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Variation in Gonadotropin Releasing-Hormone-1 Gene Expression in the Preoptic Area Predicts Transitions in Seasonal Reproductive State

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Abstract

In many seasonally reproducing animals, the experience of prolonged exposure to constant photoperiods results in the induction of a state of photorefractoriness, defined as a lack of responsiveness to a previously stimulatory photoperiod. The physiological and genetic processes that control photorefractoriness are not well understood, however, the hallmark of photorefractoriness is an endogenous change in the physiological response to a constant photoperiod. It is already known that preoptic area *gnrh1* gene expression declines during development of refractoriness to long-day stimulation in European starlings. We employed *in situ* hybridization histochemistry to characterize changes in preoptic area *gnrh1* mRNA expression during the reinstatement of photosensitivity in female starlings. Photorefractory starlings moved to short days (8L:16D) increased optical density of *gnrh1* expressing cells within ten days. Exposure to 30 short days resulted in greater visible *gnrh1* cell numbers with no detectable change in measures of ovarian follicular volume and oviduct mass. We subsequently examined the extent of *gnrh1* expression in response to photostimulation after incremental periods on short day lengths. A significant long-day-induced increase in both *gnrh1* expression and ovarian and oviduct mass occurred only after at least 30 short days. These findings demonstrate that the recovery of photorefractoriness involves an increase in *gnrh1* mRNA expression and expands upon our previous knowledge that the development of photosensitivity is associated with an increase in both the precursor proGnRH1-GAP and GnRH1 peptides in the POA. Importantly, the change in the brain sensitivity occurs well before such changes can be detected via variation in ovarian activity.

Keywords

hypothalamus; photoperiodism; bird; seasonality; HPG-axis; rhythms

INTRODUCTION

Successful timing of reproduction requires complex interactions between environmental stimuli and endogenous factors that include both the neural and endocrine systems. A key environmental stimulus many animals use to time seasonal reproduction is the annual change in day length (1–3). Photoperiodic information either acts directly to change

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hypothalamic drive on the pituitary-gonadal axis or as a *zeitgeber* to synchronize an endogenous circannual rhythm (4). The ability of an individual to use photoperiodic information to modify how the brain processes environmental information leads to adaptive seasonal variation in reproductive physiology and behavior. This process is called photoperiodism (1–2).

An important feature of vertebrate photoperiodism is photorefractoriness, when responsiveness either to the inhibitory effects of short days (e.g. rodents) or to the stimulatory effects of long days (e.g. birds) disappears (Fig. 1; 3,5–7). In most birds, long days initially stimulate gonadal recrudescence and prolonged exposure to long days also activates an inhibitory process, such that the reproductive neuroendocrine system undergoes involution (2, 6). These birds must experience non-stimulatory short days in order to regain a physiological sensitivity to the stimulatory effects of long days (2, 3, 6).

The gonadotropin releasing-hormone-1 (GnRH1) neuronal system in the septal-preoptic area (POA) provides the key link between the brain and the reproductive endocrine system. GnRH1 neurons in avian species exhibit dramatic changes following exposure to long and short day lengths (see 2, 3, 7–9 for reviews). The European starling (*Sturnus vulgaris*) is an excellent model species to examine the cellular and molecular mechanisms that govern development and dissipation of photorefractoriness due to the dramatic and reliable involution in reproductive physiology and behavior, and return of photosensitivity under short-day conditions (10, 11). Photorefractoriness in starlings is associated with a marked depression in hypothalamic *gnrh1* mRNA expression (11, 12). Previous work has shown that GnRH1 peptide in the POA increases after the transfer of starlings from long days to a short day regime (10). The increase in GnRH1 peptide associated with the dissipation of photorefractoriness may develop from 1) a change in the precursor proGnRH1-GAP (a peptide that is also known to change with photoperiodic state in starlings; 13); 2) a change in *gnrh1* mRNA expression; or 3) a consequence of both. The recent identification of the *gnrh1* genetic sequence in starlings now permits the analysis of the timing and nature of the changes in the expression of *gnrh1* expression in the POA associated with the dissipation of a photorefractory state (11,12).

In the present study we measured *gnrh1* expression in female European starlings to provide insight into when the brain switches the signals it sends the pituitary in relation to the control of photoperiodic state. First, we investigated when the removal of inhibitory day length activates the transcription of *gnrh1* expression in the POA. Second, we examined the change in *gnrh1* expression in relation to measures of ovarian and oviduct development in response to a set amount of photostimulation that was experienced by different groups of female after an increasing number of days on short day lengths. We hypothesized that the breaking of the photorefractory state by short days in female European starlings is associated with a marked increase in *gnrh1* expression. In particular it was predicted that if photorefractory birds on long days are moved to short days there will be a gradual increase in *gnrh1* expression and this expression will be proportional to the period of exposure. We predicted that 1) *gnrh1* expression will significantly increase within 30 days of exposure to short day lengths and 2) that the length of time on short day lengths will be directly related to the numbers of visible cells containing *gnrh1* and/or the density of *gnrh1* cells after stimulation by long days. Given the observation that a seasonal change in *gnrh1* expression is associated with different photoperiodic states in starlings (11), here we use *gnrh1* expression as a genetic marker to identify when the switch in the hypothalamus that drives the seasonal change from the photorefractory to the photosensitive condition occurs after exposure to short days.

METHODS

Subjects

Thirty-two female European starlings were caught using a drop down V-trap in early March on a farm in Conneautville, PA (41° 45' N lat., 80° 22' W long) and group housed on the natural day length experienced on that date (11L:13D). All birds were treated and handled in accordance with federal, state, and institutional (Johns Hopkins University) animal care use and committee guidelines.

Treatment groups

Shortly after acclimation to laboratory housing, all birds were placed on long day lengths (16L:8D) for sixteen weeks, thereby inducing a photorefractory state (2, 11). The first experiment consisted of four treatment groups (N = 4 for each): three groups on short days as described below and one control group consisted of female starlings that remained on long day lengths (16L:8D) for the duration of the experiment and were photorefractory. In order to examine the changes in *gnrh1* expression associated with the onset of photosensitivity, female starlings were transferred to short day lengths (8L:16D) for either ten (SD10); twenty (SD20) or thirty (SD30) days (Fig. 2A). The second experiment had a similar design (N = 4 for each group) except that at the end of the short day treatment, starlings were placed on a long daylength (16L:8D) for two weeks to determine the effect of photostimulation on *gnrh1* mRNA expression and gonadal recrudescence (Fig. 2B). The study was designed such that both experiments concluded on the exact same calendar day and thus, tissue from all birds was collected on the same day. This design eliminated the effects of time per se (as opposed to photoperiodic treatment) as a confounding factor on measures of neuroendocrine and peripheral reproductive physiology.

Assessment of reproductive state

Female reproductive state was determined by measuring the volume of the largest ovarian follicle, ovary state, oviduct mass, and POA *gnrh1* mRNA. At the end of the experiment the birds were killed by an overdose of secobarbital (50 mg/kg i.m.). The largest follicle diameter was measured to the nearest 0.1mm using dial calipers. The volume of the largest ovarian follicle was calculated using the formula $(4/3\pi r^3)$, where r is the radius, and the oviduct was dissected from the body and weighed. Ovary stage was assessed using the following scale: 1) smooth, no visible follicular development; 2) slightly granular appearance; 3) small follicles apparent, but no follicular hierarchy; 4) obvious follicles with evident hierarchy; and 5) large yolky follicles (14).

Brain Extraction and Sectioning

In order to examine the variation in *gnrh1* mRNA, brains were dissected out and placed directly in dry ice for 5 minutes. Brains were subsequently wrapped in aluminum foil and stored at -70°C until sectioning. Brains were sectioned on a cryostat at 20 µm thickness and mounted directly onto Superfrost Plus slides (Fisher Scientific, Santa Clara CA) and stored at -70°C. Sections were collected beginning with the tractus septomesencephalicus (TSM) split and continued every fourth section (80 µm apart) until the caudal aspect of the anterior commissure. These anatomical landmarks reliably border the rostral and caudal extents of the POA.

In situ hybridization

A plasmid containing starling *GNRH1* cDNA (GenBank Acc. #FJ178434) corresponding to the decapeptide, GNRH1 associated peptide (GAP), and the 3' UTR (approx. 350 bp) was linearized with *SacI* (sense; Promega) or *NcoI* (antisense; Promega) and purified using

GENE-CLEAN (Qbiogene, Vista, CA). Digoxigenin labeled cRNA probes (Roche Applied Sciences, Indianapolis, IN) were transcribed by SP6 (antisense) or T7 (sense) RNA polymerases using a DIG RNA labeling kit (Roche Applied Sciences, Indianapolis, IN). Unincorporated nucleotides were separated with NucAway spin columns (Ambion, Austin TX). Probe quality was verified by gel electrophoresis and spectrophotometry (Pharmacia Biotech, Ultrospec 2000).

gnrh1 mRNA expression in slide-mounted sections was analyzed by *in situ* hybridization in four separate sets and treatment groups were counterbalanced across batches. Brain sections were removed from -70°C and dried for 20 minutes at room temperature. Slides were then placed in ice cold 4% paraformaldehyde for ten minutes. Then the sections were washed three times in 1M phosphate buffered saline (PBS, Ambion cat# AM9625) for 3 minutes each. Tissue was digested with 1 $\mu\text{g/ml}$ proteinase K (Invitrogen cat# 25530) in Tris buffer pH 8.0 and 5mM EDTA. The sections were then fixed in 4% paraformaldehyde for 5 minutes at room temperature (RT). The slides were washed again in 1M PBS for three minutes at RT. Sections were acetylated for 10 minutes at RT with vigorous shaking. Sections were then placed in 1M PBS and 1% triton-X 100 for thirty minutes followed by three washes in 1M PBS for five minutes each. Then, 500 μl of hybridization solution (5X Denharts solution (Sigma D2532); 50% formamide (Roche); 5X sodium-chloride sodium-citrate (SSC, Ambion AM9763); salmon sperm DNA (Roche); yeast tRNA (Invitrogen); 1% diethyl pyrocarbonate in distilled water (DEPC)) was placed on slides and incubated in a humidified chamber containing 50 ml of formamide and 50 ml of 5X SSC for four hours at RT. Slides were then incubated in hybridization solution with approximately 300–400 ng/ml digoxigenin (DIG) RNA probe and heated at 65°C overnight in the humidified chamber. The next day, slides were placed in 5X SSC for 5 minutes at 65°C then placed twice in 0.2X SSC for 45 minutes each at 65°C . Slides were then transferred to 0.2X SSC for 5 minutes at RT, followed by tris-saline buffer solution (1M tris buffer pH 7.5, 5M NaCl, DEPC) for 5 minutes at RT. Blockade of non specific binding was accomplished by placing 500 μl of Tris-saline solution containing 10% heat inactivated sheep serum (HISS, Invitrogen) for two hours in a humidified chamber containing 50 ml tris-saline buffer at RT. Slides were then incubated in anti-digoxigenin (1:1000, Roche cat # 11093274910) in tris-saline buffer containing 3% HISS overnight at 4°C . The following morning the slides were washed in tris-saline buffer eight times for fifteen minutes at RT. Slides were then equilibrated for 5 minutes in NTMT solution (2M tris-saline buffer pH 9.5, 5M NaCl, 1M MgCl_2 , DEPC). To visualize DIG-labeled probes, slides were then incubated in NTMT solution containing a dilution of 1:200 nitro blue tetrazolium chloride (NBT, Roche) and 5-bromo-4-chloro-3-indolyl phosphate toluidine salt (BCIP, Roche), wrapped in aluminum foil and placed in a light proof container for six hours. The reaction was arrested by placing slides three times in 1M PBS (with 2ml of 5M EDTA added) for three minutes each. Slides were then fixed in 4% paraformaldehyde for ten minutes at RT to seal in the color. Slides were rinsed three times in 0.02M tris-saline buffer pH 7.5, and then air dried overnight at RT. Slides were coverslipped with permount.

Quantification and Statistical Analysis of *gnrh1* mRNA

The distribution and number of *gnrh1* mRNA expressing cells were determined at 100X magnification using a bright-field light microscope (Zeiss Axioskop, Carl Zeiss, Thornwood NY). *gnrh1* cells were manually counted beginning at the TSM split and continued through to the caudal extent of the anterior commissure. An individual *gnrh1* cell was determined by identifying the leading edge of a cell in the tissue section. Additionally, objects that were determined as *gnrh1* cells had an area greater than $7.66^{-06}\mu\text{m}^2$, but less than $3.45^{-05}\mu\text{m}^2$ (12). The entire preoptic area in each tissue section was analyzed and cells summed providing an exhaustive count of the number of *gnrh1* cells per preoptic area section. We used a sampling

procedure recommended by West (18, 19) in which an estimate of the total number of *gnrh1* neurons is determined by summing the leading edge in each tissue section and multiplying by the sampling interval. This method has previously been used in starlings (12, 20). DIG-labeled *gnrh1* mRNA expressing cells exhibited a distribution identical to those previously reported (12). The optical density (OD) of *gnrh1* mRNA expressing cells was determined using ImageJ (NIH) software. The OD of at least nine randomly-selected cells from five different tissue sections was measured for each bird. For each cell, the DIG-labeled signal was clearly discernible and consisted of an increase in OD several times greater than the background. The signal was circled and the mean grey value determined for each cell. The OD value was corrected for background by subtracting the optical density from telencephalic regions in the same section. Finally, the mean optical density was calculated by averaging *gnrh1* mRNA expressing cells. One way analysis of variance were conducted to determine significant differences in follicle volume, oviduct mass, ovary stage, *gnrh1* cells and *gnrh1* cell OD. Significance tests were conducted using Holm-Sidak analyses to determine differences in photoperiod treatments compared to the control group (i.e. photorefractory). SigmaStat 11 was employed to conduct all statistical analyses.

In order to determine the anatomically localized effect of photoperiod on *gnrh1* mRNA expressing cells, the mean number of *gnrh1* cells for each section from the TSM split to the anterior commissure was calculated across the treatment groups. There were fifteen sections in total for each starling between the TSM split and anterior commissure. The anterior commissure provides a less variable landmark compared to the TSM split across subjects and was thus selected as the point for plotting the *gnrh1* distribution. We subsequently analyzed the distribution of *gnrh1* mRNA expressing cells sequentially in fourteen sections caudal to the TSM split. This analysis provided the *gnrh1* cell distribution along the rostral-caudal axis. We conducted two-way repeated-measures ANOVA (Treatment $\times$ brain section) on the distribution of *gnrh1* cells in the rostral-caudal POA.

RESULTS

Experiment 1

A one way ANOVA showed that there was no significant treatment effect on follicle volume ($F_{3,15} = 1.71$; $p = 0.21$; $\lambda = 0.16$; Fig. 3A), oviduct mass ($F_{3,15} = 1.93$; $p = 0.17$; $\lambda = 0.19$; Fig. 3B) or ovary stage ($F_{3,15} = 1.57$; $\lambda = 0.13$; $p = 0.25$). Ovary stage (Mean $\pm$ Standard error) values were PR (1.0 ± 0.0); SD10 (1.2 ± 0.25); SD20 (1.0 ± 0.0); SD30 (1.5 ± 0.29).

There was a significant increase in *gnrh1* cell counts across the treatment groups ($F_{3,15} = 5.95$; $p < 0.05$; $\lambda = 0.78$; Fig. 4A). Holm-Sidak analyses revealed a significant difference between SD30 and PR birds ($t = 3.35$; $p < 0.01$), but not between SD20 and PR ($t = 0.08$; $p = 0.43$; *ns*), or SD10 and PR birds ($t = 0.03$; $p = 0.97$; *ns*). Moreover, there was a significant increase in *gnrh1* mRNA OD across the treatment groups ($F_{3,15} = 193.72$; $p < 0.001$; $\lambda = 0.99$; Fig. 4C). Post-hoc analysis revealed that SD10, SD20, and SD30 had significantly greater *gnrh1* mRNA OD compared to PR ($t = 6.61$; $p < 0.001$, $t = 6.21$ $p < 0.001$, $t = 8.46$; $p < 0.001$, respectively).

We found a statistically significant interaction between photoperiod and distribution of *gnrh1* cells in the rostral-caudal POA ($F_{42,210} = 2.06$; $p < 0.001$; $\lambda = 0.78$; Fig. 5A). Post-hoc analysis revealed that for section 6, SD30 birds had significantly greater numbers of *gnrh1* cells compared to PF ($t = 6.01$; $p < 0.001$); SD10 ($t = 5.71$; $p < 0.001$); and SD20 ($t = 5.71$; $p < 0.001$) birds. For section 7, SD30 birds had significantly greater numbers of *gnrh1* cells compared to PF ($t = 6.21$; $p < 0.001$); SD10 ($t = 4.96$; $p < 0.005$); and SD20 ($t = 4.2$; $p < 0.05$) birds. For section 9, SD30 birds had significantly greater numbers of *gnrh1* cells

compared to PF ($t = 8.12$; $p < 0.001$); SD10 ($t = 6.46$; $p < 0.001$); and SD20 ($t = 4.22$; $p < 0.05$) birds.

Experiment 2

A one way ANOVA revealed a significant difference in follicle volume across treatment groups ($F_{3,15} = 4.02$; $p < 0.05$; $\lambda = 0.89$; Fig. 3B). Holm-Sidak analyses revealed that SD30-LD had significantly larger follicle volumes compared to PR birds ($t = 3.03$; $p < 0.05$). There was no significant difference between SD20-LD and PR ($t = 1.13$; $p = 0.28$; *ns*), and SD10-LD and PR birds ($t = 0.33$; $p = 0.74$; *ns*). There was a significant difference in oviduct mass across treatment groups ($F_{3,15} = 4.57$; $p < 0.05$; $\lambda = 0.67$; Figure 4D). Holm-Sidak analyses showed that SD30-LD birds had greater oviduct mass compared to PR birds ($t = 3.55$; $p < 0.005$). However, there was no significant difference between SD20-LD compared to PR ($t = 1.35$; $p = 0.19$; *ns*), and SD10-LD compared to PR birds ($t = 0.37$; $p = 0.71$; *ns*). There was a significant difference in ovary stage across treatment groups ($F_{3,15} = 51.66$; $p < 0.001$; $\lambda = 0.99$). Ovary stage (Mean $\pm$ Standard error) values were PR (1.0 ± 0.0); SD10 (1.2 ± 0.25); SD20 (2.0 ± 0.0); SD30 (3.0 ± 0.0). Holm-Sidak analyses revealed that SD30-LD and SD20-LD females had significantly advanced ovary stages compared to PR ($t = 11.31$; $p < 0.001$) and SD10-LD females ($t = 5.65$; $p < 0.05$) respectively. However, there was no significant difference between SD10-LD females and PR females ($t = 1.41$; $p = 0.18$; *ns*).

A one way ANOVA revealed a significant difference in *gnrh1* cell counts across treatment groups ($F_{3,15} = 8.52$; $p < 0.005$; $\lambda = 0.99$; Fig. 4B). Holm-Sidak analyses showed that SD30-LD females had significantly greater *gnrh1* cell counts compared to PR birds ($t = 4.07$; $p < 0.005$). However there was no significant difference between SD20-LD compared to PR ($t = 0.91$; $p = 0.38$; *ns*), and SD10-LD compared to PR birds ($t = 0.05$; $p = 0.95$; *ns*). There also was a significant increase in *gnrh1* OD across the treatment groups ($F_{3,15} = 167.33$; $p < 0.005$; $\lambda = 0.99$; Fig. 4D). Post-hoc analysis revealed that SD10, SD20, and SD30 had significantly greater *gnrh1* OD compared to PR ($t = 4.00$; $p < 0.005$, $t = 3.84$ $p < 0.005$, $t = 4.84$; $p < 0.001$, respectively).

We found a statistically significant interaction between photoperiod and distribution of *gnrh1* cells in the rostral-caudal POA ($F_{42,210} = 1.69$; $p < 0.01$; $\lambda = 0.93$; Fig. 5B). Post-hoc analysis revealed that for section 7, SD30-LD birds had significantly greater numbers of *gnrh1* cells compared to PF ($t = 6.36$; $p < 0.001$); SD10-LD ($t = 5.98$; $p < 0.001$); and SD20-LD ($t = 3.98$; $p < 0.05$) birds. For section 8, SD30-LD birds had significantly greater numbers of *gnrh1* cells compared to PF ($t = 6.69$; $p < 0.001$); SD10-LD ($t = 6.52$; $p < 0.001$); and SD20-LD ($t = 4.53$; $p < 0.01$) birds. For section 9, SD30-LD birds had significantly greater numbers of *gnrh1* cells compared to PF ($t = 6.33$; $p < 0.001$); SD10-LD ($t = 5.43$; $p < 0.001$); and SD20-LD ($t = 4.71$; $p < 0.005$) birds. For section 10, SD30-LD birds had significantly greater numbers of *gnrh1* cells compared to PF ($t = 4.47$; $p < 0.01$); SD10-LD ($t = 3.08$; $p < 0.05$); and SD20-LD ($t = 3.08$; $p < 0.05$) birds. For section 11, SD30-LD birds had significantly greater numbers of *gnrh1* cells compared to PF ($t = 4.49$; $p < 0.01$) birds.

DISCUSSION

It has been known for many years that the state of photorefractoriness in seasonally breeding birds such as the European starling is dissipated by the exposure to short days. GnRH1 protein levels in starlings show a gradual increase and until now were the primary means to identify when photosensitivity develops (10,13). The increase in hypothalamic *gnrh1* expression observed here in female European starlings is a key step in the transition from reproductive photorefractoriness to photosensitivity and this dataset significantly advances

our understanding of the genetic steps involved in the avian photoperiodic response. The transfer of female starlings from long days to short days increases in *gnrh1* expression to a greater level in detectable cells and also leads to the ability to identify other POA cells that express *gnrh1* resulting in the detection of a greater number of *gnrh1* cells. We also found that the response to stimulatory long day lengths is negligible until short days have been experienced for more than ten days. A gradual increase in the number of neurons with detectable *gnrh1* expression is associated with the development of photosensitivity and subsequent photostimulation further drives *gnrh1* expression. The findings presented here identify the earliest known change in gene expression during the termination of photorefractoriness and the development of photosensitivity in birds. Furthermore, we show that a change in the expression of *gnrh1* mRNA in the avian POA is a predictor of the change of reproductive state and that the reactivation by short days provides the initial signal essential for reconnecting the brain and reproductive endocrine systems.

The gradual gonadal development observed in photosensitive female starlings is an intriguing pattern that suggests short days may facilitate ovarian function. Previous work in starlings has shown that testicular development will occur after about 4 weeks on short days (21–23). However, testicular recrudescence does not reach the sizes typical for a full breeding condition induced by stimulatory long day lengths. Thus, short days may permit a modest growth in gonadal volume, but the gonads remain in a state much smaller than what is observed during the breeding period and photostimulation by long days is required for full development (10).

Previously, the onset of photosensitivity in starlings was first identified by a slight increase in plasma LH concentrations and gonadal recrudescence after about thirty short days (10, 21–23). Indeed, the POA GnRH1 system is critical for regulating gonadotropin release and gonadal function, and an increase in GnRH1 synthesis precedes the changes in ovarian and oviduct development by several days (10). Specifically, the increase in POA *gnrh1* mRNA expression in female starlings is activated within ten days. We propose that the recovery from photorefractoriness is associated with an increase in *gnrh1* mRNA expression along with an increase in the number of visible *gnrh1* neurons. It is important to note that both the optical density of *gnrh1* cells from Experiment 1 and 2 revealed similar levels which suggests either 1) that the *gnrh1* signal was saturated due to prolonged exposure to the staining solution; or 2) that the levels of *gnrh1* mRNA expression reach a threshold early in the development of photosensitivity and maintained through photostimulation.

It has recently been hypothesized that the avian POA exhibits a functional topographic organization (24). Specifically, studies that have applied lesions (25) and employed immunocytochemistry for the immediate early gene *c-fos* (26) and GnRH1 peptide expression (20) have shown that localized regions in the POA are involved in regulating appetitive and consummatory aspects of sexual behavior. In starlings, the different photoperiodic states are associated with localized effects on the *gnrh1* mRNA (12) and protein expression (20). Here we show the gradual increase in *gnrh1* mRNA expression during the onset of photosensitivity and photostimulation. The overall pattern of *gnrh1* expression suggests that there is a gradual increase in the rostral regions of the POA with a predominant effect on the intermediate POA. The increase in *gnrh1* cells in a rostral direction from the caudal area is quite intriguing and suggests a possible diffusible factor maybe signal the increase in expression. One potential candidate is the thyrotrophin β -subunit (TSH β ; 27). We hypothesize that the removal of the inhibitory effect of long days may trigger an increase in TSH β expression in the mediobasal hypothalamus that leads to the activation of *gnrh1* expression. Then the exposure to stimulatory long days leads to greater levels of TSH β that in turn stimulates a further increase in the number of *gnrh1* cells.

It is well established that neurogenesis is widespread in the adult avian forebrain; however, it has not been identified in the hypothalamus (28,29). The change in starling *gnrh1* mRNA expression detected by *in situ* hybridization (present study; 11, 12) and the immunocytochemical detection of the precursor proGnRH1-GAP and GnRH1 protein (2, 10, 13, 20, 41), is most likely the result of variation in transcription and translation within existing cell populations. Further evidence in support of this hypothesis is derived from unpublished data collected in our laboratory that failed to identify co-labeling of the thymidine analogue bromodeoxyuridine (BrdU) in GnRH1 cells after photoinduction. A reference nucleus, the song control area HVC, (acronym used as a proper name), that is well established to exhibit neurogenesis (28, 29), had prominent BrdU labeled cells. The lack of BrdU labeled cells in the hypothalamus from previous reports (28, 29) and the absence of double labeling of BrdU in GnRH1 cells together suggest that neurogenesis is not involved in the photoinduced change in GnRH1 cell numbers.

In conclusion, the experiments presented here provide evidence that the induction of *gnrh1* expression by short days in female starlings is the initial step critical for renewed physiological responsiveness to stimulatory day lengths. The observed increase in *gnrh1* expression reported here occurred rapidly after exposure to short days and proceeded earlier than previously demonstrated at the level of proGnRH1-GAP and GnRH1 peptide expression (10). We propose that an endogenous signal develops under short day photoperiods and triggers the transcription of POA *gnrh1* expression. The endogenous signal is unknown and though melatonin concentrations increase significantly when birds are exposed to short days and has potent physiological effects that are important for regulating many avian biological rhythms (30) it does not seem necessary for photoperiodic responses in birds (6). The data also highlight the dual role of photoperiod in regulating the seasonal change in avian reproductive physiology: 1) *gnrh1* mRNA transcription/GnRH1 peptide synthesis; and 2) GnRH1 release. Overall, the data reported here provide a framework for further examination at the genetic, neural and systems components underlying vertebrate photoperiodism.

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Figure 1.

A schematic representation illustrating photoperiodism in seasonally breeding songbirds. Under natural photoperiods, the vernal increase in day length (solid line) stimulates the HPG axis leading to gonadal recrudescence (dotted line), a process called photostimulation (i.e. breeding state). After prolonged exposure to long day lengths there is a gradual decline in gonadal state leading to reproductive involution and birds are referred to as photorefractory (i.e. non-breeding). Birds become physiological responsive to stimulatory day lengths after experiencing the short winter day lengths, this change is referred to as the development of photosensitivity (i.e. pre-breeding). Arrows indicate the approximate boundaries between photoperiodic states. See (2) for a review.

A Experiment 1

B Experiment 2

Figure 2.

Diagram representing the treatment groups of the two experimental conditions. In experiment 1, female starlings were transferred from long day lengths (16L:8D) to short days for 10 (SD10), 20 (SD20), and 30 (SD30) days. Arrows indicate time when starlings were moved to short days. One group of females remained on long day lengths to maintain a photorefractory state (PR) for the duration of the experiment. In experiment 2, female starlings experience similar photoperiodic experience as experiment 1, with the additional photostimulation treatment. On day 30, birds were transferred from short day lengths to long day lengths for fourteen days. Again, arrows indicate the time when starlings were moved to short days and then long days.

Figure 3.

The peripheral reproductive measures from experiment 1 and 2. A) and B) provide the mean (+/- SEM) for follicle volumes from females in experiment 1 and experiment 2, respectively. C) and D) show the change in mean (+/- SEM) oviduct mass across experiment 1 and experiment 2 treatment groups respectively.

Figure 4.

The change in *gnrh1* cell numbers and OD in the female POA from experiment 1 and 2. A) demonstrates the mean (+/- SEM) *gnrh1* cell numbers after exposure to short day lengths for increasing periods of time. B) the mean (+/- SEM) *gnrh1* cell numbers after transfer to long day lengths once experiencing various periods on short day lengths. C) and D) show the change in *gnrh1* cell OD for experiment 1 and 2 respectively.

Figure 5.

Anatomically localized effects of photoperiod condition on *gnrl* mRNA expressing cell numbers in the starling POA. Mean (+/-SEM) *gnrl* cell numbers per POA tissue section from A) Experiment 1 – the effect of short days; 2) Experiment 2 – the effect of photostimulation after increasing periods on short days. The sections are taken at 80 μ m intervals and significance is indicated by asterisks * = $p < 0.05$; *** $p < 0.001$.