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Photoperiod-dependent regulation of gonadotropin-releasing hormone 1 messenger ribonucleic acid levels in the songbird brain

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Abstract

Annual changes in day length induce marked changes in reproductive function in temperate zone vertebrates. In many avian species, in contrast to other seasonally breeding animals, plasticity in hypothalamic gonadotropin-releasing hormone – 1 (GnRH1) expression rather than (or in addition to) release governs changes in pituitary-gonadal activity. Investigations of the cellular and molecular mechanisms that govern GnRH1 plasticity were previously hindered by a collective inability of scientists in the field to characterize the *gnrh1* cDNA in songbirds. We finally overcame this roadblock after data from the zebra finch (*Taeniopygia guttata*) genome project enabled us to rapidly clone the *gnrh1* cDNA from hypothalamic RNA of zebra finches and European starlings (*Sturnus vulgaris*). Here, we review the original data that identified GnRH1 protein plasticity in the songbird brain and discuss earlier failed attempts to clone *gnrh1* in these animals. Then, we present recent efforts, including our own, that successfully characterized *gnrh1* in zebra finch and starling, and demonstrated dynamic regulation of *gnrh1* mRNA expression, particularly in sub-populations of preoptic area neurons, in the latter. Overall, this paper highlights GnRH1 plasticity in the avian brain, and weaves into the narrative the previously untold story of the challenges to sequencing *gnrh1* in songbirds.

Keywords

LHRH; plasticity; preoptic area; photoperiod

Historical perspective

The annual change in day length, commonly referred to as photoperiod, is a key environmental signal driving seasonal breeding in many temperate (2, 9, 11, 21, 28, 30, 37,

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56, 58) and some tropical birds (22). Traditionally, the primary measure used to determine the stage of the annual breeding cycle was gonadal volume (57). In the 1970s and the 1980s, the development of radioimmunoassays for gonadotropin and sex steroid hormones in songbirds (Passeriformes) provided a method to obtain a readout as to how the brain changed seasonally in its regulation of seasonal cycles in gonadal volume and reproductive function (19, 55). Studies employing hormone measurement in combination with more traditional analyses of gonadal volume led to a clearer understanding of the way in which variation in photoperiod regulates the timing of reproduction in songbirds species such as the European starling and white-crowned sparrow (12, 32). During the 1980s, work in the starling illustrated that the neuropeptide gonadotropin-releasing hormone (GnRH1) exhibited marked changes in hypothalamic content across different photoinduced reproductive states (10). This finding provided evidence that a discrete hypothalamic neuropeptide played a significant role for the seasonal regulation of reproductive physiology.

In this review, we will discuss the relationship between photoperiodism and GnRH1 plasticity in songbirds. Then we will review some of the prior attempts by multiple laboratories to determine the genetic sequence for *gnrh1* in songbirds. Lastly, we will present recent work that successfully identified the sequence for *gnrh1* and establish the photoperiodic regulation of *gnrh1* mRNA expression in starlings.

Photoperiodic time measurement in songbirds: a focus on absolute photorefractoriness

Birds have evolved a range of photoperiodic responses (reviewed in 28). In seasonally breeding songbirds that inhabit the temperate zone, the annual change in day length provides a predictive cue for the appropriate timing of reproduction when environmental conditions are optimal (11, 44). Seasonally breeding birds exhibit dramatic involution (regression) in their reproductive systems and successful reproduction requires that they initiate gonadal growth (recrudescence) well before the appropriate conditions have occurred (11). Birds that exhibit this photoperiodic response progress through three distinct reproductive states (Fig. 1A). The vernal increase in day length stimulates gonadal recrudescence, increased gonadotropin and testosterone concentrations as well as an increase in the rate of reproductive behaviors. Birds that are in this state are referred to as *photostimulated* and are in breeding condition. Although long day lengths stimulate gonadal growth, prolonged exposure paradoxically lead to gonadal involution and ultimately renders the birds in a non-breeding state through a process referred to as *photorefractoriness*. That is, the long days that initially stimulated reproductive physiology eventually become inhibitory to hypothalamic-pituitary-gonadal (HPG) activity. Birds in this state are reproductively non-responsive to long day lengths (including constant light) as well as to other supplementary environmental cues that can stimulate gonadal growth at other times of the year (11, 13, 32). Two photorefractory responses have been described in bird: absolute and relative photorefractoriness. In the interest of brevity, this review only discusses birds that exhibit absolute photorefractoriness. There are recent views that include a description of relative photorefractoriness and GnRH1 plasticity (see 21, 28). Two criteria that have been applied to examine absolute photorefractoriness include: 1) gonadal involution after prolonged exposure to previously stimulatory long day lengths, and 2) the maintenance of gonadal regression in the presence of an even longer photoperiod (21, 32). In the laboratory, when birds are transferred from a short day (e.g., 9L:15D) to long day (e.g., 16L:8D), gonadal volumes will increase within 3 weeks and after approximately 6–7 weeks, there is an onset of the photorefractory state and gonadal involution ensues (Fig. 1B). In order to restore physiological sensitivity (i.e., responsiveness) to long day lengths, birds must first experience short days similar to those that occur naturally during the late fall and early

winter. Thus, short day lengths lead to the re-sensitization of the neuroendocrine axis; birds in this condition are termed *photosensitive* or pre-breeding.

The avian photoperiodic response is governed by the brain

Systematic experiments were performed to test whether photorefractoriness was explained by a decrease in the ability of gonadotropins to increase gametogenesis and/or steroidogenesis or via a change in sensitivity of the pituitary to hypothalamic stimulation (reviewed in 32). The conclusion derived from these studies is that the gonads and the pituitary in photorefractory birds remain responsive to exogenous treatment with, respectively, gonadotropins and gonadotropin-releasing hormone (GnRH1) so that it was assumed that the site of photorefractoriness was in the hypothalamic-preoptic region at the site of the GnRH1 system or of the upstream neural systems that regulate it (32). Studies illustrating that castrated birds responded to photoperiod in a manner identical to intact birds further supported the notion that changes in the regulation of the neuroendocrine axis at the level of the brain were critical for the control of the photoperiodic response and turned the focus away from gonadal or pituitary mechanisms (3, 32). Indeed, the annual change in pituitary luteinizing hormone (LH) concentrations emerged as the surrogate marker of hypothalamic (GnRH1) output (3). Unfortunately, follicle-stimulating hormone (FSH) remains difficult to reliably measure due to technical problems but it is unlikely that changes in pituitary sensitivity to factors regulating FSH synthesis (e.g., GnRH1, activins, or inhibins) are a major factor driving photoperiodic responses (32).

More recent studies do indicate that it is an oversimplification to merely state that the pituitary and gonadal is responsive to GnRH1 and LH/FSH respectively in birds in a reproductive or non-reproductive state (e.g., 14, 23, 24). For example, standardized injections of GnRH in dark-eyed juncos (*Junco hyemalis*) result in more robust secretions of testosterone when administered early in the breeding season than late in the breeding season. In Northern cardinals (*Cardinalis cardinalis*) injections of GnRH in non-breeding birds revealed differences in the degree to which testosterone was released with injections in March resulting in higher concentrations than those administered in December (14). These studies do indicate that endocrine tissues are not static but also change dynamically across the breeding cycle. This has always been clear to some degree but the critical point to consider here is that there is still no clear evidence that changes in the pituitary or the gonads are the primary drivers of changes in reproductive state. What is clear are changes in reproductive state are associated with changes in the brain and in peripheral tissues that affect their responsiveness to endocrine signals. The studies reviewed here that inject GnRH in a standardized manner may have sampled birds across reproductive states. For example, a bird sampled in December could still be photorefractory while a bird sampled in March is almost certainly photosensitive. Thus part of the many events that occur in association with the transitions among reproductive states involves prominent changes in peripheral tissues including the gonad. It has been shown for example that the testes in starlings exhibit remarkable changes in apoptosis over the course of the breeding cycle (60). To understand completely the timing of gonadal growth and involution in a seasonal context one must consider the hypothalamic-pituitary-gonadal (HPG) axis as an integrated system, the gonads do feedback on the GnRH system in significant ways (4).

In the vast majority of vertebrate species, GnRH1 neuronal system in the preoptic area (POA) governs the secretion of LH from the pituitary gland and is the final common pathway for the neural integration of environmental cues that regulate the timing of reproduction (18, 40). Given that gonadal function (e.g., steroidogenesis and gametogenesis) is dependent on pituitary gonadotropin secretion, which is, in turn, dependent on the

synthesis and release of GnRH1, the focus of much current research on GnRH1 in the neuroendocrine control of reproduction is logical.

Recently, several reviews have pointed out that our ability to answer questions concerning intraspecific variation in the environmental regulation of the timing of egg laying cannot be addressed merely by the measurement of changes in LH in response to changes in photoperiod (38, 54). It has long been known that ovulation, especially in wild species, requires that females be in the right photoperiodic state and that they experience the appropriate supplementary cues (i.e., the presence of a mate, good weather etc.; see 3 for a review). Photoperiod can stimulate the initial stages of ovarian development but the exponential phase of growth that occurs relatively rapidly in response to GnRH1 stimulation of the preovulatory LH surge cannot be triggered by photoperiod alone (38). As has been noted by several authors, non-photoperiodic cues can regulate multiple levels of the HPG axis in order to facilitate ovulation (38, 54). Studies of the mechanism of action of these cues (e.g., temperature, presence of a mate and nest site, etc.) are essential to address certain questions such as intra-specific variation in ovulation (38, 54). We focus on questions of the central mechanisms that regulate seasonal changes in reproductive state. Understanding this variation requires a consideration of studies that focus on the regulation of the GnRH1 neuronal system.

Identification of photoperiodic regulation of GnRH1 protein

One of the original studies that investigated the neuroendocrine control of seasonal breeding in avian species investigated the GnRH1 neuronal system in the European starling (*Sturnus vulgaris*). In this seminal work, Dawson and colleagues identified marked seasonal changes in hypothalamic GnRH1 immunoreactivity, with greater amounts during the breeding season compared to the non-breeding season (10). Additional studies of starlings made it clear that the decline in GnRH1 peptide content occurs after the gonads start to regress (11). These studies illustrated that a change in the regulation of gonadotropin release occurs prior to the inhibition of GnRH1 synthesis. Work in Japanese quail (*Coturnix coturnix*) revealed that GnRH1 plasticity (at least in terms of peptide synthesis) was minimal (20) thus indicating that in some species the seasonal regulation of gonadotropin secretion is regulated solely by changes in the release of GnRH1 (pulse frequency and/or amplitude) and not by changes in its synthesis. This latter pattern is akin to what has been described in most seasonally breeding mammals, such as sheep and hamsters. Together these findings suggested that the annual changes in photoperiod play dual roles in the seasonal regulation of reproduction: the governance of both GnRH1 synthesis and release (11). Some of the most exciting recent work on the photoperiodic regulation of the HPG axis has concerned the cellular and molecular mechanisms governing GnRH1 release (59). However, the observation of a 100-fold change in GnRH1 peptide expression in European starlings leads to the question of how light information regulates GnRH1 protein production. This question was unanswered in starlings for more than 20 years.

One potential explanation for the increase in GnRH1-immunoreactive cell numbers in breeding starlings is that the photoperiod stimulated and/or inhibited neurogenesis in a seasonally dependent manner. That is, songbirds show marked neurogenesis in adulthood and it was possible that, quite simply, GnRH neurons might be lost and gained on a seasonal basis. Indeed, neurogenesis contributes to the marked seasonal plasticity in the brain nuclei controlling song learning and production in several songbird species (6, 8). However, it is unlikely that the change in the number of GnRH1 cells occurs as a result of neurogenesis for two reasons. First, neurogenesis is widespread in the adult avian brain; however, it has not been identified in the POA to the extent that would be predicted based on the changes in GnRH1 cell numbers (33, 34). Second, injections of the thymidine analogue

bromodeoxyuridine (BrdU), a commonly used marker of cell division, failed to co-label with immunoreactive GnRH1 cells in photostimulated male Border canaries (*Serinus canaria*, unpublished observations, but see 41). Thus, the change in detectable GnRH1 cells was likely attributable to renewed transcription and translation within existing cells, though this was difficult to prove due to difficulties in cloning the *gnrh1* cDNA in songbirds.

Photoperiodic regulation of GnRH1 protein expression

Marked variation in GnRH1 protein content in the hypothalamus tends to be observed in species that exhibit absolute photorefractoriness (Fig 1C; 28, 40). Since the initial description of GnRH1 protein plasticity in European starlings (10), a number of subsequent studies in a range of songbirds revealed similar patterns across photoperiodic states (reviewed in 7, 40). The variation in GnRH1 protein expression has been proposed to have evolved as a means to restrict breeding to periods favorable for the survival of the developing offspring (21). Until very recently, it was unknown whether the marked changes in GnRH1 protein expression reflected transcriptional or post-transcriptional mechanisms. Two lines of inquiry provided indirect evidence suggesting that the photoperiod did indeed regulate *gnrh1* mRNA expression.

First, initial efforts to examine whether the *gnrh1* messenger RNA employed protein analyses, specifically an antibody that targeted the GnRH1 precursor peptide (pro-GnRH1-GAP) as an approximation. In starlings, there was pronounced variation in immunoreactivity for pro-GnRH-GAP expression with significantly lower levels in photorefractory starlings (35). This finding provided support that *gnrh1* mRNA was most likely regulated by photoperiod cues and a reduction of expression during the onset and maintenance of photorefractoriness.

Another means to assess the photoinduced variation in GnRH1 expression was to administer a neuroexcitatory glutamate analog, N-Methyl-D-aspartate (NMDA). A series of experiments in mammals illustrated that NMDA can induce acute increases in circulating LH levels (27, 47, 51, 52). This pharmaceutical agent acts directly on GnRH1 neurons and therefore provided a valuable means to directly assess whether photorefractoriness was associated with a decreased availability of hypothalamic GnRH1 content. Work in starlings demonstrated that an analog, N-methyl-D-L-aspartate, was capable of stimulating an increase in LH only in photosensitive and photostimulated starlings, and not photorefractory birds (12). Therefore, the change in photoperiodic state was indeed with a decrease in releasable stores of GnRH1, suggesting again that the *gnrh1* mRNA levels were likely decreased.

The challenge of cloning *gnrh1* in songbirds

The amino acid sequence of GnRH1 in any avian species was first identified in chickens using chromatographic and radioimmunoassay methods (26). It included the collection of nearly 250,000 chicken hypothalami for the purification of the peptide (26), reminiscent of the approach taken by Roger Guillemin and Andrzej Schally when they first purified the 10 amino acid GnRH peptide in mammals. It was found that the avian form deviated from the mammalian form at position 8 where a glutamine is substituted for an arginine. A second form of GnRH in birds, referred to as GnRH2, was also isolated at this time (29). This form deviates from the mammalian form at three of the 10 positions and does not appear to play a major role in the regulation of pituitary function.

The cDNA that encodes the pre-pro-GnRH1 in chickens was subsequently sequenced (16). This set the stage for molecular biological studies in passerine species. Studies employing high performance liquid chromatography (HPLC) methods revealed that brain extracts from

starlings possessed equal amounts of GnRH1-like and GnRH2-like peptides (39). Furthermore, there was a lack of cross-reactivity of antibodies specific to a diverse set of vertebrate forms, including mammalian, salmon and lamprey forms (39). It was therefore reasonable to assume that the decapeptide sequence of GnRH1 in songbirds, such as the starling, may have been similar to that which had been described in chickens.

Several attempts were made by a variety of labs in the 1990s to subclone *gnrh1* in songbirds. For example, two of us (GFB and MMM) in 1994 used degenerate PCR based on available sequences in chicken and other vertebrates to amplify the starling *gnrh1* cDNA from hypothalamic RNA. Despite repeated attempts using a variety of primers and PCR strategies, products corresponding to starling *gnrh1* were never obtained. Similar strategies led to the successful cloning of the turkey *gnrh1* cDNA of the gene in this species (5). Another one of us (DJB) in the late 1990s and then early 2000s attempted to clone the starling cDNA from a hypothalamic cDNA library by low stringency screening with a chicken *gnrh1* cDNA probe and later by expression cloning with a mammalian GnRH antibody. These approaches were also unsuccessful. Our failures and those of other laboratories raised questions as to whether the amino acid sequence of *gnrh1* would be indeed similar in songbirds and galliforms.

We were finally able to answer this question in conjunction with the sequencing of the zebra finch genome (53). With a combination of *in silico* (cross-species megaBLAST), degenerate PCR, and 5' and 3' RACE RT-PCR analyses, we successfully cloned the *gnrh1* cDNAs in zebra finches and starlings (45). At the same time, another group independently cloned zebra finch and starling *gnrh1* using a degenerate PCR strategy (49, 50). The open-reading frames predict proteins of 91 or 92 amino acids, consistent with the size of the prepro-hormone in other avian and non-avian species. Whereas the decapeptide in both species is perfectly conserved with chicken GnRH1, the amino acid identity in the signal peptide and GnRH associated peptide subdomains are significantly less well conserved. At the nucleotide level, the starling and zebra finch coding sequences are 87% identical to each other, but only 70 and 67% identical to chicken prepro-GnRH1. The GnRH1 decapeptide in starlings is identical to what has been described in galliformes, but the low level of nucleotide similarity within the decapeptide coding sequence likely explains our previous difficulties trying to sequence the cDNA using a PCR based strategy alone.

Photoperiodic regulation of *gnrh1* mRNA expression

Studies in galliformes were the first to investigate photoperiodic regulation of *gnrh1* mRNA production. In chicken (*Gallus gallus*), photostimulation caused an increase in hypothalamic *gnrh1* mRNA expression (15). Subsequent work in quail and turkey (*Meleagris gallopavo*) demonstrated that *gnrh1* mRNA expression was also increased by photostimulation (1, 25). However, the change in *gnrh1* mRNA was minimal compared to the variation in GnRH1 protein expression reported in songbirds (7).

In starlings, *gnrh1* mRNA was found to exhibit marked photoperiodic regulation (45, 49, 50; Fig. 2A). Photosensitive starlings had modest levels of *gnrh1* expression, and exposure to long days increased expression, followed by a marked decrease to almost non-detectable levels in photorefractory birds (45; 46). This work was pivotal for establishing that declines in *gnrh1* mRNA levels may be a critical step required for the development of photorefractoriness. The significant inhibition of *gnrh1* mRNA expression in photorefractory birds is only removed after starlings experience at least 10 consecutive short days (41). Therefore, exposure to short days removed the inhibitory 'lock' on *gnrh1* mRNA expression induced by the photorefractory state. However, the ability of long days to initiate *gnrh1* expression to breeding state levels requires exposure to short days for several more

weeks. Together, the findings provide a framework in which long days both stimulate *gnrh1* mRNA expression, but also initiate an inhibitory process leading to photorefractoriness. A similar phenomenon may occur upon exposure to short days, one in which the initial exposure to short days initiates *gnrh1* re-expression, but also initiates another series of events necessary for the subsequent receptivity to stimulatory long days. The exact signal produced during the exposure to short days that gives rise to the resumption of *gnrh1* mRNA expression is currently unknown, but may include either an endogenous timing system or the removal of direct light stimulation during long days.

There are two potential phenomena that could underlie the marked changes in *gnrh1* mRNA: 1) an increase in *gnrh1* expression throughout the entire POA; or 2) anatomically localized variation in expression. When the POA was subdivided based upon functional regions, the rostral and intermediate medial POA exhibited the largest changes in the number of *gnrh1* expressing cells (Fig. 2B). The intermediate lateral POA also exhibited a significant increase in photostimulated compared to both photosensitive and photorefractory males (Fig. 2C). These findings suggest that changes in the *gnrh1* system may exhibit functional topography in response to photoperiodic cues.

The vast majority of work has focused on the variation in *gnrh1* mRNA expression in starlings. However, similar findings have also been reported in other photoperiodic species. For example, Perfito and colleagues (36) demonstrated that wild populations of Great tits (*Parus major*) showed marked differences in photoinduced *gnrh1* mRNA expression. Great tits that inhabit a higher latitude exhibited a marked increase in *gnrh1* mRNA expression shortly after photostimulation, while *gnrh1* mRNA in a population at a lower latitude remained at a lower level of expression (36). Indeed there are most likely latitudinal differences in the degree of *gnrh1* plasticity. For example, in the tropically breeding rufous-collared sparrow (*Zonotrichia capensis*), *gnrh1* mRNA expression is also relatively constant across pre-breeding and breeding states in both males and females (Fig. 3; 42). However, there was a marked sex difference in the levels of *gnrh1* mRNA. The lower level of *gnrh1* mRNA in female rufous-collared sparrows may be attributable to the observation that females generally require additional supplementary cues in order to attain full reproductive development.

Conclusions

Though marked seasonal plasticity in the GnRH1 system in starlings was known for more than a quarter century, it was only in the last few years that we finally confirmed that variation in *gnrh1* mRNA expression underlies these changes. New answers always generate additional questions and that is certainly the case here. For example, do changes in *gnrh1* reflect alterations in mRNA transcription or stability? How do long days first stimulate and later suppress *gnrh1* gene expression? How do short days relieve this inhibitory process? In recent years, a novel peptide referred to as gonadotropin-inhibitory hormone (GnIH) was demonstrated to inhibit GnRH1 and pituitary function (48). This interesting peptide may turn out to be an important regulator of GnRH1 and gonadotropin release especially on a fast time scale (48). Microarray studies investigating seasonal variation in gene expression in the hypothalamus (31) and POA (43) have identified a number of other biological pathways, including DNA methylation and/or histone modifications that could be involved in regulating *gnrh1* mRNA expression on a seasonal basis. With the rapid advancements at the cellular and molecular levels in songbirds, the future of avian neuroendocrinology looks bright and the next few years will undoubtedly yield significant advances in our understanding of mechanisms of photorefractoriness and the regulation of *gnrh1* expression.

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Highlights

1. This paper discusses the relationship between photoperiodism and GnRH1 plasticity in songbirds.
2. Reviews prior attempts by multiple laboratories to determine the genetic sequence for *gnrh1* in songbirds.
3. Presents recent work that successfully identified the sequence for *gnrh1* and established the photoperiodic regulation *gnrh1* mRNA expression in songbirds.

Figure 1.

Photoperiodic regulation of reproduction. A) The vernal increase in day length stimulates gonadal development. In many avian species, the onset of photorefractoriness is associated with gonadal involution, even in the presence of stimulatory days. The late fall and early winter short days are required for the termination of a photorefractory state and the development of a photosensitive state. B) In laboratory settings, long days will stimulate gonadal recrudescence in photosensitive birds. Continued exposure to long days results in photorefractoriness and gonadal involution. C) Photosensitive birds have moderate levels of GnRH1 expression that are further increased after stimulation. The onset of photorefractoriness is associated with a decrease in GnRH1 expression. The lack of parallel patterns between gonadal state (B) and GnRH1 content (C) is due to the dual role of the photoperiod: regulating GnRH1 synthesis and release. Data was adapted from Dawson et al., 2001.

Figure 2.

The annual change in photoperiod regulates *gnrh1* mRNA expression. A) Mean ($\pm$ SEM) *gnrh1* cells in photosensitive, photostimulated, and photorefractory starlings. Photoperiodic states are associated with variation in *gnrh1* mRNA expressing cells in anatomically distinct regions of the POA. B) Mean ($\pm$ SEM) *gnrh1* mRNA cells in the rostral and intermediate regions in the medial POA increased after photostimulation and subsequently decreased to negligible levels in photorefractory starlings. C) A similar pattern was observed in the lateral POA with a greater change in the intermediate region across the three photoperiodic states. Data were taken from Stevenson et al., 2009b.

Figure 3.

Seasonal profile of *gnrh1* mRNA expression in a tropical breeding species. The rufous-collared sparrow did not exhibit a significant change in *gnrh1* optical density (OD) between pre-breeding and breeding states. There was a significant difference observed between males and females. Data taken from Stevenson et al., 2012d.