMSO in DH_2O (vehicle) or YM116 (C17,20 lyase inhibitor; a kind gift of Dr. Hisataka Shikama, Yamanouchi Pharmaceuticals, Japan) [13,14] were placed subcutaneously in the back (after [9]). YM116 is a potent and specific inhibitor of DHEA synthesis. Based on rodent studies [14] and our pilot songbird studies, the dose was set at ~ 10 mg/kg body weight/day. Silastic implants (7 mm long, 0.76 mm i.d., 1.65 mm o.d.) were packed with crystalline DHEA (Steraloids Inc., Newport RI) or left empty as a control. Subjects were randomly assigned to receive YM116, YM116+DHEA, or control treatment ("CON"). Subjects' cages were then distributed throughout the housing complex as described above.

One week following implantation, a blood sample was collected from the alar wing vein of each subject. Steroids were extracted from plasma (~ 75 μl) using 2 ml diethyl ether. Extracts were dried under nitrogen and reconstituted in assay buffer. DHEA was measured using a specific and sensitive radioimmunoassay (Diagnostic Systems Laboratories, Webster, TX; DSL-8900), modified to increase sensitivity [15].

Testing and perfusion

Testing and perfusion were performed between 7:30 and 11:30 AM, November 9-29 2003, 12-13 days after implantation. Three of the nine CON subjects were assigned to a silent control group; all other subjects were exposed to STI. Immediately prior to STI or silent control, a video camera was set up for filming the subject's behavior (< 1 min required for set-up). A cage containing a stimulus male (or an empty cage for silent control) and a tape player were then placed next to the subject's cage. The tape player broadcast male song sparrow songs at a rate of 5/min, with switches between song types occurring once per minute (two song types per tape). Two different tapes and two different stimulus males were employed. The order of STIs on a given test day was counterbalanced across treatment groups, as was the use of the stimulus

males and playback tapes. STI and filming were terminated at 45 min, and all equipment was removed. Animals were weighed and perfused 90 min after initiation of STI.

Behavioral observations

We scored behavior from videotapes for the first 10 min of each STI, focusing on two behaviors that could be unambiguously quantified: 1) *Proximity Time*, the amount of time that the subject spent in the half of the cage next to the stimulus (measured by 15-sec time samples), and 2) *Contacts*, the number of times the subject made full contact with the wire separating the two cages (both feet on the barrier; beak contact alone was not scored). Song sparrows rarely sing when placed in immediate proximity to other birds in captivity (personal observations), and an insufficient number of subjects sang during STI for analysis.

Immunocytochemistry and data quantification

Subjects were overdosed by isoflurane vapor and transcardially perfused with 0.1M phosphate buffered saline followed by 4% paraformaldehyde (both cooled to 4° C). Brains were removed, postfixed overnight, transferred to 0.1M phosphate buffer for short-term storage at 4° C (<4 weeks), and then sunk in 30% sucrose prior to sectioning on a cryostat. Three coronal series were cut at 40 µm, two of which were transferred into phosphate buffer for the present experiments. One tissue series was labeled for Zenk (egr-1 antibody raised in rabbit, Santa Cruz Biotech, Santa Cruz CA) and the second series was labeled for Fos (Fos antibody was kindly provided by Els D'Hondt). Both primary antibodies were used at a concentration of 1:1000 and were labeled using a goat anti-rabbit secondary antibody conjugated to Alexa Fluor 594 (Molecular Probes). Details of our immunocytochemical procedure, including materials and concentrations, have been published elsewhere, as have our methods for IEG quantification [1,16]. Specificities of the primary antisera have also been fully addressed [1,17].

IEG quantification and the delineation of brain regions were conducted using the methods previously employed for song sparrows [1] and the chemoarchitectural parcellation of the LS recently established for songbirds (including song sparrows) [16]. IEG quantification was performed blind to subject groups. Photomicrographs (10×) were generated for regions of interest using a Zeiss Axioscop microscope and an Optronics Magnafire digital camera linked to a Macintosh G4 computer. For each region, 1-4 sections were photographed bilaterally (dependent upon size). A standardized box outline was created in Photoshop 5.5 for each region, and the same box was used for that area in all subjects. The boxes were superimposed on the photomicrographs, and all IEG-positive profiles within the boxes were quantified by placing dots over each profile and then counting the dots in NIH Image 1.63 (W. Rasband, National Institutes of Health). Raw cell counts were then converted into the number of profiles per 100 µm².

We quantified IEG responses within the following areas: taenial amygdala (TnA), POA, AH, BSTm (commissural and posterior band), lateral BST (BSTl, commissural), paraventricular nucleus of the hypothalamus (PVN), VMH (medial and lateral subdivisions), and multiple zones of the commissural and post-commissural LS (rostral division, LSr, combining the medial and dorsolateral zones, and the ventral, ventrolateral and lateral zones of the caudal division, LSc). The identification of these areas has been addressed [1,16]. Our chemoarchitectural studies of the LS [16] have allowed us to generate methods for localizing the various zones based upon major landmarks (e.g., anterior commissure, pallial commissure, septomesencephalic tract and ventricular morphology), and we previously generated polygon outlines for LS zones in sparrows [1], which were employed here.

Results

Regional IEG responses to STI

In order to examine IEG responses to STI, we compared the silent CON group to the STI CON group (see next section for data on DHEA manipulations). As shown in Table 1, numerous areas exhibited significant IEG responses to STI ($p < 0.05$, unpaired t-tests). STI produced a significant Fos response in the ventrolateral LSc and AH. A significant Zenk response was observed for the ventrolateral LSc, LSr and commissural BSTm.

Effect of DHEA manipulations on behavior and IEG response

Plasma DHEA levels differed significantly across groups (Kruskal-Wallis $H = 11.130$; $p < 0.005$) and were as follows: CON, 1164 ± 410 ng/ml; YM116, 738 ± 81 ng/ml; and YM116 +DHEA, 4443 ± 578 ng/ml. Plasma DHEA was significantly higher in the YM116+DHEA group (Mann-Whitney, $p < 0.01$ in both cases). We detected no effects of treatments on behavioral or IEG responses to STI (all ANOVA p 's > 0.05). However, given the broad range of DHEA levels, we correlated plasma DHEA levels with IEG cell counts. There was a significant negative correlation with Fos-ir nuclei in the commissural BSTm (corrected Rho = -0.524 , tied $p < 0.05$) and near-significant negative correlations with Fos-ir nuclei in the BSTl (corrected Rho = -0.477 , tied $p = 0.06$) and Zenk-ir nuclei in the LSc.v (corrected Rho = -0.483 , tied $p = 0.06$).

IEG correlations with behavior

These analyses included all animals exposed to STI (CON, YM116, and YM116+DHEA). Contacts were natural log transformed to produce data that were normally distributed. Proximity Time and Contacts were positively correlated ($r = 0.684$, $p < 0.005$). In contrast, the number of other contacts (with walls other than the one separating the two birds) was not correlated with Proximity Time ($r = 0.094$, $p > 0.7$) or Contacts ($r = 0.029$, $p > 0.5$). Hence, Proximity Time and Contacts appear to provide meaningful measures of agonistic response that are not correlated with general activity.

Proximity Time was negatively correlated with Fos-ir cell counts in the PVN ($r = 0.538$, $p < 0.05$). As shown in Figure 1A-D, Contacts were negatively correlated with the numbers of Fos-ir nuclei in multiple zones of the LSc (ventral, ventrolateral, and dorsal) and in the PVN. The correlation between Contacts and the number of Fos-ir nuclei in the AH also approached significance (Fig. 1E). Zenk-ir cell counts were negatively correlated with Contacts in the LSr, ventrolateral and lateral LSc, and PVN (Fig. 1F-I).

Discussion

The present data provide several novel insights into basal forebrain circuits that are relevant for the regulation of agonistic behavior in birds, including the first data on 1) Zenk responses to agonistic stimuli during the non-breeding season, 2) basal forebrain Fos responses to agonistic stimuli, 3) the relationships between IEG activity and aggressive behavioral responses to STI, and 4) the relationship between plasma DHEA and IEG responses to STI.

Zenk responses to STI across seasons

We recently examined Zenk responses to STI and nonsocial stress (handling and restraint) during the breeding season in male song sparrows [1]. Zenk responses to both STI and nonsocial stress were obtained in the LSr, LSc.l, and lateral VMH. In addition, a selective response to STI was found for the LSc.vl and commissural BSTm. The present data from the non-breeding season are very similar, although not identical. Thus, we observed a significant Zenk response in the LSc.vl, LSr and commissural BSTm, but not for the LSc.l and lateral VMH (although a

strong trend was observed for Fos in the lateral VMH; see Table 1). In addition, we did not find a significant Zenk response in areas that previously showed only a Zenk response to nonsocial stress – the LSc.d, LSc.v, BSTl, PVN POA and AH [1]. Note that the two regions that respond selectively to STI in spring (LSc.vl and commissural BSTm) also showed Zenk responses in autumn, supporting the hypothesis that these regions are specifically involved in the control of aggression. It is possible that responses to STI in the LSc.l and lateral VMH require elevated plasma T and are specific to the breeding season. Our findings here for the BSTm are also comparable those in other birds, which have also shown that IEG responses to social stimuli are localized to the commissural level of the avian BSTm, but not to post-commissural levels [1,18].

Fos responses to STI

Previous experiments demonstrate that Fos provides a marker of neural response that is complementary to and only partially redundant with Zenk [4,5], and a similar result was obtained here. Thus, we observed a significant response in the LSc.vl with both Fos and Zenk, but in the AH, a significant response was observed only for Fos.

Comparisons with other species

The present results are extensively consistent with recent experiments in estrildid finches and waxbills [5], in which we exposed males and females of four species to a same-sex conspecific. These species are matched for important aspects of behavior and ecology, but differ strongly their species-typical group sizes (we employed one territorial, one modestly gregarious, and two highly gregarious species). The testing paradigm limited behavioral performance, thus species differences in IEG response likely reflect divergence in perceptual and/or motivational processes. The territorial species exhibited significantly greater IEG responses to the social stimulus within the LSc.vl, LSc.l, AH, and lateral VMH. Fos responses within the extended medial amygdala (TnA and BSTm), were also negatively correlated with gregariousness. Hence, these areas may be particularly relevant for the expression of territorial behavior.

In mammals, agonistic encounters elicit significant Fos responses within a similar suite of areas – the extended medial amygdala, LS (particularly the ventrolateral LS), AH, and lateral VMH/ventrolateral hypothalamus [19-21]. Although these areas show increased IEG expression following agonistic encounters, it is not necessarily the case that these areas promote aggressive behaviors. For instance, subordinate male hamsters show substantially greater Fos responses than do dominant animals within the LS, lateral VMH and AH [21]. Here we found that aggressive behavior correlates negatively with IEG responses in a very similar group of brain regions, with the exception of the lateral VMH. Interestingly, the overall pattern is also extraordinarily similar to that observed during pup avoidance in female rats [22]. Hence, increased activity in these sites likely does not reflect aggressive motivation *per se*, but rather social aversion and avoidance.

DHEA and responses to STI

Previous data on wild non-breeding song sparrows demonstrate that DHEA increases agonistic singing in response to STI, but not other agonistic behaviors (e.g., flights and approach) [11]. Here, we examined whether DHEA influences IEG responses to STI in brain areas that express sex steroid receptors and/or enzymes that metabolize DHEA to sex steroids [12,23-25]. We found no effect of DHEA manipulations on behavior, although our current testing paradigm did not elicit a song response (likely due to the close range of interaction). However, we did observe a significant negative correlation between plasma DHEA and Fos-ir cell counts in the BSTm, and near-significant negative correlations with Fos-ir nuclei in the BSTl and Zenk-ir nuclei in the LSc.v. Overall, these observations are consistent with the profile of more aggressive birds (see Figure 1).

Conclusion

IEG responses to agonistic encounters have not been extensively studied in birds. Two studies in free-living song sparrows showed that STI elicits a Zenk response in the midbrain central gray and areas related to audition and song (the basal forebrain was not examined) [2,3], and data from captive song sparrows were collected from subjects that were concurrently exposed to a handling stressor [1]. All three of these studies were conducted during the breeding season. Despite the differences between our previous and present studies in season (spring vs. autumn) and testing conditions (handling vs. no handling), we obtained similar results for Zenk. In addition, the present experiments provide novel data on Fos responses to STI and provide the first demonstration that aggressive behavior in birds correlates with IEG expression in the basal forebrain. Finally, we observed a pattern of IEG response that is consistent with patterns of IEG response that have been observed in rodents [19–21], suggesting that neural circuitry relevant to aggression has been strongly conserved during vertebrate evolution.

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Figure 1.

A-E. Correlations between aggressive behavior ($\ln$ the number of barrier contacts during a 10-min observation) and Fos-ir cell counts in the ventral, ventrolateral and dorsal zones of the caudal lateral septum (LSc.v, LSc.vl and LSc.d; **A-C**, respectively), paraventricular hypothalamus (PVN; **D**), and anterior hypothalamus (AH; **E**) of male song sparrows exposed to a simulated territorial intrusion ($n = 16$). **F-I.** Correlations between barrier contacts and Zenk-ir cell counts in the rostral LS (LSr; **F**), LS.vl (**G**), lateral zone of the LSc (LSc.l; **H**), and PVN (**I**). Cell counts are shown as the number of immunoreactive nuclei per $100 \mu\text{m}^2$.