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Photostimulation Induces New Gene Transcription in Gonadotropin-releasing Hormone Somata in a Seasonally Breeding Songbird

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Abstract

Photostimulation Induces New Gene Transcription in Gonadotropin-releasing Hormone Somata in a Seasonally Breeding Songbird

By Said S. Saab

Birds use a variety of environmental cues, such as day length, temperature, and social interactions, to time reproductive efforts. For most seasonal breeders, day length is the most important cue and takes precedence over all others. In both males and females housed on short days, exposure to a single long day induces a robust release of gonadotropin-releasing hormone (GnRH) from the hypothalamus. The mechanisms underlying this response are only beginning to be understood. Previous research has shown that one long day causes striking upregulation of immediate early gene expression in regions of the mediobasal hypothalamus that contain GnRH axons and terminals. This upregulation is thought to represent the activation of tanycytes and astrocytes in the median eminence as well as neurons located in the infundibular nucleus, which may play a role in the retraction of glial processes that surround GnRH terminals. Although the photoperiodic response of the mediobasal hypothalamus has been well-studied, photo-induced activity in the GnRH neurons themselves has never been described. In this study, we used immunohistochemistry to assay the expression of the immediate early genes c-fos and egr-1 in the GnRH somata of male and female white-throated sparrows exposed to a single long day. We found that the protein products of both genes increased in GnRH neurons of the septo-preoptic area of the hypothalamus by 26 hours after dawn on the long day. These results suggest that photostimulation does in fact stimulate new gene transcription in the GnRH neurons on a relatively rapid time scale. Further research is required to determine whether the GnRH somata are themselves integrating photic cues, or whether they are simply responding to an increased demand for GnRH synthesis.

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Introduction

Over time, seasonal reproduction in birds has evolved to ensure optimal survival of the offspring. As it would not be advantageous, for example, for certain bird species to hatch during the cold winter season, birds synchronize their reproductive response with favorable environmental conditions (Wingfield & Kenagy, 1991). Because breeding is costly and requires an unusually high supply of resources, it is limited to certain times of the year. As food is the ultimate factor affecting survival and thus a key determinant of environmental favorability, its abundance is crucial for successfully raising the young. Therefore, most species breed when food supplies are highest and days are the longest, during the spring and summer seasons (reviewed by Sharp, 2005). Since it would be energetically unfavorable to maintain a reproductive system year-round, the gonads are in a regressed state during the non-breeding season and recrudesce, or increase notably in size, in preparation for the breeding season each year.

Gonadal recrudescence in birds is under hormonal control. Gonadotropin releasing hormone (GnRH) neurons in the septo-preoptic area of the hypothalamus release GnRH into the portal vasculature at the median eminence (Fig. 1). The median eminence is part of the mediobasal hypothalamus (MBH), which is further divided into infundibular nucleus (IN) and other ventral hypothalamic structures. Astrocytes and tanycytes in the median eminence, along with neurons located in the infundibular nucleus, play a role in the retraction of glial processes that surround GnRH terminals and allow for the release of GnRH (Yamamura et al., 2004). The GnRH then travels to the anterior pituitary through the bloodstream, via the portal vasculature at the base of the brain, where it binds to receptors and leads to the release of luteinizing hormone (LH)

and follicle stimulating hormone (FSH). FSH and LH bind to receptors in the gonads, subsequently affecting their functioning. FSH stimulates the maturation of ovarian follicles in females and promotes sperm production in males, leading to a notable enlargement of the gonads. LH controls the release of estradiol in females, and testosterone in males. Overall, these sex steroids (i) control gonadal function, (ii) aid in the development of secondary sex characteristics, and (iii) directly affect reproductive behavior (reviewed by Johnson, 2000; Kirby & Froman, 2000).

Although a small number of bird species have shown endogenous circannual rhythms responsible for dictating reproductive state in unpredictable or temporally constant environments, the majority of bird species rely on environmental cues to accurately time gonadal recrudescence (Gwinner, 1996; Gwinner & Dittami, 1990; Farner, 1985; Wingfield, 1983; Wingfield & Kenagy, 1991). The number of environmental cues birds can use is vast and can be broken down into four categories proposed by Wingfield (1983): (i) *initial predictive information* (e.g. day length) that both triggers gonadal maturation in anticipation of the breeding season and demarcates the general times during which reproduction may occur, (ii) *local predictive information* (e.g. rainfall, ambient temperature, food availability) that allows for the fine-tuning of the reproductive state to the local environment, (iii) *synchronizing and integrating information* (e.g. hearing a conspecific's song), consisting mainly of social cues that allow for the coordination of breeding efforts, and (iv) *modifying information* (e.g. flash flood, forest fire) that may disrupt or terminate reproductive behavior when conditions are highly unfavorable. Although all of these cues play an important role in final gonadal maturation and nesting onset, here we focus on an initial predictive cue, day length, as its

high predictability makes it take precedence over others in demarcating the appropriate time for reproduction (Ball & Hahn, 1997). Although other environmental signals aid in the fine-tuning of this period, it is this dependence on photoperiod that has allowed many bird species to accurately time their reproductive efforts.

The timing of reproduction in most birds is especially sensitive to day length, a reliable external cue that affects the reproductive system. As days lengthen, the levels of reproductive hormones increase and the gonads begin to recrudesce. Although there are variations in the minimum day length necessary to induce "photostimulation," above a certain threshold the changes in hormone levels and gonadal size are notable. In many avian species, for example, the release of GnRH and LH occurs in response to long days. In starlings (*Sturnus vulgaris*) housed on short days, exposure to long days results in an immediate increase in plasma GnRH and LH levels. Over weeks, this increase in hormone levels is associated with gonadal recrudescence (reviewed by Dawson, 2001). The initial increase in reproductive hormones occurs rapidly—LH can be detected in quail (*Coturnix coturnix japonica*) and in white-throated sparrows (*Zonotrichia albicollis*) after exposure to a single long day (Perera & Follett, 1992; Meddle & Follett, 1997; Maney et al., 2007).

To identify the specific brain regions involved in the response to photocues, many researchers have relied on markers of cellular activity, most commonly immediate early genes (IEGs). IEGs are gene sequences with rapid induction—their mRNAs can be detected as early as three minutes following neuronal stimulation by electrophysiological activity or second messenger systems. The mRNAs code for various products, among them transcription factors that control gene expression. These transcription factors then

bind other regulatory elements and attach to specific DNA sites in the promoter region of the gene to be transcribed (reviewed by Herdegen & Leah, 1999). Using immunocytochemistry, one can detect the presence of IEGs and subsequently formulate hypotheses regarding cellular activity.

Experiments using IEGs have been useful in identifying brain regions activated by increases in day length. Specifically, photostimulation induces dramatic IEG induction in the MBH, where the GnRH terminals are located. In quail, Meddle and Follett (1997) reported induction of FOS in glial cells within the median eminence, and in neurons in the IN. Similarly, Peczely and Kovacs (2000) reported IEG expression around the GnRH terminals of mallards (*Anas platyrhynchos*) in the MBH and median eminence after exposure to one long day.

Although the well-studied IEG response in the MBH occurs simultaneously with massive GnRH release, few researchers have asked whether the GnRH neurons themselves express IEGs during photostimulation. In mallards, Peczely and Kovacs (2000) reported no IEG induction in the GnRH somata of the septo-preoptic area in response to photostimulation. This is surprising, since GnRH somata are crucial in initiating the hormonal cascade that results in gonadal recrudescence and would therefore be expected to directly respond to changes in day length. Meddle et al. (1999) claimed that photostimulation does not induce IEG expression in the GnRH somata of quail, but the study they described has never been published.

Regardless of its accuracy, the idea that the GnRH somata do not themselves express photo-induced IEGs has contributed toward a model wherein these neurons play a rather passive role in the response to long days. This model is primarily based on

publications by Meddle et al. (Meddle & Follett, 1995; 1997; Meddle et al., 1999) and Yoshimura et al. (Yamamura et al., 2004; Yoshimura, 2005), which suggest that new gene transcription is not necessary in the somata of GnRH neurons prior to the release of GnRH from the axon terminals. Instead, other cell populations in the MBH that synapse onto the axon terminals of the GnRH-producing neurons are thought to control GnRH secretion. Examination of the MBH has unveiled a high concentration of deep brain photoreceptor cells, responsible for sensing light penetrating through the skull and converting that energy into an electrochemical signal (Silver et. al., 1988; reviewed by Sharp, 2005). Furthermore, lesion studies of the MBH in which the GnRH fiber terminals were spared resulted in the loss of gonadal response to photocues, thereby directly implicating the cells in the IN in photo-induced GnRH release (Sharp & Follett, 1969; Davies & Follett, 1975; Juss, 1993). In combination, these findings have contributed to a model wherein the GnRH somata do not respond directly to changes in day length.

In this study, we tested the hypothesis that GnRH somata *do* in fact respond to photic cues with new protein synthesis, thereby playing an active role in the photoperiodic response. In order to investigate whether these neurons respond rapidly to photostimulation, we quantified the induction of two immediate early genes, Egr-1 and FOS, in GnRH somata after a single long day in both male and female white-throated sparrows, a highly seasonal songbird (Falls & Kopachena, 1994). If the GnRH neurons in these birds do respond to photostimulation with new protein synthesis, we expected to observe IEG induction in their somata after exposure to a long day.

Methods

Animals

All procedures involving animals were approved by the Emory University Institutional Animal Care and Use Committee. A total of 15 male and 21 female white-throated sparrows were collected in mist nets on the Emory University campus, Atlanta, GA, during the months of November and December in 2005 (n=10 females), 2006 (n=6 females), and 2007 (n=5 females; n=15 males). Sex was determined by PCR analysis using a blood sample (Griffiths et al., 1998). Birds were housed in walk-in flight cages (4′ × 7′ × 6′), 6–15 birds per cage in the animal facilities. Food and water were supplied *ad libitum*. Day length was kept constant at 10 hours of light, 14 of darkness (10L:14D), corresponding to the shortest day a bird would experience in Georgia during the winter season. Birds were kept on this light schedule for at least 12 weeks prior to photostimulation (Shank, 1959; Wolfson, 1958).

Photostimulation and Tissue Collection

Birds were transferred to individual cages (15" x 15" x 17") that were placed inside sound-attenuated booths (Industrial Acoustics, Bronx, NY). The day prior to tissue collection, 19 birds (8 males, 11 females) were photostimulated (16L:8D) and 17 (7 males, 10 females) were kept on the short day schedule (10L:14D). The following morning, two hours after dawn, birds were deeply anesthetized and rapidly decapitated. In a subset of birds, a blood sample was collected from the jugular vein for LH assay prior to decapitation. Brains were immersion fixed in 5% acrolein solution for 2.5 hours,

washed three times in phosphate buffered saline (PBS), sunk in 30% sucrose for two days at 20˚C, and frozen at -80˚C until sectioning on a microtome.

LH Assay

To determine whether photostimulation had the desired effect, an LH assay was run on subset of females (n=5 long day, n=5 short day). LH was measured by a postprecipitation, double-antibody radioimmunoassay (RIA) using a homologous chicken LH RIA technique (Follett el al., 1972) that has been validated for songbirds (Dawson & Goldsmith, 1982) and has been used in this species (Spinney et al., 2006). The assay uses highly purified chicken LH for standard curves and for radioiodination. Goat anti-rabbit γglobulins were used as the secondary antibody. Further details of the LH assay are described by Wingfield et al. (1991).

Immunocytochemistry (ICC)

Brains were cut into two sets of 50-μm coronal sections. In 2006 and 2008, one set was labeled for FOS and GnRH and the other for Egr-1 and GnRH. In 2007, one set was labeled for Egr-1 and GnRH and the other set was not used in the study. Sections were double labeled for Egr-1 and GnRH or FOS and GnRH as follows: Free-floating sections were washed in PBS, incubated in 0.1% sodium borohydride for 15 minutes, and washed again in PBS. They were then rinsed in 0.5% H_2O_2 for 30 minutes and washed in PBS with 0.3% Triton (PBST). Sections were then blocked at room temperature in 20% normal goat serum (NGS) in PBST for 1 hour, after which point they were transferred to Egr-1 antibody (Santa Cruz Biotechnology, Santa Cruz, California, USA) diluted 1:8000

in PBSTN (0.3% Triton, 2% NGS) for 2 days at 4˚C. Following a rinse in PBSTN, sections were labeled using a biotinylated goat anti-rabbit IgG (1:250 in PBSTN, 1 hour at room temperature) and avidin-biotin complex (1 hour; 1:400 avidin, 1:400 biotin; Vector Laboratories, Burlingame, CA). Labeling was visualized using diaminobenzidine (DAB) enhanced with nickel as follows: Sections were washed in PBS and rinsed in 0.1M acetate buffer for 5 minutes. They were then transferred to a glucose oxidase-NiDAB solution (2.5% nickel ammonium sulfate, 0.025% DAB, 0.4% glucose oxidase, .08% ammonium chloride) for 3-5 minutes (Shu et al., 1988). The reaction was stopped in 0.1M acetate buffer.

The second ICC used to label the cGnRH-I cell bodies was identical to the one used to label Egr-1, except that labeling was visualized using diaminobenzidine without nickel enhancement. H. Urbanski donated the GnRH antibody (HU60). Sections were incubated with HU60 diluted 1:5000 in PBT with 2% NGS. For the FOS and GnRH double label ICC, sections were incubated with FOS antibody (Santa Cruz Biotechnology, Santa Cruz, California, USA), diluted 1:18,000 in PBSTN. All other steps were identical to those in the double Egr-1 and GnRH ICC. Double-labeled GnRH neurons appeared as a dark bluish-black nucleus (Egr-1 or FOS) surrounded by a light brown cytoplasm (GnRH).

Quantification of GnRH Neurons

GnRH-immunoreactive somata were counted in each 50-μm coronal section between the septo-mesencephalic tract (TSM) and the anterior commissure (AC). The GnRH somata were localized in three distinct populations: in a main central cluster and in two lateral

clusters, one dorsal and one ventral to the main cluster (Fig. 2). The number of cells double-labeled for FOS and GnRH, or Egr-1 and GnRH, was expressed as a percentage of total GnRH immunoreactive cells in each of the GnRH cell populations counted.

Statistical Analysis

Plasma levels of LH were compared between the control and photostimulated birds using a t-test. A MANOVA was conducted to determine the effects of sex and day length on the percentage of GnRH neurons expressing FOS or Egr-1. Because the 2007 females were not labeled for FOS, they were excluded from this MANOVA. Following the MANOVA, F-tests were performed to assess the effects of sex and day length on the expressions of each IEG individually. The 2007 females were included in the Egr-1 F-test. Pairwise comparisons (t-tests) were performed within day length between males and females, and within sex between the long-day and short-day groups. Pearson's correlation tests were run to determine whether the level of FOS expression in GnRH neurons was related to the level of Egr-1 expression. These correlations were run on the short-day and long-day groups separately. The effect of sex and day length on the number of GnRH neurons was assessed by ANOVA.

Analysis of the GnRH Promoter

As genomic resources for analyzing promoter regions are not yet available in the sparrow, we used that of another passeriforme, the zebra finch (*Taeniopygia guttata*), to search for Egr-1 and FOS binding sites upstream of the GnRH gene. The sequence for cGnRH1 published by Stevenson et al. (2009) was used to search the zebra finch genome

using the Blast-Like Alignment Tool (BLAT) available at http://genome.ucsc.edu. The genome browser feature was used to identify the 5' end of exon 1, which codes for the 5' untranslated region. The 1,000 base pair sequence preceding exon 1 was then entered into the Transcription Element Search Software (TESS) program, which identified all possible binding sites for known transcription factors.

Results

Plasma LH

The plasma assay revealed that a single long day increased blood LH concentrations—there was a significant difference between the control (Mean = 0.186 ng/mL; Std. Error = 0.102) and the photostimulated (Mean = 0.935 ng/mL; Std. Error = 0.091) birds (P = 0.0006).

Immediate Early Gene Induction in GnRH neurons

As there were no double labeled cells in the dorsal and ventral lateral clusters (Fig. 2; B and C), statistical analyses were conducted for the main cluster only (Fig. 2; A). A MANOVA revealed a significant effect of day length (Wilks' λ $F_{2,25} = 7.293$; $P = .0032$) and a trend for the effect of sex (Wilks' λ $F_{2,25} = 2.908$; $P = .0732$) on the percentage of GnRH neurons expressing IEGs. There was no interaction between sex and day length (Wilks' λ $F_{2,25} = 2.515$; $P = .1011$).

Egr-1 Induction in GnRH Neurons

Exposure to a single long day induced Egr-1 expression in the main cluster of GnRH neurons (Fig. 3), but not in the lateral clusters (Fig. 4). Post-hoc F-tests revealed a significant effect of sex ($F_{1,32} = 6.926$; $P = .0130$) and day length ($F_{1,32} = 20.062$; $P = < 0.0001$) on the percentage of neurons expressing Egr-1, as well as a significant interaction between sex and treatment ($F_{1,32} = 6.336$; $P = .0170$). Individual pairwise comparisons between treatment groups revealed that the long day males had a significantly higher percentage of double-labeled GnRH neurons than the short day males ($P = 0.006$; Fig. 5). Within the females, the difference in percentage of double-labeled cells between long-day and short-day birds also reached statistical significance, with the long-day females having more double labeling than those on short days ($P = 0.015$). Within-treatment pairwise comparisons between the sexes revealed that within the long-day group, males had a significantly higher percentage of double-labeled GnRH neurons than females ($P = 0.014$). This was not the case for the short-day group, in which both males and females had a similar percentage of neurons co-expressing Egr-1 ($P = 0.600$).

FOS Induction in GnRH Neurons

Exposure to a single long day induced FOS expression in the main cluster of GnRH neurons (Fig. 6), but not in the lateral clusters. Post-hoc F-tests revealed a significant effect of sex ($F_{1,26} = 5.856$; $P = .0228$) and day length ($F_{1,26} = 5.504$; $P = .0377$) on the percentage of GnRH neurons expressing FOS, as well as a significant interaction between sex and treatment ($F_{1,26} = 4.795$; $P = .0377$). Within-sex pairwise comparisons between treatment groups revealed that the long day males had a significantly higher percentage

of double-labeled GnRH neurons than the short day males ($P = 0.041$; Fig. 7). Within the females, the difference in percentage of double-labeled GnRH cells between long-day and short-day birds did not reach statistical significance ($P = 0.193$). Pairwise comparisons between the sexes revealed that within the long-day group, males had a significantly higher percentage of double-labeled GnRH neurons than females ($P = 0.027$). This was also the case for the short-day group ($P = 0.001$).

Correlated FOS and Egr-1 Expression in GnRH Neurons

Pearson's correlation tests revealed that the percentage of GnRH neurons expressing Egr-1 was correlated in the long-day group with the percentage expressing FOS ($r^2 = .626$; $P = < .0001$; Fig. 8). This correlation was not, however, apparent in the short-day group ($r^2 = .005$; $P = .814$).

Number of GnRH Neurons

An ANOVA showed no effect of sex ($F_{1,32} = 0.002$; $P = .9610$) or day length ($F_{1,32} = 1.182$; $P = .2851$) on the total number of GnRH neurons counted. There was no interaction between sex and day length ($F_{1,32} = 1.534$; $P = .2245$). The number of GnRH neurons was similar in all groups (Fig. 9).

IEG Binding Sites in the Zebra Finch GNRH Promoter

In order to bind DNA, FOS must dimerize with JUN, the protein product of another IEG (reviewed by Herdegen & Leah, 1998). Together, this dimer binds to a sequence known as AP-1. In the 1,000 base pairs upstream from the 5' UTR of the zebra finch GnRH

gene, three potential AP-1 sites were identified. Two of these sites, however, were on the reverse strand and are thus not in a position to regulate GnRH synthesis. The remaining site was "AP-1-like" in that the sequence will bind JUN/JUN but not FOS/JUN dimers (Newell et al., 1994). Because this site does not bind FOS, it was not considered relevant to the FOS induction we saw. No Egr-1 binding sites were found.

Discussion

In this study, we tested the hypothesis that GnRH somata respond to photic cues with new protein synthesis. After a single long day, Egr-1 and FOS expression increased in the GnRH somata of the septo-preoptic area. This cell population projects to the ME, where GnRH is released in turn leading to gonadal recrudescence. The increase in IEG expression levels in these somata following photostimulation is suggestive of their involvement in the photo-induced rise in plasma GnRH.

Previous studies of photo-induced GnRH release have led to a somewhat different model wherein the somata of the septo-preoptic GnRH system play a rather passive role in the response to photic cues. In this model, photo-induced GnRH release is controlled entirely at the level of the GnRH terminals in the MBH, and the somata in the preoptic area are not actively involved. Meddle and Follett (1995a, 1997) showed robust induction of FOS within the basal tuberal hypothalamus and ME of Japanese quail following photostimulation, providing support to the view that the cell populations in this region control the release of GnRH. Saldanha et al. (2001) later showed a close association among encephalic photoreceptor terminals, tanycytes, and GnRH terminals in the MBH of adult ring doves (*Streptopelia roseogrisea*). The researchers hypothesized that the

photosensitive cells form synapses onto the GnRH terminals, thereby controlling GnRH release at the MBH. More recently, Yamamura et al. (2004) examined the ultrastructure of the ME, revealing the encasement of GnRH terminals by glial endfeet under short day but not under long day conditions. The researchers proposed that the observed morphological changes were involved in the regulation of GnRH release—when the encasements are retracted, the terminals can contact the portal vasculature and GnRH can get into the bloodstream. The retraction of the glial endfeet is thought to be controlled by neurons in the basal tuberal hypothalamus (Yamamura et al., 2004), which may explain the FOS induction in that region noted by Meddle and Follett (1995a, 1997). In combination, these studies have lent support to the hypothesis that the release of GnRH is controlled at the level of the MBH.

In contrast to the aforementioned model, our data contribute toward a model wherein the GnRH somata play an active role in the response to photostimulation. Whereas the authors of all previous work reported the absence of photo-induced IEG expression in GnRH somata (Peczely & Kovacs, 2000; Meddle et al., 1999), we found clear evidence that both Egr-1 and FOS are in fact induced in this population of neurons. Other researchers have also shown evidence that GnRH cells in this region could be involved in the integration of photic cues. Saldanha et al. (2001) observed that the terminals of photoreceptor cells form synapses onto the dendrites of GnRH somata in the septo-preoptic area of the hypothalamus and the lateral septum. This direct link with photoreceptors might provide the GnRH neurons with information useful in coordinating hormonal release with long day length.

The possibility remains that the IEG induction we observed is not caused directly by photic cues, but rather by an increased demand for GnRH synthesis. If the synthesis of new GnRH requires the activation of these IEGs, we would expect to find FOS and Egr-1 binding sites in the promoter of the GnRH gene. In mice, the GnRH gene has an Egr-1 binding site in its promoter (reviewed by DiVall et al., 2007). In humans, binding sites for FOS have been identified on the GnRH promoter (Nelson et al., 1998). As genomic resources for analyzing promoter regions are not yet available in the white-throated sparrow, we used that of another passeriforme, the zebra finch, to search for Egr-1 and FOS binding sites upstream of the GnRH gene and found none. Although we cannot draw definite conclusions regarding the relationship between the observed IEG induction and GnRH synthesis in the white-throated sparrow, the lack of Egr-1 and FOS binding sites in the zebra finch GnRH promoter suggests the transcription of the GnRH gene does not appear to directly involve either one of these IEGs. Thus, the IEG response may be more related to the actual photostimulation than to simply replacing the GnRH that is secreted in response to photostimulation.

In this study, a long day induced a greater increase in IEG expression in male than in female sparrows, which is suggestive of differences between the sexes in their response to environmental cues. Ball and Ketterson (2005) argued that male birds are affected more by initial predictive cues such as day length, whereas female birds are affected more by supplementary information such as temperature and food availability. Day length is highly predictable and thus can be relied on far in advance of the breeding season, unlike temperature and food availability, which are more immediate cues. By relying on day length birds can predict the breeding season far in advance and be more

prepared when it arrives. Male white-throated sparrows arrive at the breeding grounds one to two weeks earlier than the females and establish their territories in preparation for the arrival of potential mates (reviewed by Falls & Kopachena, 2004). Therefore, being able to predict the breeding season well in advance is adaptive for them. Day length can provide predictive information only about the time of year, however, and cannot provide information on immediate local conditions, such as temperature and food availability, which may vary from year to year and from location to location. Because the investment in egg production in females is far greater than that necessary for the production of sperm in males, preparing for reproduction when local conditions are not optimal would be far more costly for females. The recovery time necessary to gather new resources and generate new eggs would be considerably longer than that needed by males. For females, being more finely-tuned to supplementary information allows for egg laying during the most optimal conditions.

Given the greater sensitivity to supplementary information in females, it is important to note that the overall difference in IEG protein levels between the sexes may have resulted from being in captivity. Females generally require a large number of supplementary environmental cues, and in an experimental setting may be less receptive than normal to changes in day length (reviewed by Ball & Ketterson, 2005). Moore (1983) demonstrated that in white-crowned sparrows (*Zonotrichia leucophrys*), in contrast to photostimulated captive males, photostimulated captive females had low reproductive hormone levels and gonads that were not fully developed. This effect of captivity on the female response could have resulted in a sex difference by artificially lowering an IEG response that may have otherwise been at the level seen in males.

Whereas the sex difference in Egr-1 induction was simply a matter of degree, the sex difference in FOS induction was more dramatic in that males responded with FOS and the females did not. The fact that the females had an Egr-1 response but not a FOS response is suggestive of a qualitative difference between Egr-1 and FOS induction. It is important to note that FOS and Egr-1 are structurally different and as a result bind to DNA at unique sequences (reviewed by Herdegen & Leah, 1998; Thiel & Cibelli, 2002). Any given gene may or may not have binding sites for either Egr-1 or FOS in its promoter. Under the assumption that IEGs are transcribed only when needed, it could be the case that the females responded to photostimulation by turning on genes that have an Egr-1 but not a FOS binding site in their promoters, whereas the males turned on genes that have Egr-1, FOS, or binding sites for both. There are circumstances under which one IEG is transcribed and others are not, depending on the specific function that cell must perform—it is not the case that when one is transcribed others are too. For example, conspecific song induces FOS but not Egr-1 induction in the hippocampus of juvenile female zebra finches, whereas in the males, the same stimulus induces Egr-1 but not FOS induction (Bailey & Wade, 2003). The expression of the two IEGs is, therefore, not necessarily related.

Although the expression of these two IEGs is not always correlated, we found that in this particular population of neurons, the induction of one may be related to that of the other. As FOS and Egr-1 expression were related in long day animals following photostimulation (Fig. 8), a common mechanism of action may be responsible for simultaneously upregulating both IEGs. McMahon and et al. (1990) noted similarities in *fos* and *egr-1* mRNA expression levels in the developing mouse skeleton. In mouse

embryos, *egr-1* and *fos* expression were induced at similar levels in multiple areas. Similar patterns of *fos* and *egr-1* induction have been noted in several other cell types, including immune cells and in bone marrow cells responding to stimulation (reviewed by McMahon et al., 1990). In combination, these observations support the hypothesis that FOS and Egr-1 expression may be related at a mechanistic level in some cases.

Despite the close relationship between Egr-1 and FOS induction in the GnRH neurons of the septo-preoptic system, we found that the two proteins were not induced at the same level—Egr-1 expression was higher than FOS expression (Figs. 5, 7). It is perhaps not surprising that Egr-1 is expressed in higher quantities in this cell population, as this IEG is generally expressed at higher levels than FOS throughout the brain (Maney, unpublished). If Egr-1 induction is generally higher than FOS induction following a stimulus, given that the females seem to be less responsive than the males to photostimulation, the FOS response of the females might have been present but too low to detect with the number of birds in our study. As only a few cells were double-labeled for both GnRH and FOS in the long day females, utilizing a larger number of birds may have better enabled us to detect small differences in IEG induction.

It is also important to note that the induction of Egr-1 and FOS may follow different time courses, which could have accounted for our inability to detect a FOS response in the females. Research by Meddle and Follett (1997) suggests that photo-induced IEG transcription peaks at different time points in different populations of neurons. As we sampled IEG induction at only one time point, it is possible that we might have missed the FOS response in the females, while capturing some of the Egr-1 response. This could have happened if the two IEGs were induced in separate populations

of cells. We believe, however, that this is likely not the case in our study, as Egr-1 and FOS induction occurred in the same cluster of cells. In order to explore the photoperiodic response of GnRH neurons more thoroughly, future experiments should include samples taken at multiple time points. The full characterization of the time course of FOS and Egr-1 induction, in combination with studies focusing on the identification of genes transcribed in response to photostimulation, would provide for a more thorough understanding of the response to photocues in the GnRH somata of the septo-preoptic area.

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Figures and Captions

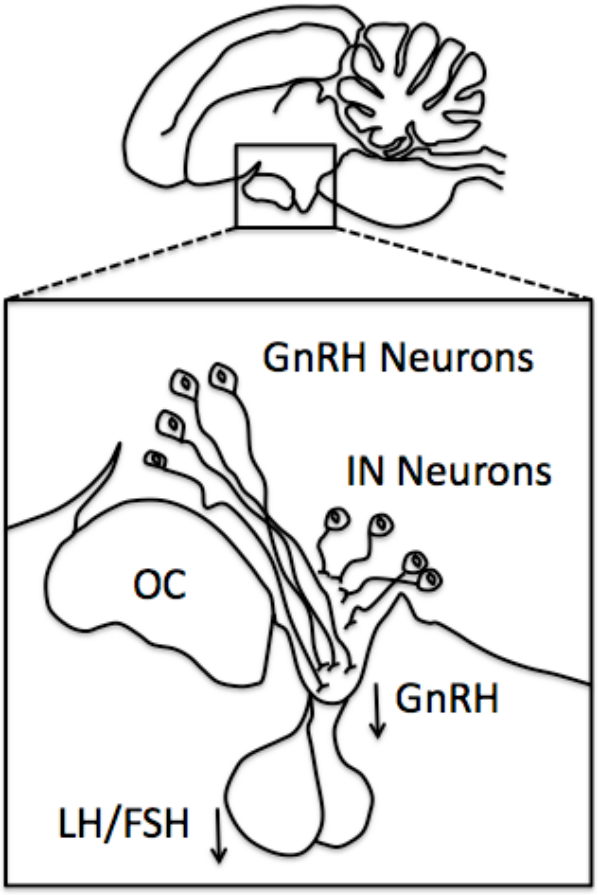

Fig. 1. Diagram depicting a sagittal view of the hypothalamic-pituitary-gonadal axis. Axons of GnRH somata project to the median eminence, where GnRH is released. GnRH reaches the anterior pituitary via the portal vasculature, leading to the release of LH and FSH, which in turn act on the gonads. IN, infundibular nucleus; OC, optic chiasm; LH, luteinizing hormone; FSH, follicle stimulating hormone.

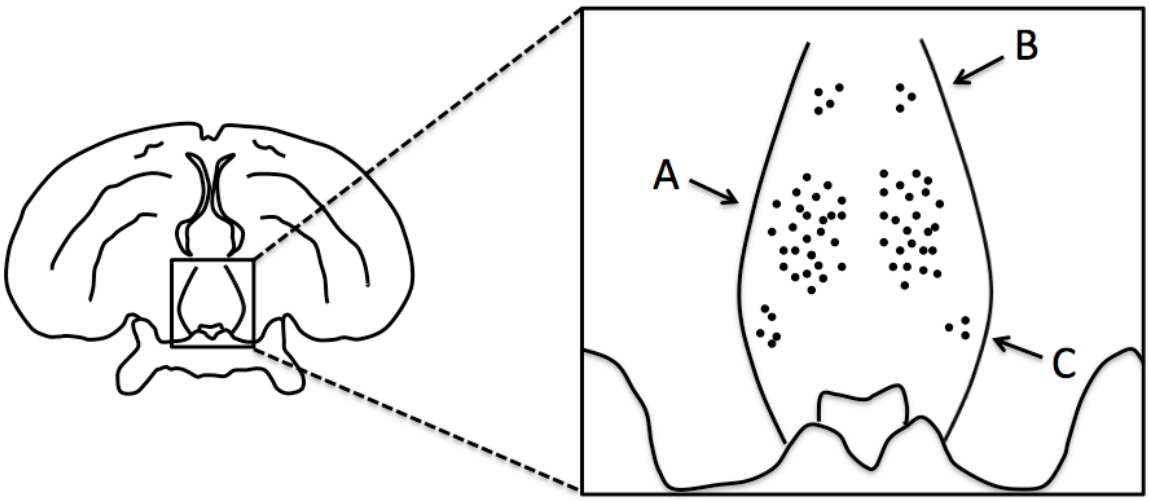

Fig. 2. GnRH neurons were counted in three distinct populations (A, B, C) along the midline between the septo-mesencephalic tract and the anterior commissure.

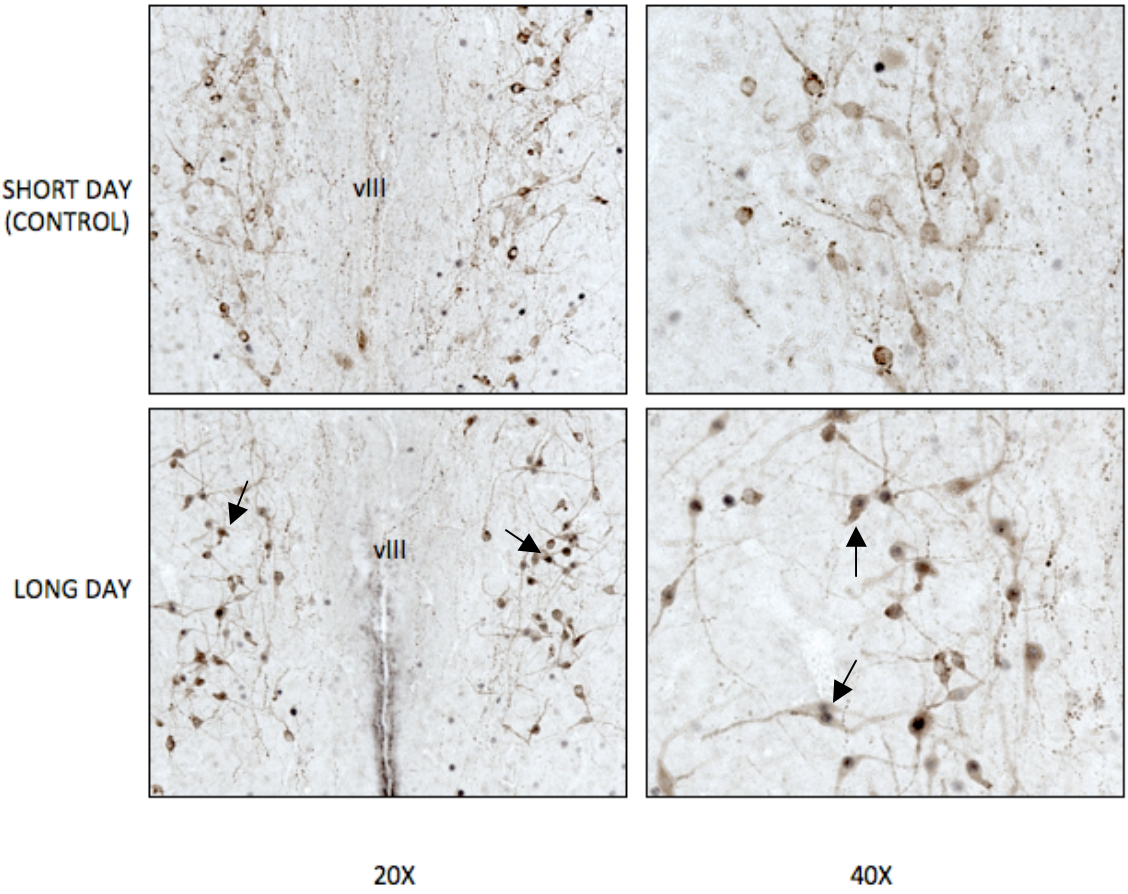

Fig. 3. Photomicrographs taken with a 20X (left) and 40X (right) objectives show induction of Egr-1 in GnRH neurons in long-day birds (bottom) relative to short day birds (top). The arrows point to neurons that are immunoreactive for both GnRH (brown cytoplasm) and Egr-1 (bluish-black nucleus). *vIII*, Third ventricle.

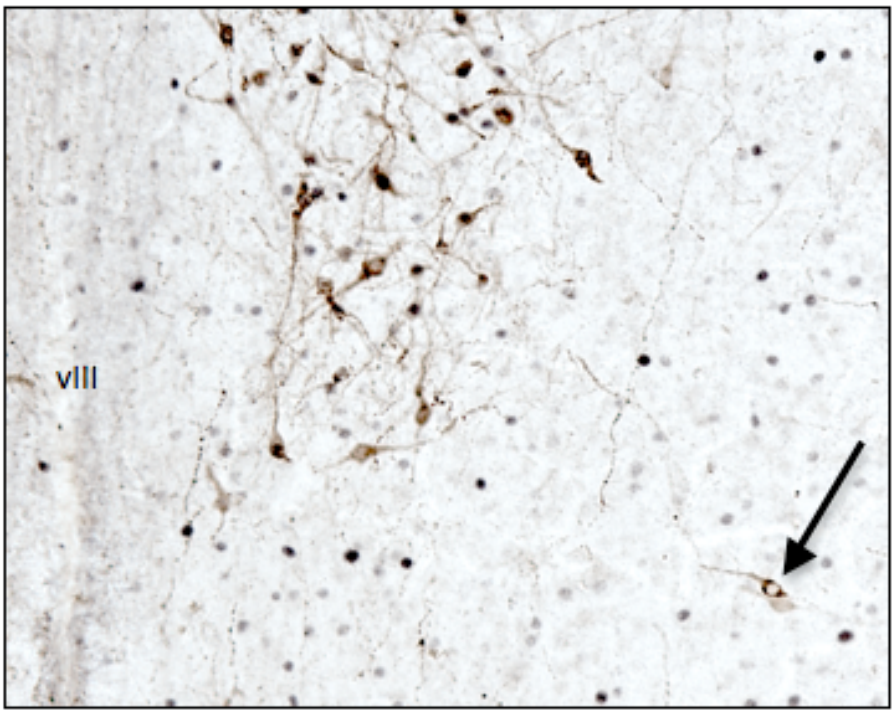

Fig. 4. Photomicrograph taken with a 20X objective showing that whereas the main cluster of GnRH-IR cells expressed Egr-1, the lateral cluster did not. Cells in the lateral cluster (black arrow) did not express Egr-1. *vIII*, Third ventricle.

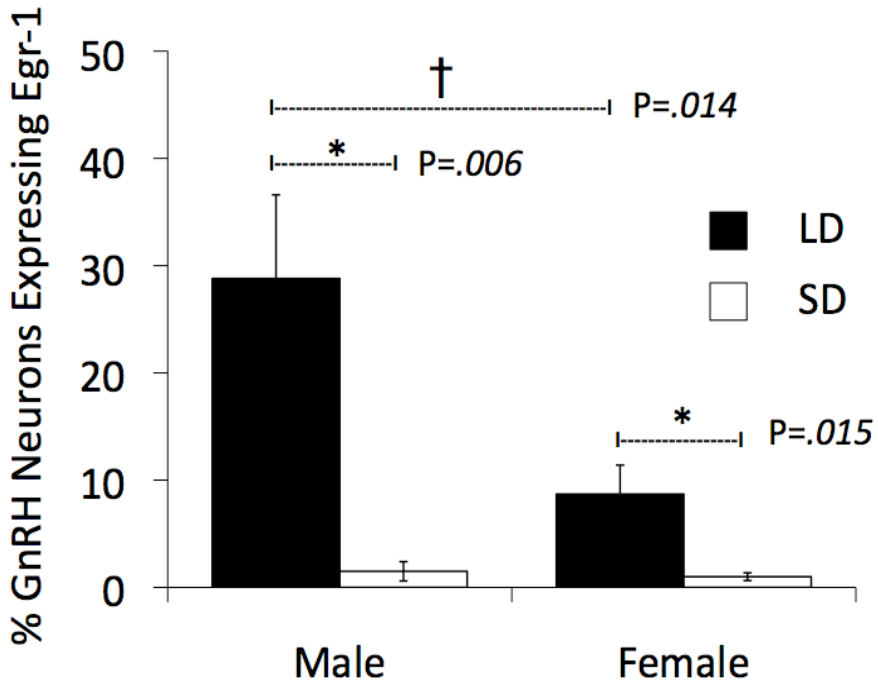

Fig. 5. Percentage of GnRH neurons expressing Egr-1 in males and females. Exposure to a single long day significantly increased Egr-1 expression in GnRH neurons in both males and females. There was a significant sex difference in Egr-1 expression in LD animals. *Effect of day length within sex; † Effect of sex.

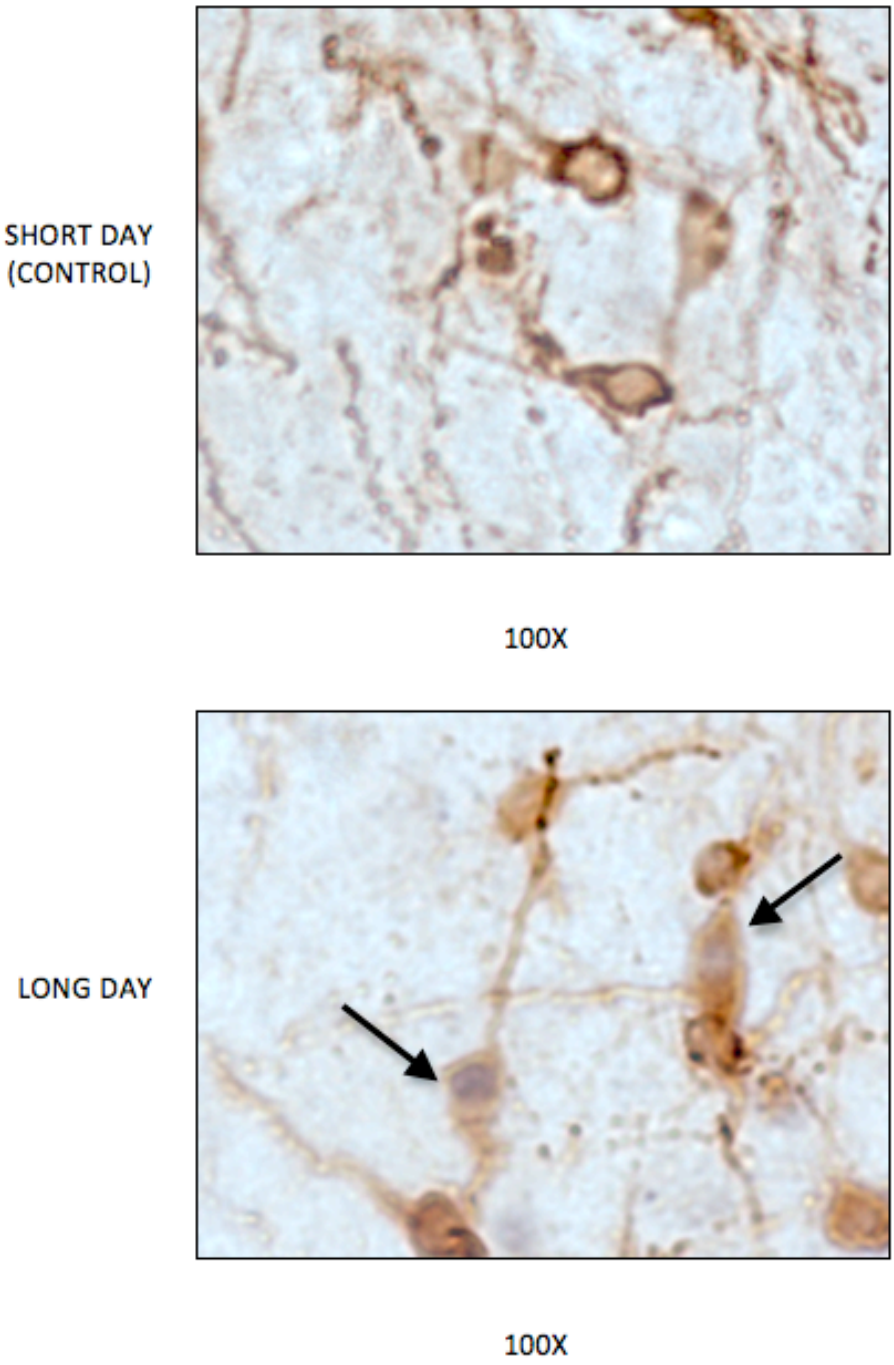

Fig. 6. Photomicrographs from a control bird (top) and a long day bird (bottom) taken with a 100X objective. The bottom panel depicts neurons that are immunoreactive for both GnRH (brown cytoplasm) and FOS (bluish-black nucleus).

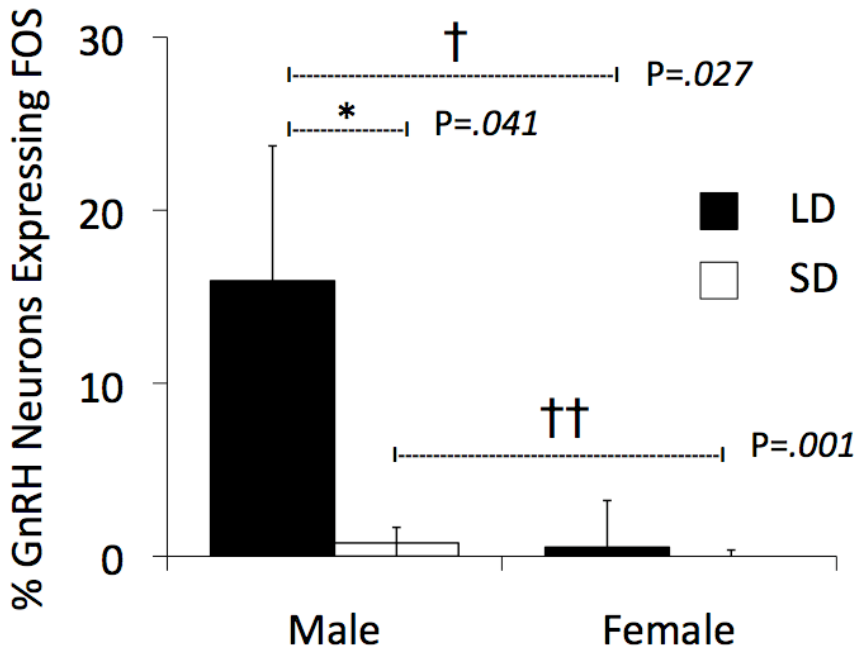

Fig. 7. Percentage of GnRH neurons expressing FOS in males and females. Exposure to a single long day significantly increased FOS expression in GnRH neurons in males but not in females. There was a significant sex difference in FOS expression in the LD and the SD animals. *Effect of day length in males; † Effect of sex in long day animals; †† Effect of sex in short day animals.

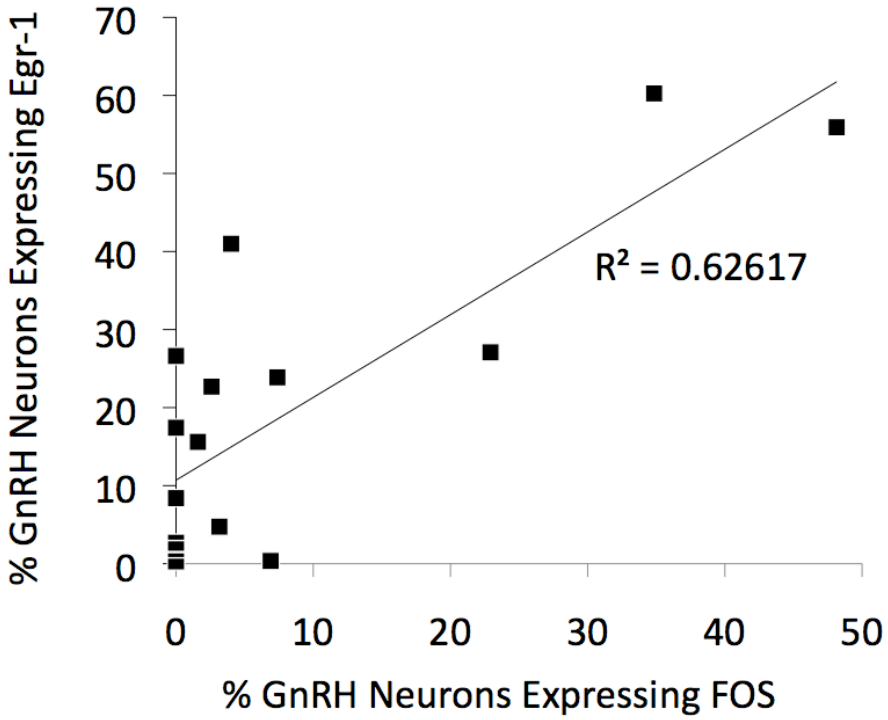

Fig. 8. The proportion of GnRH-IR cells that expressed Egr-1 was related to the proportion that expressed FOS in long day animals. SD animals not shown—all clustered around zero.

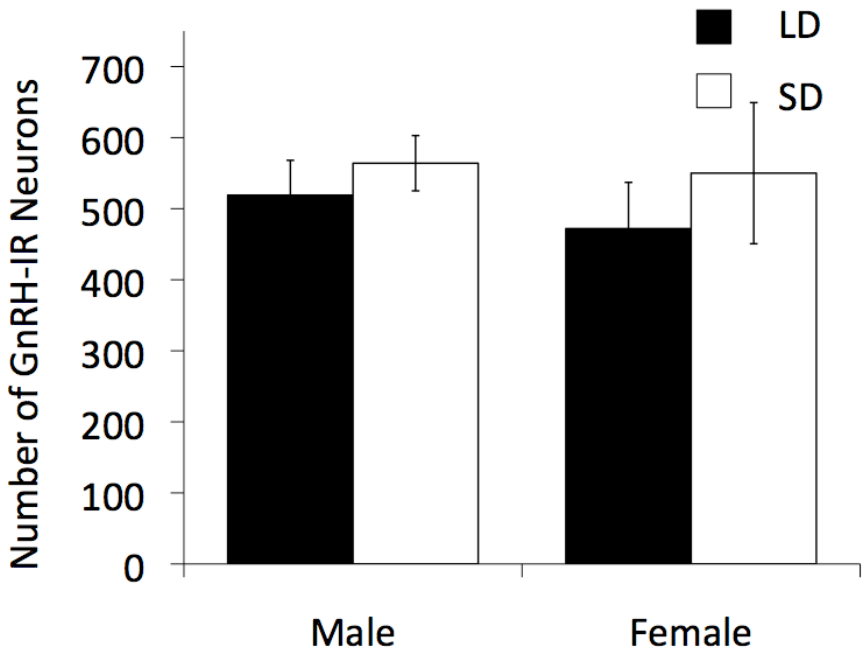

Fig. 9. The number of GnRH-IR neurons in the main cluster did not differ significantly between males and females (P= 0.628) or treatment groups (P= 0.175).