Neural and Neuroendocrine Processing of a Non-Photic Cue in an Opportunistically-Breeding Songbird

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ABSTRACT

Recent studies of the onset of breeding in long-day photoperiodic breeders have focused on the roles of type 2 and 3 iodothyronine deiodinases (DIO2 and DIO3) in the conversion of thyroxine (T4) to triiodothyronine (T3) and subsequent activation of the reproductive axis. It has been hypothesized that an increase in DIO2 and reciprocal decrease in DIO3 causes the release of gonadotropin-releasing hormone (GnRH) from the hypothalamus, setting off a reproductive cascade, and that this DIO mechanism for GnRH release is conserved across vertebrate taxa. We sought to test whether social cues that are known to stimulate reproductive behaviors can activate the DIO system to initiate reproduction in a non-photoperiodic bird, the zebra finch (*Taeniopygia guttata*). Isolation of males and subsequent presentation of females did not increase DIO2 or GnRH expression in the hypothalamus, nor did it decrease gonadotropin-inhibitory hormone (GnIH) or DIO3. Males receiving a female stimulus showed significantly higher mRNA expression and immunoreactive cell count of the immediate early gene early growth response protein 1 (EGR-1) than isolated males, indicating hypothalamic activation in response to a female. Cells immunoreactive for EGR-1 were not co-localized with those immunoreactive for GnRH. Reproductive behaviors (singing, copulation attempts, and overall activity) were significantly higher in males receiving a female stimulus. This study presents a social effect on behavior and EGR-1 expression in the hypothalamus of males in response to females, but more research is needed to determine if the DIO2 system and the GnRH system are responsive to social stimulation in this species.

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INTRODUCTION

The physiological mechanisms allowing animals to time breeding appropriately in response to environmental cues have been the subject of research for almost a century. Recent studies of the onset of breeding in long day photoperiodic breeders have focused on the role of type 2 iodothyronine deiodinase (DIO2) in the conversion of thyroxine (T4) to triiodothyronine (T3) and subsequent activation of the reproductive axis. In seasonally-breeding Japanese quail, *Coturnix japonica*, long day lengths induce an increase in the expression of the thyroid stimulating hormone beta subunit (*TSHβ*) followed by an increase in local expression of *DIO2* and a decrease in type 3 iodothyronine deiodinase (*DIO3)* in the hypothalamus (Yoshimura et al. 2003, Nakao et al. 2008). In this DIO model the local photo-induced increase in expression of *DIO2* and decrease in *DIO3* is viewed as a "reciprocal switch", causing an increase in locally-available T3 in the hypothalamus. While it has not been demonstrated directly, this model suggests the increase in hypothalamic T3 causes a release of gonadotropin-releasing hormone (GnRH) from the hypothalamus, thus increasing expression and release of the gonadotropins follicle stimulating hormone (FSH) and luteinizing hormone (LH) from the pituitary and activating the gonads.

The reciprocal switching of *DIO2* and *DIO3* is hypothesized to be a conserved mechanism of reproductive activation in all vertebrates (Nakane and Yoshimura, 2014), though this has only been tested in photoperiodic breeders (Yoshimura et al. 2003, Revel et al. 2006, Hanon et al. 2008). If the DIO system is an evolutionarily-conserved mechanism that regulates GnRH release then it is likely that it responds to cues other than day length, given that multiple cues can influence GnRH release. Species that do not respond reproductively to changes in day length and those that live in areas where day lengths do not differ throughout the year could instead activate the DIO system to cause a release in GnRH in response to stimulatory cues such as availability of mates, food, or nesting sites.

Non-photoperiodic breeders that breed whenever conditions are favorable are termed opportunistic breeders (Dawson et al. 2001). To time breeding appropriately, these species - such as zebra finch, *Taeniopygia guttata* - respond to and integrate proximate cues of food and water availability, mating opportunities, and other resources to time reproduction (Perfito et al. 2007, 2008, Zann et al. 1995, Hahn et al. 1995). The mechanism(s) underlying reproductive activation in response to food,

water, or mate availability remain(s) unknown, thus we sought to investigate a potential role for the DIO system in reproductive activation in this species

The hypothalamic neuropeptides GnRH and gonadotropin-inhibitory hormone (GnIH) are two likely key integrators of environmental stimuli for reproduction. As such, they are also likely to interact with the DIO system to control breeding. While breeding zebra finches do not differ from non-breeders in hypothalamic immunoreactivity of GnRH and GnIH (Perfito et al. 2011), the DIO system could be acting to regulate GnRH release when breeding commences. This, together with the proposed role of DIO in activation of breeding in photoperiodic species, led us to test if the DIO, GnRH and GnIH systems respond to a social stimulus in zebra finches. Specifically, we sought to test whether there is a reciprocal switch between *DIO2* and *DIO3* expression following social stimulation of an opportunistic breeder, similar to that seen in response to long days in a photoperiodic species (Yasuo et al. 2005). When males were housed in isolation and deprived of social cues, we expected to see a decrease in *DIO2* expression, an increase in *DIO3* expression and decreases in GnRH, LH, and testosterone (T). Following presentation of a potential mate to these isolated males, we expected to observe an increase in *DIO2* expression, a decrease in *DIO3* and *GnIH* expression and increases in GnRH, LH and T. We expected to see these changes in association with an increase in reproductive behaviors in response to a new female and sought to characterize activity of the DIO system in a social paradigm.

RESULTS

Real-time quantitative PCR

Hypothalamic expression of *DIO2* and *DIO3* did not differ across groups (Fig 1 A, B) (ANOVA, DIO2: $F_{(2,21)}=0.264$ $p=0.599$, DIO3: $F_{(2, 19)}=0.624$, $p=0.547$). Expression of GnRH and GnIH was also similar across groups (Fig 1 C, D) (ANOVA, GnRH: $F_{(2,21)}=1.907$, $p=0.173$, GnIH: $F_{(2,21)}=2.321$, $p=0.123$). Expression of the housekeeping gene *18S* did not differ across groups ($F_{(2,21)}=0.117$, $p=0.256$). Early growth response protein 1 (*EGR-1)* expression was greater in the group receiving females as compared to the isolated group (ANOVA, $F_{(2,21)}=3.507$, $p<0.049$), while the group exposed to males had an intermediate level of *EGR-1* expression. Tukey's multiple comparisons test produced a mean difference of -1.035 between the isolated

group and the group receiving females, indicating a significant difference between these two groups (Fig 2 A). Pituitary expression of the beta subunit of FSH (FSHβ) did not differ across groups (ANOVA, $F(2,21)=1.080$, $p=0.358$). GnIH expression in the gonads was also not significantly different between groups (ANOVA, $F(2,21)=1.528$, $p=0.240$).

Immunocytochemistry

Analysis of immunocytochemistry revealed no significant differences in GnRH immunoreactive cell number (GnRH-ir) between groups ($F(2,21) = 0.606$, $p=0.555$) (Fig 4). In sets of tissue double-labeled for EGR-1 and GnRH, EGR-1 was not found to be co-localized with GnRH-ir cells. Analysis of single-label EGR-1 ICC showed that number of EGR-1 labeled cells was significantly greater in the hypothalamus in the female stimulus group as compared to the isolate group and the male stimulus group (Figure 2B, $F(2,21) = 6.686$, $P<0.04$, ANOVA, Tukey's multiple comparisons test where $p<0.05$ as indicated by letters a, b).

Circulating Plasma Hormones and Testicular Volume

Enzyme immunoassay (EIA) revealed circulating testosterone was significantly higher in males receiving a male stimulus and those receiving a female stimulus as compared to isolated males (Fig 5A, Multiple comparison t-tests with Sidak-Bonferroni correction, $p<0.012$ for isolated vs. male stimulus, $p<0.021$ for isolated vs. female stimulus). The male stimulus group did not differ in circulating testosterone level from the female stimulus group (Multiple comparison t-tests with Sidak-Bonferroni correction, $p=0.848$)

Luteinizing hormone (LH) did not differ across groups (Fig 5B, $F(2,21)=1.958$, $p=0.166$), nor did circulating corticosterone (Fig 5C, $F(2, 14)=0.555$, $p=0.586$). The RIA was run first followed by the EIAs for testosterone and corticosterone, thus plasma was not available from all birds for measurement of testosterone (total of 8 samples in the isolated and female stimulus groups and 7 in the male stimulus group) and corticosterone (total of 6 samples for isolated and female stimulus groups and 5 for male stimulus group). Testis volume was not significantly different across groups ($F(2,21)= 0.985$, $p=0.211$).

Behavior

Prior to presentation of a stimulus, animals in all three groups had similar levels of activity (defined as time resting subtracted from the total time) ($F_{(2,21)}=0.212$, $p=0.81$). The isolate and male groups maintained levels of activity similar to that of their baseline recording, while males presented with a female were more active ($F_{(2,21)}=14.42$, $p<0.0002$) (Fig. 6A). Males in the presence of a female also showed significantly more copulation attempts ($F_{(2,21)}=14.11$, $p<0.0001$) (Fig. 6B) and greater number of songs than isolated males and those in the presence of a male ($F_{(2,21)}=4.74$, $p<0.02$, ANOVA) (Fig. 6C).

DISCUSSION

In this experiment we sought to test whether social cues activate the DIO system in male zebra finches. If the mechanism of DIO-induced GnRH release is evolutionarily conserved across vertebrate taxa, and if there is no separate or additional mechanism for regulating GnRH release, we would expect *DIO2/DIO3* expression in non-photoperiodic species to respond to stimulatory cues other than photoperiod. We predicted that the HPG axis would be activated in male zebra finches that were presented with a female after isolation. Thus, we also predicted that there would be reciprocal switching of *DIO2* and *DIO3* expression in the hypothalamus, precipitating other indicators of reproductive activation (increased GnRH expression, increased circulating LH or T, increased reproductive behaviors). In our non-photoperiodic model and within this experimental paradigm, no changes in the DIO system were observed with a social stimulus.

Males receiving a female stimulus showed increased levels of activity, song behavior, and copulation attempts compared to isolates or those receiving a male stimulus, suggesting males were responding to females with appropriate reproductive effort. Males with a female stimulus also showed a physiological response to these females in the form of an increase in *EGR-1* expression and EGR-1 protein immunoreactivity in the hypothalamus. It is unknown, however, what cell type is being activated in response to the social stimulus. In double-label immunocytochemistry EGR-1 was not found to be co-localized with GnRH. While this indicates that GnRH-ir cells might not be directly activated with presentation of a female, EGR-1-ir cell count in the hypothalamus did increase, including in the pre-

optic area where GnRH cells are located. A similar pattern in immediate early gene immunoreactive (IEG-ir) cells surrounding but not co-localized with GnRH cells was observed in white-crowned sparrows following treatment with NMDA (Meddle et al. 1999). In that study increased C-FOS immunoreactivity was associated with an increase in LH release, suggestive of GnRH release. While the activation of hypothalamic cells shown with an increase in EGR-1 indicates a hypothalamic response to female stimulus, theoretically this increase in expression could be associated with the change in behavior seen with the presence of a female as opposed to causing an increase in activity in the reproductive axis.

Males exhibited a behavioral response to the presence of a female, but other reproductive parameters usually associated with reproductive activation, including increased GnRH expression and increased circulating LH, did not change. Likewise, no changes in expression of *DIO2* or *DIO3* were found. While thirty minutes is considered sufficient time for changes in expression to be seen, it is possible that dynamic changes of *DIO2, DIO3,* and *GnRH* expression were missed due to the analysis of a single time point. Experiments inducing *DIO2* expression in photoperiodic breeders with a photic stimulus show great variation in timing of *DIO2* expression after stimulus presentation. Japanese quail show a change in expression a few hours into the first long day (Yoshimura et al. 2003), while Syrian hamsters showed increases in *DIO2* expression 8 days after transfer from short to long day lengths (Yasuo et al. 2009). *DIO2* expression can be stimulated by social cues in photoperiodic breeders several days after stimulus presentation (Perfito et al. 2015), and thus an experiment with many time points after social stimulation would be required to provide a definitive conclusion as to whether the *DIO* system responds to the presence of a potential mate in zebra finches. Alternately, a time-course study of zebra finches could show an increase in *GnRH* expression and release independently from changes in *DIO2* expression, as was found in European starlings (Bentley et al. 2013). Testosterone was significantly higher in males receiving a female stimulus than isolated males. Testosterone was also higher in males receiving a male stimulus, indicating an increase in testosterone with all social stimuli, not with female stimulus only.

In summary, this study provides evidence that male zebra finches respond to potential mates with increased reproductive effort and increased activity of the hypothalamus. The lack of changes seen in *DIO2, DIO3, GnIH* and *GnRH* expression

across groups indicate that either these components of the reproductive axis are not influenced by social cues in this species, or that our experimental time-course was not sufficient to reveal the influence of social cues reproductive activation. If the DIO system is truly an evolutionarily conserved mechanism in the initiation of vertebrate reproduction, then further experiments over different time-frames will elucidate changes in the DIO response to social cues.

MATERIALS AND METHODS

Study Animals

Adult male zebra finches were bred and housed in mixed-sex free-flight aviaries at the Field Station for the Study of Behavior, Ecology, and Reproduction at the University of California, Berkeley. Birds are considered adult at 90 days post-hatch. Birds were exposed to natural changes in day length that were supplemented with full-spectrum artificial light to create a minimum 12L:12D photoperiod. Birds were supplied with water and German millet mixed with canary seed *ad libitum*. Food was supplemented with cuttlebone, grit, and lettuce weekly. All animal care and procedures were approved by the University of California Office of Laboratory Animal Care and conducted in accordance with local animal welfare laws and policies.

Study Design

Adult male zebra finches were caught from the colony between 10 and 11 am and transferred to an isolation cage (18”x8”x12”) inside a custom-made temperature and light-controlled insulated box. Within each box, males did not receive any visual or auditory input from the colony or adjacent isolation boxes. For the two days of isolation males were maintained on the 12L:12D photoperiod they had experienced in a colony setting and had *ad libitum* access to water and German millet mixed with canary seed.

After two days of isolation birds (n=8 per group) were randomly assigned to one of 3 treatments: 1) maintained in isolation (isolated), 2) presented with a novel female for 30 minutes (female stimulus), or 3) presented with a novel male for 30 minutes (male stimulus). Behavior was recorded for 30 minutes before and 30 minutes after stimulus presentation for all treatments. The novel stimulus birds came

from separate colony rooms, ensuring that the experimental males had had no previous experience with these stimulus animals.

After treatment, experimental males were deeply anesthetized with isoflurane (Phoenix Pharmaceuticals Inc., Burlingame, CA, U.S.A.) and rapidly decapitated, at which time trunk blood was collected. Brain and testis tissue were frozen immediately on dry ice (Experiment 1). Heads with pituitary tissue intact were frozen on dry ice and the pituitaries were subsequently extracted under a dissection microscope.

This experiment was repeated with six birds in each group. In this second experiment the brains were fixed in a 4% paraformaldehyde solution (PFA) for three days before being cryoprotected in 30% sucrose in 0.1 M phosphate-buffered saline (PBS) and frozen (Experiment 2).

Tissue Processing

Trunk blood was spun in a centrifuge at 4 ºC at 1500 g for 10 minutes to separate blood plasma. Plasma was stored at -80 ºC before assay for LH, testosterone, and corticosterone. Pituitaries were extracted from the skull and placed into 1 ml of TRIzol reagent (Invitrogen by Life Technologies, Grand Island, NY, U.S.A.) and homogenized. One testis from each bird was placed into 1 ml of TRIzol and homogenized.

Brains were cut into 40-µm thick slices on a cryostat (cm3050s, Leica Microsystems, Buffalo Grove, IL, U.S.A.) and mounted directly on to slides. Brain tissue was mounted onto slides beginning with the appearance of the tractus septomesencephalicus (TrSM), a neuroanatomical landmark anterior to the hypothalamus, and all slices were mounted until the appearance of the cerebellum. 3mm punches were taken through the hypothalamus from alternating sections and placed in TRIzol. All sections were mounted on slides for subsequent immunocytochemistry (ICC). The brains put in PFA immediately after collection were also cut into 40-µm thick slices, but were stored in antifreeze prior to ICC. Prior to freezing, length and width of testes were measured to assess fresh testis volume. Volume was calculated as $V=4/3\pi a^2 b$, where a is half the width and b is half the length (long axis) of the testis.

RNA was extracted from hypothalamus, pituitary, and gonadal tissue using chloroform (as described in Perfito et al. 2012). RNA extracts were treated with a DNAse (Invitrogen by Life Technologies, Grand Island, NY, U.S.A.) to digest any single- and double-stranded DNA and subsequently reverse transcribed using M-MLV reverse transcriptase (Promega Corporation, Madison, WI, U.S.A.) to create cDNA from each tissue.

Real-time quantitative PCR

Real-time quantitative polymerase chain reaction (qPCR) on cDNA obtained from hypothalamic punches was run for a number of genes: *DIO2, DIO3, GnRH, GnIH,* and *EGR-1*, an immediate-early gene (IEG). This IEG was taken as an indicator of general activation of neurons (Hoffman et al. 1993, Morgan and Curran 1989, 1995). The reference gene *18S* was used as a control gene in hypothalamic, pituitary, and gonadal tissue as its expression did not change with treatment in any tissue. cDNA was diluted 1:25 with water treated with diethylpyrocarbonate (DEPC). Primers were designed for each gene from previously published sequences in the zebra finch genome (see Table 1 for accession numbers**)** using Primer3 software (simgene.com). qPCR was performed using manufacturer's instructions for SYBR green reagent (Applied Biosystems by Life Technologies, Grand Island, NY, U.S.A.). Post-qPCR products were cloned, sequenced, and compared to GenBank to confirm identification of target genes. Raw fluorescent data were analyzed with the RT-PCR Miner program (Zhao and Fernald, 2005) and cycle thresholds were obtained from this program. Expression values were calculated as $1/(1+E)^{\wedge}Ct$, where E is the average PCR efficiency for the gene of interest as calculated by a standard curve and Ct is the cycle threshold. Data are shown as fold-change, which was calculated by dividing the expression of the gene of interest (corrected for the reference gene *18S*) by the average expression for the isolate group.

Immunocytochemistry

Immunocytochemistry (ICC) was performed on series of brain tissue to label GnRH and EGR-1 proteins. One series of brains from experiment 1 (mounted on slides) were single-labeled for GnRH and another series was double-labeled for GnRH and EGR-1. EGR-1 single-label ICC was conducted on one series of brain tissue from

experiment 2 (free-floating brain sections) that had been fixed at the time of collection. For GnRH single-label and GnRH/EGR-1 double-label slides were fixed in 4% paraformaldehyde (PFA) for 20 minutes and washed in 0.1M phosphate-buffered saline (PBS, pH 7.4) three times. Slides were then incubated in 0.03% hydrogen peroxide in methanol for 30 minutes followed by three PBS washes and one hour of incubation in 2% normal goat serum (Vector Laboratories, Burlingame, CA, U.S.A.) in 0.2% phosphate-buffered saline with Triton x-100 (PBS-T). Slides were incubated in GnRH primary antibody (HU60, a gift from Dr. Henryk Urbanski, Portland, OR, U.S.A.) 1:5000 in PBS-T for one hour at room temperature and subsequently for 48 hours at 4 ºC. After incubation in the primary antibody slides were washed with PBS, incubated in the secondary antibody biotinylated goat anti-rabbit (1:250 in 0.2% PBS-T, Vector Laboratories, Burlingame, CA, U.S.A.) for one hour, washed in PBS-T, and incubated in avidin/biotinylated enzyme complex (PK-6100, Vectastain Elite ABC Kit (Standard), Vector Laboratories, Burlingame, CA, U.S.A.) as per manufacturer's instructions. Slides were washed in PBS and incubated with DAB peroxidase substrate kit (SK-4100, Vector Laboratories, Burlingame, CA, U.S.A. For double-label slides were then incubated in the primary antibody for EGR-1 (c-19, sc-189, Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) 1:1000 in 0.2% PBS-T for 24 hours. Slides were then washed and incubated as described above with biotinylated goat anti-rabbit secondary antibody, then ABC, then DAB with nickel sulfate. For an EGR-1 single-label all steps were the same as described above on free-floating tissue, then brain tissue was mounted onto slides. All slides were then dehydrated with graded ethanol, cleared in xylenes and coverslipped with Permount (SP 15-100, Fisher Scientific Inc., Waltham, MA, USA).

Quantification of Immunocytochemistry

Cell counts for all immunocytochemistry were obtained by a researcher unaware as to experimental treatment. All cells immunoreactive (-ir) for GnRH were counted for each animal and assessed for co-localization with EGR-1. Single-label EGR-1 was counted at 20x magnification centered around the midline with the base of the brain just visible in the microscope field from the pre-optic area (POA) through the appearance of the cerebellum. All EGR-1-ir cells in this space were counted for each individual and averaged over the total number of sections counted for analysis.

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Hormone assays

Concentrations of testosterone and corticosterone in plasma were measured using enzyme immunoassay kits (EIA, Enzo Life Sciences, Farmingdale, NY, U.S.A.). These kits have been validated and optimized for zebra finches **(**Lynn et al. 2007, 2010**)**. Protocols for both hormones were followed as specified by Lynn et al. 2010. Plasma was diluted 1:40 for testosterone and corticosterone EIA. All samples were run in duplicate on one plate per hormone, with a standard curve also in duplicate for comparison.

Radioimmunoassay for Luteinizing Hormone

Plasma was assayed in 15 µl duplicate samples in a single assay using a micro-modification of the radioimmunoassay originally devised by Follett and colleagues (1975). Intra-assay coefficient of variation was 3.4% and the lower detection limit was 0.07 ng/ml.

Analysis of Behavior

Video recordings were taken before and after presentation of the stimulus animal with security cameras (SWADS-120CAM-US, Swann Communications, Santa Fe Springs, CA, U.S.A.) and recorded with a 4-channel DVR (Q-See, Anaheim, CA, U.S.A.). Animals were recorded for 30 minutes prior to stimulus presentation and 30 minutes during stimulus presentation. Individuals receiving no stimulus animal had their cages opened and handled in a similar fashion to those receiving a stimulus animal. Behavior was recorded for each individual animal by counting copulation attempts and number of songs and by timing the duration of total time resting. Activity levels were calculated by subtracting the time resting from total time of recording. Number of songs was determined by counting song bouts with five or more seconds between each song. Due to the low number of songs, directed (towards the stimulus animal) and undirected (with no obvious intended recipient) songs were combined and taken as a measure of total song activity. The first five minutes of each video were not included in the analysis to allow animals time to adjust to the experimenter opening the cage. Analysis of video was conducted by researchers with no explicit knowledge of the experimental treatment of animal or experimental design. Analysis of video could not be completed entirely blind, as the researcher analyzing the video could also

see the stimulus animal. The researcher was given 30 minute videos to watch, and was not told whether it was a video of the animal prior to stimulus or a control animal that received no stimulus.

Statistical Analysis

One-way analysis of variance (ANOVA) tests were performed for immunoreactive cell counts, gene expression as measured by qPCR, and hormone concentrations from RIA and EIA tests. One-way ANOVA tests were also performed for behavioral measures of total time active, copulation attempts, and number of songs. Significance was set at $p<.05$. In cases of statistical significance, Tukey's test of multiple comparisons was used to assess which groups were significantly different. All statistical analyses were performed using Prism 6 (GraphPad, La Jolla, CA, U.S.A.).

Experiment 1 had 8 animals per group, though there are some data sets where all animals could not be used for analysis. For circulating plasma hormones, radioimmunoassay for luteinizing hormone was performed first, followed by EIAs for testosterone and corticosterone. Thus, there was not enough plasma collected from some birds to run all three assays. For analysis of real-time quantitative PCR (qPCR), samples with expression levels 3 or more standard deviations above the mean were considered outliers and were excluded from analysis. This occurred for two samples for expression of *DIO3*, one from the male stimulus group and one from the female stimulus group giving a sample size of 8 for the isolate group and 7 each for the male stimulus and female stimulus groups.

List of symbols and abbreviations:

ANOVA Analysis of variance
DEPC Diethylpyrocarbonate
DIO Deiodinase system of DIO2 and DIO3
DIO2 Type 2 iodothyronine deiodinase
DIO3 Type 3 iodothyronine deiodinase
EGR-1 Early growth response protein 1
EIA Enzyme immunoassay
FSH Follicle stimulating hormone
FSHβ Follicle stimulating hormone beta subunit
ICC Immunocytochemistry
IEG Immediate early gene

LH Luteinizing hormone
GnIH Gonadotropin-inhibitory hormone
GnRH Gonadotropin-releasing hormone
GnRH-ir Gonadotropin-releasing hormone immunoreactive cells
PBS 0.1 M phosphate-buffered saline
PFA 4% paraformaldehyde solution
qPCR Real-time quantitative polymerase chain reaction
T Testosterone
T3 triiodothryonine
T4 thyroxine
TrSM Tractus septomesencephalicus
TSHβ Thyroid stimulating hormone beta subunit

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Author contributions: D.E. and G.B. designed the study. D.E. conducted the study and carried out all laboratory work: hormone assays, qPCR, and ICC. D.E. performed all data analysis. D.E. and G.B. drafted the manuscript and gave final approval for publication.

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Figures

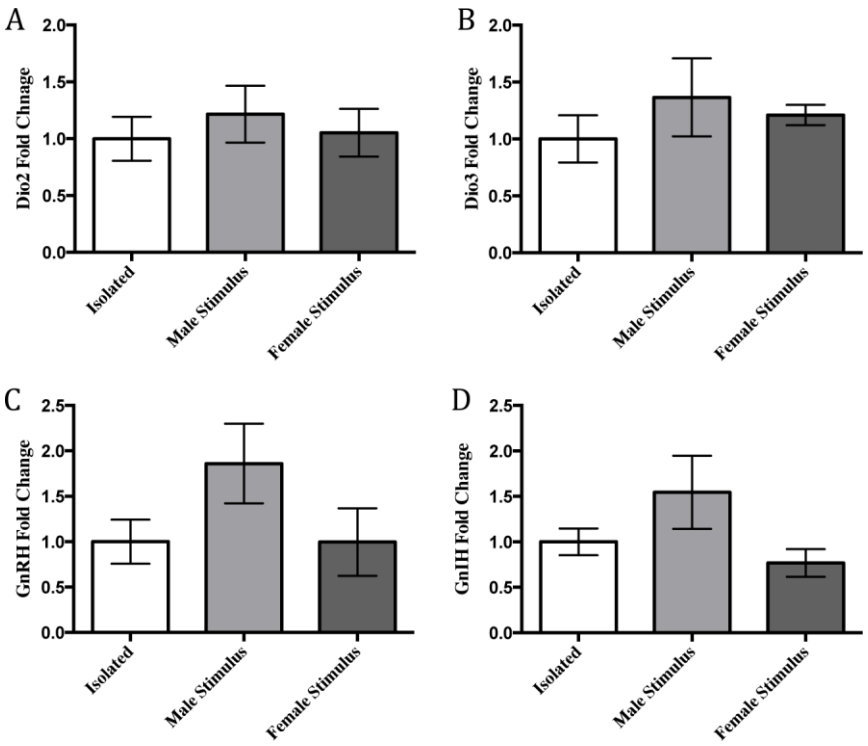

Figure 1. Fold change in mRNA expression of *DIO2* (A), *DIO3* (B), *GnRH* (C), and *GnIH* (D). The isolated group is shown in white, group given a male stimulus in light gray, and group given a female stimulus in dark gray. Fold change across groups (n=8 per group) after 30 minutes of treatment was not significantly different for any of these genes (ANOVA, *DIO2*: F (2,21)=0.264 p=0.599, *DIO3*: F (2, 19)=0.624, p=0.547, *GnRH*: F (2,21)=1.907, p=0.173, *GnIH*: F (2,21)=2.321, p=0.123). Data is shown as mean +/- s.e.m. This experiment was completed once.

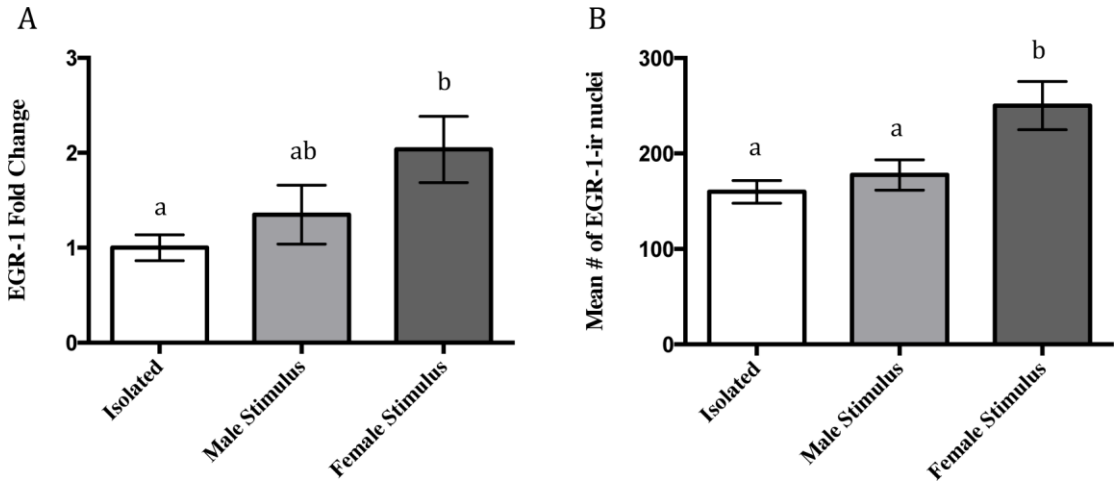

Figure 2. EGR-1 mRNA expression (A) and protein (B). A. Fold change in immediate-early gene *EGR-1* mRNA expression (mean +/- s.e.m.) in the hypothalamus was significantly different across groups (ANOVA, $F(2,21)=3.507$, $p<0.049$). Tukey's multiple comparisons test produced a mean difference of -1.035 between the isolated group and the group receiving females, indicating a significant difference between these two groups, shown here where *a* is significantly different from *b* Birds given a male stimulus show an intermediate amount of *EGR-1* expression, not significantly different from the level of expression in the isolates or those receiving a female, indicated by *ab*. B. There were significantly more EGR-1-ir cells in the hypothalamus of males receiving a female as compared to isolates and those receiving a male stimulus (ANOVA, $F(2,21) = 6.686$, $P<0.04$). Data is shown as mean +/- s.e.m. This experiment was completed once.

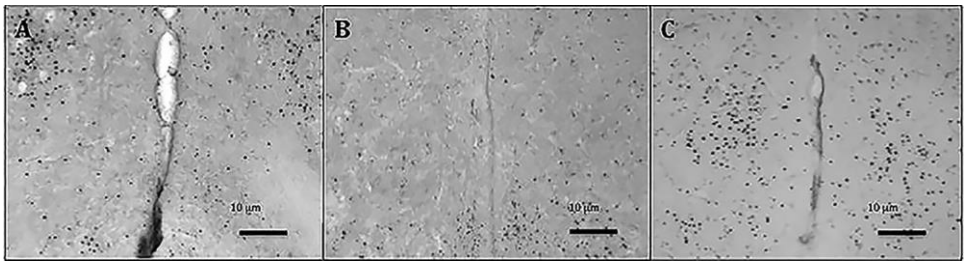

Figure 3. Single-label EGR-1 immunocytochemistry. Representative photographs of EGR-1 positive cells in the pre-optic area (POA) of the hypothalamus of isolated males (A), males receiving a male stimulus (B), and males receiving a female stimulus (C).

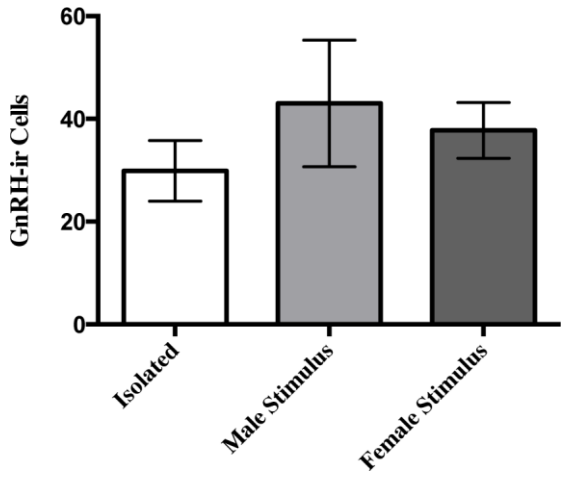

Figure 4. Single-label GnRH Immunocytochemistry. No significance difference in GnRH-ir cell count was found between groups ($F(2,21) = 0.606$, $p=0.555$). Data is shown as mean +/- s.e.m. This experiment was completed once.

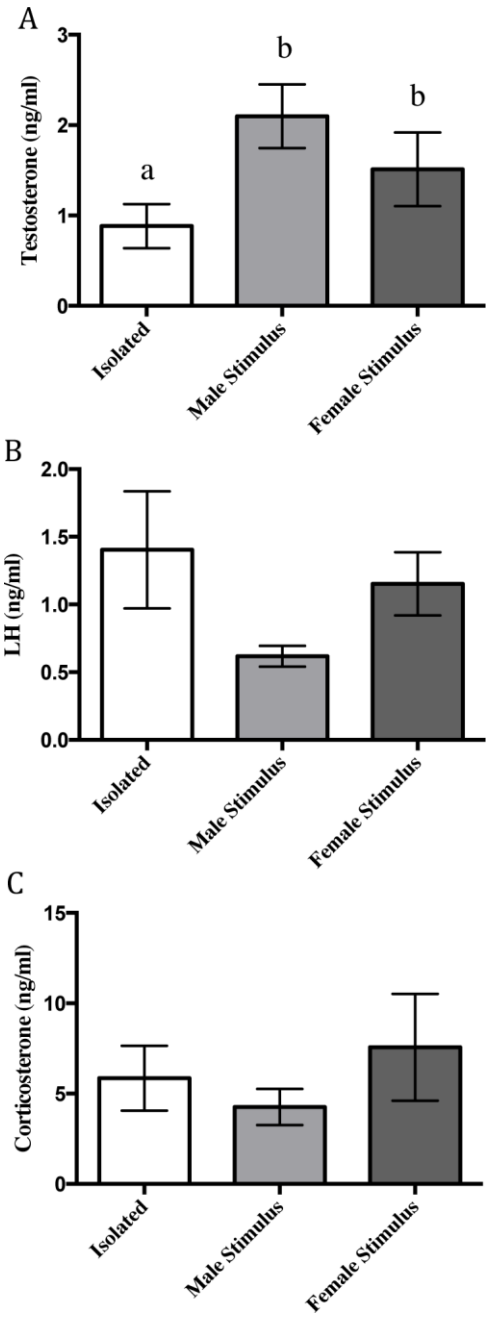

Figure 5. Circulating concentrations of testosterone (A), luteinizing hormone (B), and corticosterone (C). Testosterone (mean +/- s.e.m.) was significantly higher in males receiving a male stimulus and those receiving a female stimulus as compared to isolated males (Multiple comparison t-tests with Sidak-Bonferroni correction, $p<0.012$ for isolated vs. male stimulus, $p<0.021$ for isolated vs. female stimulus). The male stimulus group did not differ in circulating testosterone level from the female stimulus group (Multiple comparison t-tests with Sidak-Bonferroni correction, $p=0.848$). Luteinizing hormone (mean +/- s.e.m.) did not differ across groups (F

(2,21)=1.958, p=0.166), nor did circulating corticosterone (mean +/-s.e.m., F (2, 14)=0.555, p=0.586). This experiment was completed once.

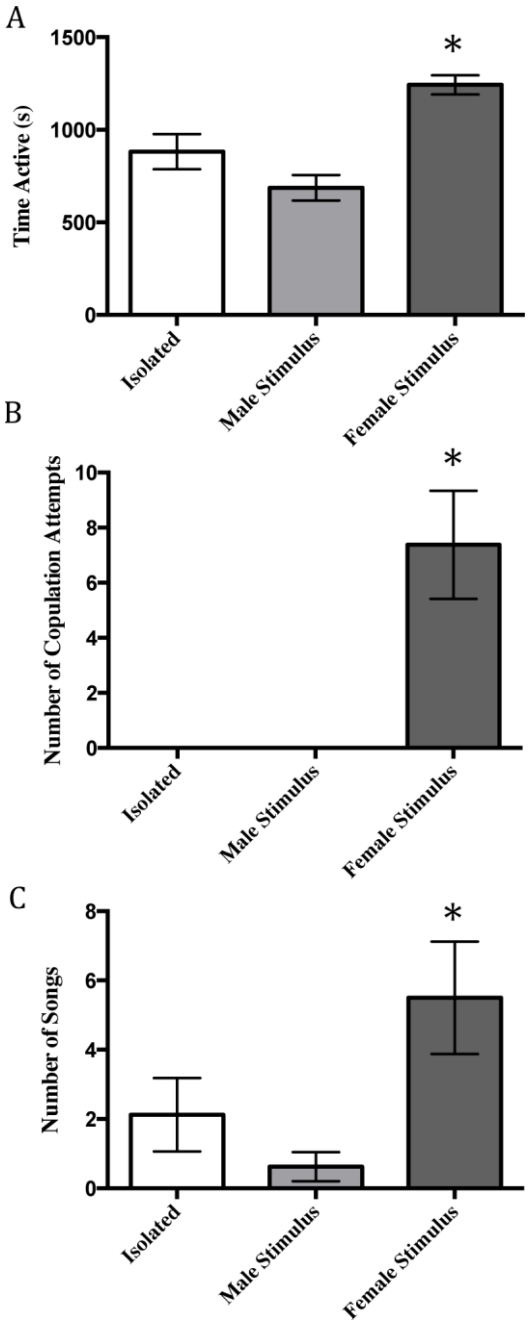

Figure 6. Behavioral Output. In the presence of a female, males were more active and exhibited increased sexual behavior. Males given a female stimulus for 30 minutes showed increased activity (A) ($F(2,21)=14.42$, $p<0.0002$), number of copulations (B) ($F(2,21)=14.11$, $p<0.0001$), and number of songs (C) ($F(2,21)=4.74$, $p<0.02$, ANOVA) as compared to isolated males and those given a male stimulus. Data is shown as mean +/- s.e.m. This experiment was completed once.

Tables

Gene	Forward Primer	Reverse Primer	bp	Genbank Accession #
Dio2	CAGGTCAAACTGGGAGGAGA	CACACTTGCCACCAACATTC	103	NM_001270969
Dio3	TACAACATCCCCAAGCACCA	TCTGCCTCCCTGGTACATCA	186	XM_004174551
GnRH	ACTCCACAACCTCTCTCAGG	CTCTGCTGCTCCTCCTCTAA	209	NM_001142320, XM_002197400
GnIH	CCCTGAGATTTGGAAGAGC	CAGATTGACAGGCAGTGAC	152	AB522971.1
EGR-1	AACGAGAAAACCCTGCCAGA	TCCACTGACGAGGCTGAAGA	147	NM_001080957.1
18S	CCATCCAATCGGTAGTAGCG	GTAACCCGTTGAACCCCATT	151	HQ873432.1

Table 1. Primer sequences used for real-time quantitative PCR in hypothalamic tissue and anticipated size of amplified products.