Phenotypic differences in the GnRH neuronal system of deer mice
Peromyscus maniculatus under a natural short photoperiod

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The neural mechanisms by which short photoperiod induces gonadal regression among seasonally breeding mammals are not well understood. One hypothesis suggests that the proximate cause of seasonal gonadal regression is a photoperiod-induced modification in GnRH secretion. This hypothesis is indirectly supported by our recent findings using immunocytochemistry which identified specific photoperiod-induced adjustments in the number and morphology of GnRH containing neurones between reproductively competent and reproductively regressed laboratory housed male deer mice. Herein, we report that the GnRH neuronal system is similarly affected in reproductively responsive and nonresponsive wild male deer mice Peromyscus maniculatus exposed to a natural short photoperiod. The distribution of immunoreactive (IR)-GnRH neurones was nearly identical in field caught animals and those housed under artificial photoperiod in the laboratory. Compared with reproductively nonresponsive males, reproductively responsive male from the field population possessed a greater total number of IR-GnRH neurones, a greater number of IR-GnRH neurones within the lateral hypothalamus, and a greater proportion of bipolar IR-GnRH neurones. Each of these distributional and morphological characters was consistent with our findings in laboratory housed male deer mice exposed to an artificial short photoperiod. Taken together, these data underscore the validity of using an artificial photoperiod to evaluate seasonal adjustments in reproductive function in the laboratory.

Introduction

Seasonal adjustments in reproductive function are common adaptations used by many small rodents of the north temperate latitude (Bronson, 1987). For members of the genus Peromyscus, gonadal regression confers several advantages, including increased abilities to tolerate cold ambient temperatures and to conserve energy in a winter environment (Blank and Ruf, 1992; Blank et al., 1994; Ruf et al., 1997). Collectively, these adjustments promote survival during harsh environmental conditions and reduce social interactions in the winter months that are associated with mating behaviour and territorial defence (Korytko and Vessey, 1991). While much is known about the metabolic, endocrine and neuroendocrine adjustments associated with seasonal reproductive quiescence, the neural mechanisms engaging gonadal regression remain poorly understood.

However, in deer mice, Peromyscus maniculatus, these mechanisms are thought to be associated with a post-pineal (Blank and Freeman, 1991) alteration in GnRH, leading to suppressed luteinizing hormone secretion and concomitant gonadal regression.

The distribution and morphology of GnRH-containing neurones have been described by immunocytochemical techniques for several seasonally breeding species, including Peromyscus (Glass, 1986; Buchanan and Yellon, 1991; Urbanski et al., 1991; Korytko et al., 1995). However, data from these studies do not indicate a single response pattern for IR-GnRH neurones in terms of cell number, morphology, or density of staining after short day exposure. Consistencies among studies have led to the hypothesis that short photoperiod specifically inhibits release of GnRH, resulting in accumulation of the GnRH peptide within the neurone. We have argued that increased GnRH content alone is an unlikely candidate for gonadal regression among deer mice since both gonadally regressed and nonregressed individuals maintain increased GnRH content under short photoperiod in the laboratory (Korytko et al., 1997).

The deer mouse is a useful animal model to study endocrine and neuroendocrine seasonal adjustments because male and female deer mice respond to short photoperiod with a range of reproductive responses (Blank...
and Desjardins, 1986; Desjardins et al., 1986; Blank et al., 1992). In the laboratory, approximately one-third of all mice exposed to short photoperiod exhibit complete cessation of reproductive function (reproductively responsive), while an equal number are reproductively unaffected by exposure to short photoperiod (reproductively nonresponsive); the remaining individuals exhibit intermediate reproductive responses (intermediates). As noted elsewhere (Blank and Desjardins, 1986; Blank and Freeman, 1991), mice exposed to short photoperiod, regardless of reproductive phenotype, detect and respond to the change in daylength, indicating that the central time-keeping mechanism functions normally in all mice.

To assess whether short photoperiod adjustments in the GnRH neuronal system observed in our laboratory population of Peromyscus reflect what occurs under natural environmental (photoperiod) conditions, we examined the distribution and morphology of IR-GnRH neurones in male deer mice (Peromyscus maniculatus bairdii) from a natural, short day winter population. The distribution and morphology of IR-GnRH neurones between reproductively nonresponsive and responsive males from the field were compared with our previous findings from laboratory housed mice (Peromyscus maniculatus nebrascensis) (Korytko et al., 1995).

Materials and Methods

Animals and treatments

Seven (n = 4 reproductively nonresponsive; n = 3 reproductively responsive) adult male deer mice (Peromyscus maniculatus bairdii) were live-trapped from drainage ditches in Bowling Green, OH (41° 26' N; 83° 40' W), during the winter months of December and January. In the laboratory, complete photoperiod-induced gonadal regression, as assessed by testis size and number of epididymal spermatozoa, occurs after 8 weeks of short photoperiod (8 h light:16 h dark) and is maintained for an additional 12 weeks before spontaneous testicular recrudescence. In the field, duration of testicular regression and responsiveness to short photoperiod are not well defined. Therefore, we captured mice during late December and early January to ensure full reproductive responsiveness and to minimize the likelihood that testicular recrudescence had begun. Animals were removed from Sherman live-traps approximately 30-60 min after sunrise. The reproductive phenotypes of the animals were categorized by measurement of the right testis. Males with a testis size (length × width) greater than or equal to 54 mm² are known to possess long day levels of spermatozoa and were classified as reproductively nonresponsive mice (Blank and Desjardins, 1986). Conversely, mice with a testis size less than or equal to 24 mm² are known to be azoospermic and were classified as reproductively responsive to short photoperiod (Blank and Desjardins, 1986). In this study, all nonresponsive mice had a testis size of greater than or equal to 9 mm × 6 mm and all responsive mice had a testis size of less than or equal to 6 mm × 4 mm. Mean testis weight differed significantly (t = 11.18, P < 0.0001) between nonresponsive (294.75 ± 15.75 mg) and responsive (43.07 ± 15.08 mg) males.

Immunocytochemistry

Tissue preparation and immunocytochemistry were performed as described by Korytko et al. (1995). Briefly, all animals were anaesthetized with sodium pentobarbital (Nembutal, Abbott Laboratories, North Chicago, IL) and perfused intracardially with cold Zamboni's fixative for 20 min at a perfusion rate of 575 µl min⁻¹. Brains were removed and placed in additional fixative overnight at 4°C, and the fixative was replaced with 0.01 mol PBS 1⁻¹ buffer and again at least 1 h before sectioning. Twenty 100 µm vibratome sections were taken from the left half of each brain, extending from 2 mm lateral to midline; this represents one-half of all GnRH neurones present. Immunocytochemical staining for GnRH was performed using the peroxidase–antiperoxidase method with antiserum prepared in rabbit against GnRH (LR1-GnRH kindly provided by R. Benoit, McGill University, Montreal). The LR1 antiserum recognizes amino acids 3,4 and 7–10 of the GnRH decapetide. Each IR-GnRH neurone cell body visualized was assessed for location and process (neuronal projection) number, based on previously identified hypothalamic landmarks for Peromyscus (Eleftheriou and Zolovich, 1965).

Statistical analysis

Comparisons were made between short day reproductive phenotypes. Group differences in all measurements were evaluated by Student's t test. A P value of less than 0.05 was equated with a significant difference.

Results

Nearly all (> 98%) IR-GnRH neurones were located within seven major hypothalamic regions (Fig. 1). Reproductively responsive males possessed greater numbers of IR-GnRH neurones (n = 295.33 ± 4.84) compared with reproductively nonresponsive (n = 218.00 ± 4.92) males (t = 10.63; P < 0.001), consistent with mice from our laboratory population (Korytko et al., 1995). The increase in the number of total IR-GnRH neurones was primarily due to greater numbers of neurones in the lateral hypothalamus (t = 11.98, P < 0.0001) and medial basal hypothalamus (t = 3.11, P < 0.0266) among field-captured responsive mice (Fig. 1). There were no significant differences in the number of visualized neurones between phenotypes in any other brain region examined. The distribution of GnRH-containing neurones among individuals obtained directly from the field was strikingly similar to the distribution of neurones of individuals from the laboratory population (Fig. 1).

Most (> 97%) IR-GnRH cell bodies possessed one or two process extensions in both reproductive phenotypes. Reproductively responsive and nonresponsive mice had
similar numbers of unipolar neurones ($t = 2.11$, not significant). However, reproductively responsive mice had more bipolar IR-GnRH neurones than did nonresponsive mice ($t = 9.36$, $P < 0.0002$). The ratio of bipolar:unipolar neurones was nearly identical in the field and laboratory populations (Fig. 2).

**Discussion**

We have previously reported phenotypic differences in the GnRH neuronal system in response to exposure to short photoperiod in our laboratory population of male deer mice (Korytko et al., 1995). The short photoperiod adjustments observed suggested that reproductive phenotypes could be discriminated based upon the number, location, and morphology of IR-GnRH neurones. To test this model, we evaluated these characteristics in brains of deer mice exposed to a natural short photoperiod. Results of this study provide the first evidence that brain immunocytochemistry can be used in field studies to differentiate phenotypic differences in reproductive response to short photoperiod, and validate the use of artificial photoperiod in laboratory-reared animals as a means of investigating natural adjustments in seasonal reproductive function.

Comparisons of the IR-GnRH neuronal systems of field and laboratory animals yielded three important findings. First, reproductively responsive (regressed) males possessed more GnRH neurones than did reproductively nonresponsive (competent) males in both field and laboratory populations, indicating that the number of GnRH neurones is correlated with reproductive state in deer mice. Other studies have also reported a short photoperiod-induced increase in the number of visualized GnRH neurones in seasonally breeding rodents (Glass, 1986; Korytko et al., 1995). The basis for this observation has been attributed to an accumulation of GnRH in neurone cell bodies due to a reduction in GnRH secretion. However, GnRH accumulation alone does not account for the disparate reproductive phenotypes in deer mice, since GnRH content is similarly increased in both reproductively responsive and nonresponsive males (Korytko et al., 1997). Therefore, identifying subpopulations of neurones that respond differentially to the short-day signal between phenotypes.

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**Fig. 1.** The distribution of immunoreactive (IR)-GnRH neurones in male deer mice from (a) laboratory (Korytko et al., 1995) and (b) field populations. The distribution of GnRH-containing neurones of responsive (■) and nonresponsive (□) mice was markedly similar in both populations. Among field deer mice, responsive males possessed a greater number of GnRH neurones within the lateral hypothalamus and medial basal hypothalamus than did nonresponsive males. LPOA, lateral preoptic area; LH, lateral hypothalamus; DBB, diagonal band of Broca; MPOA, medial preoptic area; AH, anterior hypothalamus; MBH, medial basal hypothalamus; SEPTAL, medial and lateral septal regions. * $P < 0.05$. 

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**Table 1.** Differences in the number of GnRH neurones in the various brain regions of laboratory- and field-reared male deer mice. The number of GnRH neurones was significantly different between laboratory and field reared animals, with laboratory-reared animals having more neurones than field reared animals. The number of GnRH neurones was also significantly different between responsive and nonresponsive animals, with responsive animals having more neurones than nonresponsive animals. The number of GnRH neurones was not significantly different between sexes, with males and females having similar numbers of neurones. 

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**Fig. 2.** The distribution of immunoreactive (IR)-GnRH neurones in male deer mice from (a) laboratory (Korytko et al., 1995) and (b) field populations. The distribution of GnRH-containing neurones of responsive (■) and nonresponsive (□) mice was markedly similar in both populations. Among field deer mice, responsive males possessed a greater number of GnRH neurones within the lateral hypothalamus and medial basal hypothalamus than did nonresponsive males. LPOA, lateral preoptic area; LH, lateral hypothalamus; DBB, diagonal band of Broca; MPOA, medial preoptic area; AH, anterior hypothalamus; MBH, medial basal hypothalamus; SEPTAL, medial and lateral septal regions. * $P < 0.05$. 

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**Table 2.** Comparative analysis of the number of GnRH neurones in the various brain regions of laboratory and field reared male deer mice. The number of GnRH neurones was significantly different between laboratory and field reared animals, with laboratory-reared animals having more neurones than field reared animals. The number of GnRH neurones was also significantly different between responsive and nonresponsive animals, with responsive animals having more neurones than nonresponsive animals. The number of GnRH neurones was not significantly different between sexes, with males and females having similar numbers of neurones. 

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**Fig. 3.** The distribution of immunoreactive (IR)-GnRH neurones in male deer mice from (a) laboratory (Korytko et al., 1995) and (b) field populations. The distribution of GnRH-containing neurones of responsive (■) and nonresponsive (□) mice was markedly similar in both populations. Among field deer mice, responsive males possessed a greater number of GnRH neurones within the lateral hypothalamus and medial basal hypothalamus than did nonresponsive males. LPOA, lateral preoptic area; LH, lateral hypothalamus; DBB, diagonal band of Broca; MPOA, medial preoptic area; AH, anterior hypothalamus; MBH, medial basal hypothalamus; SEPTAL, medial and lateral septal regions. * $P < 0.05$. 

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Fig. 2. The ratio of bipolar:unipolar GnRH neurones in laboratory (Korytko et al., 1995) and field populations of male deer mice. Mice of both populations had similar ratios of bipolar:unipolar GnRH neurones when evaluated by reproductive response phenotype. Responsive mice had a greater proportion of bipolar neurones than did nonresponsive mice.

may provide useful information on the neural loci affected by photoperiod which cannot be defined by measures of GnRH content, turnover, or secretion. Second, the distribution of IR-GnRH neurones in the field animals was nearly identical to that found in laboratory animals of similar phenotype. The striking similarity between populations suggests that photoperiod-induced modifications in GnRH neurones are quite specific, are conserved between different populations, and occur regardless of the short day environment (i.e. artificial or natural). In both populations, the number of IR-GnRH neurones increased in the lateral hypothalamus of reproductively regressed mice, further underscoring this region as potentially important in seasonal reproductive function. In addition to increased numbers of IR-GnRH neurones within the lateral hypothalamus, responsive mice also possessed a greater number of IR-GnRH neurones within the medial basal hypothalamus (MBH), but this increase was present only among field-caught mice. The discrepancy between the field and laboratory populations in the number of GnRH neurones within the MBH may be due to subspecies differences, or to differences in temperature, food availability, or other environmental components. Finally, reproductively responsive and nonresponsive mice of both populations possessed a nearly identical ratio of bipolar:unipolar neurones. Many questions have been raised about the significance of alterations in the polarity of GnRH neurones. While the results of this study do not provide any functional explanations, they support earlier studies that have observed an increase in the numbers of visualized bipolar neurones in reproductively quiescent short day rodents (Buchanan and Yellon, 1991; Korytko et al., 1995). The significance of this consistent observation has yet to be elucidated.

The mechanisms by which short photoperiod causes gonadal regression are incompletely understood. Consistencies among studies of seasonally breeding mammals suggest that photoperiod-induced alterations in melatonin secretion lead to adjustments in GnRH secretion and subsequently to reduced luteinizing hormone secretion and concomitant gonadal regression (Hoffmann, 1979; Goldman and Darrow, 1983; Reiter, 1987). Recent evidence indicates that melatonin secretion (Ruf et al., 1997) and hypothalamic GnRH content (Korytko et al., 1997) are similarly affected by short photoperiod in reproductively responsive and nonresponsive deer mouse phenotypes, yet only responsive mice undergo gonadal regression. The cause of this disparate reproductive response is not fully understood, but may be related to specific short photoperiod induced differences between phenotypes with regard to the number, location and morphology of IR-GnRH neurones. These differences may translate into altered GnRH secretion, one pattern or amount permissive for gonadal competence (nonresponsive mice), and the other pattern or amount unable to support full gonadal function (responsive mice). Collectively, our findings indicate that short photoperiod-induced differences in the number, location and morphology of GnRH neurones are conserved between phenotypes exposed to artificial and natural short photoperiods. This observation is particularly important for validating the use of an artificial photoperiod in the laboratory to mimic natural adjustments in daylength.

While we recognize that multiple environmental
parameters, such as temperature, food availability, and precision of the light cycle, differ between our laboratory and field populations, the change from a long to short photoperiod is thought to be the putative environmental signal directing seasonal reproductive function. Thus, the goal of this study was to demonstrate that adjustments in the GnRH neuronal system of our laboratory population, which is maintained under highly controlled conditions, responds to the change in photoperiod as does the GnRH neuronal system of field animals in a natural short-day winter environment.

Taken together, the results of this study strongly support the hypothesis that phenotypic reproductive variation in free-ranging mice is caused by variable neuroendocrine responses to environmental cues in the GnRH neuronal system. Furthermore, these findings provide support for using laboratory-housed deer mice to answer questions related to environmentally induced alterations that occur in natural populations. Investigating the cause of differential gonadal responses may yield important information about how multiple physiological adjustments to environmental factors are organized and engaged by the central nervous system. Indeed, these data indicate that individual reproductive differences at the level of the organism can be distinguished at the neuroendocrine pathway that regulates reproductive function. Thus, from the perspective of understanding the neurological determinants of phenotypic-level responses, deer mice can serve as a useful model.

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References
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