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Microevolution of neuroendocrine mechanisms regulating reproductive timing in *Peromyscus leucopus*

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Synopsis A key question in the evolution of life history and in evolutionary physiology asks how reproductive and other life-history traits evolve. Genetic variation in reproductive control systems may exist in many elements of the complex inputs that can affect the hypothalamic–pituitary–gonadal (HPG) or reproductive axis. Such variation could include numbers and other traits of secretory cells, the amount and pattern of chemical message released, transport and clearance mechanisms, and the number and other traits of receptor cells. Selection lines created from a natural population of white-footed mice (*Peromyscus leucopus*) that contains substantial genetic variation in reproductive inhibition in response to short winter daylength (SD) have been used to examine neuroendocrine variation in reproductive timing. We hypothesized that natural genetic variation would be most likely to occur in the inputs to GnRH neurons and/or in GnRH neurons themselves, but not in elements of the photoperiodic pathway that would have pleiotropic effects on non-reproductive functions as well as on reproductive functions. Significant genetic variation has been found in the GnRH neuronal system. The number of GnRH neurons immunoreactive to an antibody to mature GnRH peptide under conditions maximizing detection of stained neurons was significantly heritable in an unselected control (C) line. Furthermore, a selection line that suppresses reproduction in SD (photoperiod responsive, R) had fewer IR-GnRH neurons than a selection line that maintains reproduction in SD (photoperiod nonresponsive, NR). This supports the hypothesis that genetic variation in characteristics of GnRH neurons themselves may be responsible for the observed phenotypic variation in reproduction in SD. The R and NR lines differ genetically in food intake and iodo-melatonin receptor binding, as well as in other characteristics. The latter findings are consistent with the hypothesis that genetic variation occurs in the nutritional and hormonal inputs to GnRH neurons. Genetic variation also exists in the phenotypic plasticity of responses to two combinations of treatments, (1) food and photoperiod, and (2) photoperiod and age, indicating genetic variation in individual norms of reaction within this population. Overall, the apparent multiple sources of genetic variation within this population suggest that there may be multiple alternative combinations of alleles for both the R and NR phenotypes. If that interpretation is correct, we suggest that this offers some support for the evolutionary ''potential'' hypothesis and is inconsistent with the evolutionary ''constraint'' and ''symmorphosis'' hypotheses for the evolution of complex neuroendocrine pathways.

Introduction

A major question in evolutionary ecology asks how life-history traits evolve (Roff 2002). Field studies answer this question by identifying patterns of traits associated with particular selective forces. From a physiological perspective, however, this answer is incomplete. Life-history traits such as age at first reproduction, the timing of reproduction, or number/size of offspring per reproductive attempt are caused by the combined effect of secreted neuronal or hormonal chemical messages, receptors for the messages, and the responses of cells to receptor activation (Ricklefs and Wikelski 2002; Hau 2007; McGlothlin and Ketterson 2008). In higher animals, regulatory control is complex because multiple types of secretory cells release different chemical messages that travel different distances, are cleared away at different rates, act through their own individual receptors on multiple types of target cells, and induce different responses in different targets. Genetic variation in DNA sequence, heritable DNA methylation, transcription to mRNA, and post-transcriptional modification in any or all elements of these complex systems might affect the ultimate phenotype of animals.

An evolutionary ecologist can legitimately combine all of these complex effects to consider a single life-history trait as having a population

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reaction norm of phenotypes (i.e., the range of phenotypes/ages at first reproduction possible in a population given the range of possible environments), along with the possibility of genetic variation among individuals in their individual norms of reaction (the range of phenotypes/ages at first reproduction possible for that individual given the range of possible environments) (Roff 2002; Lessells 2008). In contrast, an evolutionary physiologist must examine multiple interacting potential variables: genetic variation in any or all of the many physiological traits that contribute to a single life-history trait (Williams 2008), including the potential for genetic variation in norms of reaction (Lessells 2008). To add even greater complexity, many cells and chemical messengers contribute to more than one life-history trait; hormones that may affect age at first reproduction (e.g., growth hormone, thyroxin, sex steroid hormones, and others) often have known multiple affects on body size and shape, physiology, or behavior that are themselves subject to selection, leading to multiple potential life-history trade-offs (Stearns 1989; McGlothlin and Ketterson 2008). Attempting to discover patterns and processes of evolutionary physiology that link genetic variation in neurons, glands, hormones, and receptors to phenotypes subject to selection is challenging, but critical to understanding the evolution of life histories and the causes and consequences of variation (Ricklefs and Wikelski 2002; Williams 2008).

The studies we summarize here were undertaken in part to answer evolutionary physiological questions. We ask how genetic variation in neuroendocrine traits affects phenotypes that are subject to natural selection, how selection might act on phenotypes to alter neuroendocrine traits within a population, how much genetic variation in neuroendocrine traits exists within natural populations, how does that genetic neuroendocrine variation cause phenotypic variation, and can we predict how perturbations to a population from natural or anthropogenic sources will affect physiology and therefore phenotype? Sophisticated molecular genetics and cell biology are uncovering more and more about heredity and cell function related to these questions, and new approaches in ecology and evolutionary biology are explaining how organismal interactions and selection result in changes in populations over time. Evolutionary physiology is a necessary link between the two. Medically, it is important to understand individual genotypic and phenotypic variation applied to human reproductive health and life history (Bittner and Friedman 2000). At present, we suggest that the most important missing information concerns the sources of natural genetic variation in neuroendocrine traits that affect specific phenotypic traits. To address this question, we chose to test for neuroendocrine genetic variation in a single life-history trait, winter reproductive timing, which is known to be genetically variable in multiple populations of multiple species of temperate-zone rodents (Bronson and Heideman 1994; Prendergast et al. 2001).

Environmental heterogeneity in reproductive timing

Winter reproductive timing is adjusted by mechanisms that use environmental and endogenous information to suppress or stimulate the reproductive axis. The evolution of reproductive timing is a response to predictable seasonal changes or nonseasonal changes in resource availability, temperature, and risk that occur in many environments (Bronson 1989; Prendergast 2005). Reproduction is costly, and physiological strategies that maximize fitness in response to variation in environmental conditions should be favored by natural selection (Horton and Rowsemitt 1992). Selective pressures are often variable over time and space, resulting in individual phenotypes and genotypes that are favored or disfavored variably depending upon the season, year, or location (Bell 1997; Mitton 1997). High variation in environmental conditions could, in principle, allow high phenotypic and genetic variation in reproductive timing within wild populations (Nelson 1987; Blank 1992; Heideman et al. 2005). Even in the absence of environmental variability, mutation, genetic drift, and genetic variation due to alleles with low effects on fitness could result in high levels of within-population genetic variation. Genetic variation in reproductive timing from any cause must be based on underlying neuroendocrine variation in mechanisms that alter reproductive timing.

Genetic variation in reproductive timing

Studies on natural genetic variation in neuroendocrine life-history traits have stringent requirements: (1) known genetic variation in well-characterized populations either still in the wild or recently derived from nature, (2) moderate to large sample sizes, and (3), methods for collection of tissues and data that are usually time consuming (Heideman 2004). Experiments applying artificial selection to wild-derived populations provide a method to study genetic variation in neural and endocrine traits within populations (Gibbs 1999; Garland 2002).

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Genetic variation in such populations can be inferred to have been largely or entirely present in the source population, allowing cautious inference about how microevolution might occur in nature due to natural selection or genetic drift. It is important to recognize that variation in long-captive populations, highly inbred populations, and domesticated species is helpful, but limited, for understanding natural genetic variation and microevolution of neuroendocrine regulatory systems (Heideman 2004; Smale et al. 2005). In order to understand the evolution of reproductive timing, we must understand the natural variation in neuroendocrine traits that affect reproductive timing.

In this article, we describe natural genetic variation in neuroendocrine systems that regulate reproductive timing in a population of a widespread species of North American mouse, *P. leucopus* (the white-footed mouse). In the series of studies we integrate here, natural variation in a single population was isolated in different lines of mice by artificial selection on a single phenotypic trait, winter reproduction. The variation is natural in the sense that the few generations of laboratory selection have not permitted sufficient time to accumulate and spread new mutations, and thus differences between lines are likely to be based on variable alleles that exist in the wild population.

Animal model

The following description of our animal model and selection lines is summarized from Avigdor et al. (2005) and Broussard et al. (2009). White footed mice are small rodents (16–24 g adult body weight) found through much of southern, central, and eastern North America. The timing of reproduction is highly variable among populations, including year-round breeding in some southern latitudes, breeding mostly in the spring and summer months in more northern latitudes, and many additional patterns of timing (Bronson and Heideman 1994). Females produce multiple litters per year. After a 3-week gestation period, females produce litters ranging in size from two to eight offspring. Females wean offspring to independence at an age of 3–4 weeks. Males and females reach full adult body size at age 70 days but become sexually mature at the age of about 46–60 days under favorable conditions.

Because of high mortality due to predation, average longevity in nature is generally in the range of several months, and very few individuals reach 1 year in age. Thus, at the beginning of a temperate-zone winter, mice have a relatively low probability of surviving until spring, creating a potential life-history tradeoff in which winter reproduction might provide an immediate increase in fitness, but at the cost of higher energetic requirements and increased predation risk (Heideman et al. 2005). Wild populations of *P. leucopus* include individuals with varying reproductive responses to short photoperiod (Lynch and Gendler 1980; Heideman and Bronson 1991; Heideman et al. 1999a). Some members of a population respond strongly to short-day photoperiods typical of the winter months by exhibiting gonadal regression or significantly delayed reproductive development, while others appear capable of reproducing at all times of the year and under any photoperiod. Individuals also may express intermediate responses to short photoperiod. In samples from the natural source population of our colony collected in different years, ~20–50% of adults captured have been reported to be in winter reproductive condition during mid-winter (Terman 1993; Heideman et al. 1999a). The evidence suggests that there is widespread, genetically based variability in the photoneuroendocrine pathway that regulates reproduction of this species (Heideman and Bronson 1991; Heideman et al. 1999a; Prendergast and Nelson 2001).

Selected and control lines of mice

Two selected lines and an unselected control (C) line of mice used in this study were produced by artificial selection for reproductive responses to short photoperiod on a population of *P. leucopus* founded from mice captured in 1995 near Williamsburg, Virginia, USA (Lat 37°N, long 76°W) (Heideman et al. 1999a). Forty-eight wild-caught mice bred successfully in the laboratory to establish a parental laboratory generation of 104 breeding pairs. The unselected C line was founded from the parental laboratory generation of males and females paired at random. To establish selection lines either reproductively inhibited in short days (Responsive, R) or not reproductively inhibited by short days (Nonresponsive, NR), offspring of the parental generation were conceived and born in long photoperiod (16L:8D; LD), transferred to short-day photoperiod (8L:16D; SD) within 3 days of birth, weaned at 21–23 days of age, and singly housed in polyethylene cages with wire tops and pine shavings until 70 ± 3 days of age. Mice were examined at 70 days of age and assigned a reproductive index based on testis size or the size of the ovaries, uterine diameter, and presence or absence of visible corpora lutea (Heideman et al. 1999a). Females with ovaries ≤ 2 mm in length, lacking visible corpora lutea, and uterine diameter of

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≤0.5 mm were classified as reproductively inhibited and thus responsive (R) to short photoperiod. Females with large ovaries, large visible follicles or corpora lutea, and uterine diameter >1 mm were classified as nonresponsive (NR). Males with a testis index (TI = length times width of testis) <24 mm^2 were classified as R, those with a TI > 32 mm^2 were classified as NR (Heideman and Bronson 1991; Heideman et al. 1999a). In some of our studies, data on size of testes have been presented as estimated testis volume (ETV = $width^2 \times length \times 0.523$); a TI of 24 mm^2 is approximately equivalent to ETV of 50 mm^3; a TI of 32 mm^2 is approximately equivalent to ETV of 90 mm^3. These measures are highly correlated with mass of testes (Heideman and Bronson 1991; Broussard et al. 2009). After founding, each line in each generation included 20–50 successful breeding pairs. Within three generations in the laboratory, most young mice from the R line had suppressed reproductive systems in SD, while the C line (not subject to selection) continued to produce a distribution of reproductive phenotypes similar to the parental generation (Heideman et al. 1999a). In the 10th and subsequent generations, occasional individuals (~5–10%) with a fully responsive phenotype occur in the NR line, and occasional individuals (~5–10%) with a fully nonresponsive phenotype occur in the R line (Heideman, unpublished data). In these later generations, our subjective impression is that unintentional domestication is beginning in all three lines, noticeable primarily in a slight subjective increase in tameness of these jumpy and sometimes aggressive mice. Additional details on the selection lines and on R, NR, and intermediate (I) phenotypes are provided elsewhere (Heideman et al. 1999a, 2005; Broussard et al. 2009). In typical experiments, mice were born in LD and either retained in LD or transferred to SD within 3 days of birth. In some experiments mice were retained in SD until age 70 days and then retained in SD or transferred to LD.

Strategy for testing neuroendocrine traits as potential sources of variation in reproductive timing

The regulation of seasonal timing could, in principle, have dozens to hundreds of possible locations for genetic variation between R and NR mice. Any neuroendocrine trait that is altered in SD relative to LD and can stimulate or inhibit gonadal development is a potential source of genetic variation in reproductive photoresponsiveness. There are many possibilities, including any element of the pathway that transmits information about daylength to the hypothalamic–pituitary–gonadal (HPG) axis or "reproductive axis" (Fig. 1) that controls reproduction (Ebling and Cronin 2000; Goldman 2001). Input runs from the retina in the eye along retinohypothalamic tracts to the hypothalamus, through the suprachiasmatic nucleus (SCN) and paraventricular nucleus, then out of the brain to the superior cervical ganglia, and back via sympathetic neurons to the pineal gland. The pineal gland produces its hormone, melatonin, only at night, and melatonin is, therefore, a hormonal signal for night. A short-day pattern of melatonin acts, apparently indirectly via thyroid hormone (Nakao et al. 2008; Ono et al. 2008), on pulsatile GnRH secretion into the pituitary portal system from the median eminence in the hypothalamus, which in turn regulates secretion of follicle stimulating hormone and luteinizing hormone from the pituitary, and gametogenesis and production of sex steroid hormones, respectively, in the gonads. In principle, any allelic variant that blocked the photoperiodic portion of this pathway would make an individual nonresponsive to photoperiodic cues. However, most such effects would have pleiotropic effects on other traits that would likely be disfavored by natural selection in a

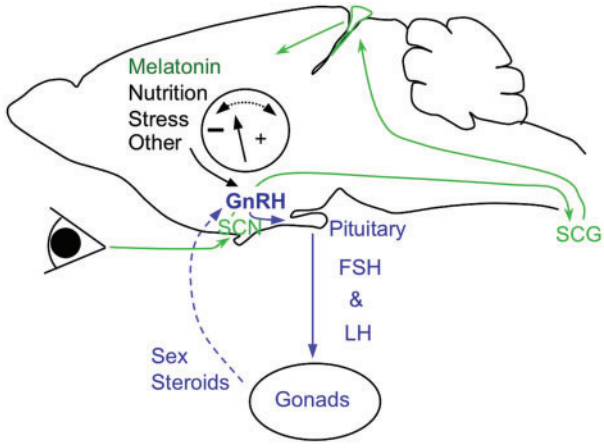

Fig. 1 The HPG axis (in blue) of the reproductive system is regulated by multiple inputs, including season (photoperiod pathway, in green), nutrition and stress (in black). Genetic variation could occur in the photoperiodic pathway, in nonphotic inputs to GnRH neurons, in GnRH neurons themselves, and/or elsewhere in the HPG axis, and each might be altered by microevolutionary change. At the neuroendocrine level, microevolutionary changes may include numbers of neurons or hormone-secreting cells, activity of these cells, abundance of receptors or binding of receptors in target cells, and strength of response to receptor binding, represented in the figure by an adjustable rheostat.

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natural population. Altered function of the circadian clock in the SCN or melatonin secretion could create R and NR individuals and does so in some inbred, captive populations (reviewed by Majoy and Heideman 2000), but disrupted circadian rhythms or melatonin rhythms would disrupt many important aspects of rhythmic behavior and physiology (DeCoursey et al. 2000).

For natural populations, we have examined three hypotheses for natural genetic ''rheostats'' that could tend to increase or decrease the sensitivity of the reproductive axis to SD (Fig. 1). First, we hypothesized that genetic variation in elements of the photoperiodic pathway that have functions and effects distinct from the reproductive axis would not be sources of the natural genetic variation found in a wild population (H_1). We hypothesized two likely sources of natural variation that could affect seasonal reproduction without disrupting other functions. First is variation in the GnRH neuronal system that affects the response to seasonal signals (H_2), and the second is variation in the strength of stimulatory and inhibitory seasonal inputs to the GnRH neuronal system (H_3). Neuroendocrine variation of either type would either stimulate or inhibit the entire reproductive axis as a unit, with minimal indirect effects on other aspects of physiology and behavior. In the studies that we summarize below, we tested for variation in neuroendocrine traits that would affect other responses (H_1) and for variation in neuroendocrine traits that may be specific to seasonal inputs to the HPG axis (H_2 and H_3).

Tests of H_1: Phenotypic and neuroendocrine traits predicted not to vary between lines

There are well-characterized laboratory populations in which nonresponsiveness to photoperiod is caused by partial disruptions to the circadian clock (Puchalski and Lynch 1986; Carlson et al. 1989; Puchalski and Lynch 1991; Freeman and Goldman 1997a). We found that while both males and females differ significantly between R and NR lines in some parameters of circadian rhythms, the differences appear not to be the cause of variation in photoresponsiveness (Majoy and Heideman 2000). In a test for general responsiveness to melatonin, both lines were found to have neurons in the preoptic area of the hypothalamus that altered neuronal firing rate in response to melatonin treatment *in vitro*, and responses of neurons from the two lines were similar (Fetsch et al. 2006). Thus, there was no evidence for inability to either detect or respond to melatonin in the NR line. Both lines decreased in body mass and intake of food identically in SD (Heideman et al. 2005) and both lines had higher brown adipose tissue (BAT) in SD (Reilly et al. 2006), indicating that both lines have the capacity to detect and respond functionally to an SD physiological signal.

Interestingly, both the R and NR lines have lower masses of testes and seminal vesicles when raised in SD than when raised in LD (Avigdor et al. 2005; Heideman et al. 2005). Thus, most or all males from the NR line are at least slightly reproductively photoresponsive. The functional difference is that, despite reproductive suppression by SD, NR males develop high average testis size in SD, matching the LD testis size of males in the C line and the parental generation (Heideman et al. 1999b, 2005; Avigdor et al. 2005). Furthermore, NR mice in SD appear to be fertile (P.D. Heideman, unpublished data). In contrast, males from our R line are strongly reproductively suppressed by SD, with most R mice that are raised in SD having testes of a size that indicates either oligospermy or azoospermy (see Broussard et al. 2009). We also found no evidence that the lines differ in response to manipulation of NMDA receptors with exogenous agonists or antagonists (Tatum and Heideman, unpublished data), suggesting that this general input to GnRH neurons is not variable. Because NMDA receptors are widespread in the brain and serve many functions, this input is another that one might predict as unlikely to be variable in a natural population. All of these results are consistent with H_1.

Tests of H_2: Variation in GnRH neurons, a simple story

In a study on GnRH neuronal staining, NR mice had 50% higher counts of immunoreactive (IR) GnRH than did R mice (Avigdor et al. 2005). In the unselected C line, the number of IR-GnRH neurons was significantly heritable, with a broad-sense heritability of 0.72 (Heideman et al. 2007). Both results are consistent with an interpretation of high amounts of genetic variation in GnRH neurons in the source population. Preliminary data supports a functional outcome from the variation in GnRH neurons. NR females that were ovariectomized with estradiol replacement had higher LH levels than similarly treated R females in both LD and SD (P.D. Heideman et al., manuscript in preparation). An ongoing study is examining differences between NR and R males in sexual behavior in SD and LD. The results suggest a simple source of variation between R and NR mice that potentially accounts for the phenotypic variation between the selection

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lines: a functional difference at the level of GnRH neurons that results in differences in pituitary regulation of the gonads.

The most appropriate interpretation of counts of IR-GnRH neurons is still uncertain because the regulation of GnRH mRNA expression, translation to pro-GnRH protein, and post-translational modification to mature decapeptide GnRH for release are all unknown. GnRH mRNA is translated in the rough ER to produce pro-GnRH. Pro-GnRH is processed in the Golgi-apparatus into secretory vesicles, with final processing to mature GnRH occurring within vesicles that are then transported to nerve terminals for release (Yin and Gore 2006). GnRH neurons secrete maximally only during the GnRH surge of females, in which secretion may be 100-fold higher than at other times for reproductively active females or males. Male mammals are capable of producing an artificially induced GnRH surge (e.g., McPherson and Mahesh 1982), but never do so naturally. Thus, GnRH neurons in reproductively active males are secreting GnRH at perhaps a few percent of maximal capacity, suggesting that mature GnRH may never be depleted significantly in male mammals. Depending upon how GnRH mRNA and protein products are regulated, counts of IR-GnRH neurons might be total neuron counts (if sufficient target peptide is present in each GnRH neuron and if ICC conditions are such as to label every neuron above background staining) or might be related to GnRH neuronal activity (if some neurons have too little GnRH to label above background). Our studies (Avigdor et al. 2005; Heideman et al. 2007) optimized ICC and counting criteria to maximize detection of GnRH neurons. The estimates we obtained for total number of IR-GnRH neurons approximate the number of GnRH neurons expected from a rodent with this size of body and brain, suggesting that the counts of IR-GnRH neurons might estimate the total for GnRH neurons (Avigdor et al. 2005). However, further work is necessary to determine whether genetic variation in counts of IR-GnRH neurons indicates genetic variation in some aspect of activity, function, or neuron number. It is important to recognize that studies using antibodies to different GnRH peptide targets and with differently optimized ICC conditions might provide counts that reflect the intensity of stimulation of GnRH neurons, GnRH neuronal secretory activity, or total number of GnRH neurons.

Evidence for functional significance of the number of GnRH neurons is provided by studies of ''split rhythm'' female hamsters. In this model, the two suprachiasmatic nuclei (SCN) each control the timing of a GnRH surge, causing two GnRH surges separated by 12 h (Swann and Turek 1985), each involving only half of the GnRH neurons: those ipsilateral to the controlling SCN (Kriegsfeld and Silver 2006). In these hamsters, the two preovulatory LH surges that result each produce half of the LH secretion of a normal single surge (de la Iglesia et al. 2003), suggesting that the amount of LH secreted is related to the number of actively secreting GnRH neurons.

While variation in the number of IR-GnRH neurons appears to have functional significance to the HPG axis in this population, the number of IR-GnRH neurons was not correlated with testis size in either the NR or the R line in either photoperiod (Avigdor et al. 2005). Furthermore, the number of IR-GnRH neurons was not correlated with testis size in SD in the unselected C line (Heideman et al. 2007). This suggests that while variation in the number of IR-GnRH neurons is involved in variation in reproductive photoresponsiveness, the overall size of the testes that develop is controlled by some independent factor.

Tests of H_3: A more complex story, variation in inputs to GnRH neurons

Variation in regional binding of iodomelatonin

While the two lines did not differ in neuronal firing in response to melatonin treatment in the preoptic area/anterior hypothalamus (Fetsch et al. 2006), the lines differed in iodomelatonin binding in regions of the brain that might be involved in reproductive regulation. Binding of iodomelatonin (IMEL) occurs in brain regions of both R and NR mice in a pattern similar to that in other rodents (Heideman et al. 1999b). In the SCN, which has melatonin receptors that appear to be involved in regulation of circadian rhythms, binding of IMEL is similar in the NR and R lines. However, in the medial preoptic area (MPOA) and bed nucleus of the stria terminalis (BNST), areas which are implicated in reproductive regulation (Meisel and Sachs 1994; Hill et al. 1996), binding of IMEL was significantly higher in NR than R mice (Heideman et al. 1999b). There were no differences in the binding of IMEL detected in the pars tuberalis, paraventricular nucleus, or dorsomedial hypothalamic nucleus (Heideman et al. 1999b). Because it is not yet fully understood how melatonin acts on inputs to GnRH neurons, the functional significance of variation in the MPOA and BNST is not known, but suggests genetic variation in

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the processing of melatonin signals to the reproductive axis.

Variation in food intake and phenotypic plasticity of reproductive photoresponsiveness

Males in the NR line were found to eat ~45% more food than did males in the R line in both LD and SD (Heideman et al. 2005). This suggests both a need for higher intake of food to support reproduction in winter in the NR line and a cost that occurs in LD as well as in SD (Heideman et al. 2005). *Ad libitum* intake of food was found to be correlated with testis size in SD, but not in LD, suggesting that intake of food is important for maintaining reproductive condition in SD, but not in LD. Furthermore, males in the NR line became more sensitive to reproductive suppression by SD when subjected to mild restriction of access to food (Reilly et al. 2006). In the study by Reilly et al. (2006), in order to rule out the possibility of responses to food that were simply due to nutritional insufficiency, a paradigm of food restriction was developed that allowed mice to increase body mass at the same rate as same-aged mice fed *ad libitum*. The restriction method gave alternate nights with 70% of the *ad libitum* intake measured at the start of the experiment and *ad libitum* access to food (Reilly et al. 2006). Under this paradigm, mice were able to compensate for reduced food on nights when food was restricted by eating more on the alternate nights of *ad libitum* availability. This resulted in a reduction of overall intake of food by ~5–10%. Mice in food-restricted treatments gained weight at the same rate as controls with *ad libitum* feeding, indicating that food-restricted mice were not energy-limited, although they were presumably hungry on the nights food was restricted. Control mice in both LD and SD had large testes, and mice in the food-restricted LD group did not differ from these in testis size. Importantly, testis size decreased significantly only in the food-restricted group in SD (Reilly et al. 2006). This suggests that the high intake of food of NR males is necessary to support reproduction in SD, but not in LD. Because the other genetically defined subset of the population, the R line, is reproductively suppressed in SD even on an *ad libitum* diet, while the NR line has a phenotypically plastic response to SD that varies with intake of food, there must be genetic variation for the reaction norm of responses to food and photoperiod.

Phenotypic plasticity with respect to age

The tendency to suppress reproduction in SD decreases with age (Broussard et al. 2009). Males in the R line are more strongly reproductively suppressed in SD at age 70 days than after exposure to LD when older, followed by retesting in SD at age 240–330 days (Broussard et al. 2009). In males from the C line that were similarly reproductively suppressed by SD at 70 days, retesting in the same manner at 240–330 days also indicated reduced sensitivity to reproductive suppression with age, with an increased proportion failing to suppress testis size as older adults (Broussard et al. 2009). This result indicates phenotypic plasticity in response to photoperiod that varies with age and with line, suggesting additional genetic variation in this population in the reaction norms of response to photoperiod.

Quantitative phenotypes and potential causes of variation from single versus multiple alleles

Neither selection line is uniform in the reproductive phenotype in SD, as noted in the description of our lines above, which suggests that our lines are not genetically uniform for physiological traits that contribute to photoresponsiveness. Furthermore, none of our evidence implies that either population is uniform in the neuroendocrine mechanisms that underlie variation in the SD breeding phenotype. Reproductive phenotypes cannot be predicted in our mice solely by IR-GnRH neuron counts, binding of IMEL in the MPOA and BNST, or intake of food, as there is substantial overlap between lines in the ranges of these measures (Heideman et al. 1999b; Heideman, unpublished data). However, this lack of evidence for any single source of genetic variation resulting in R or NR phenotypes is not sufficient to conclude that there must be multiple sources of genetic variation in photoresponsiveness. It is difficult to propose a single gene for which variability would alter binding of melatonin, intake of food, and number of GnRH neurons, but some single source of variation might be able to have a pleiotropic effect on these traits. At present, however, we suspect one of two possibilities. First, it may be that individuals in a line share alleles of two or more genes that combine to make them strongly photoresponsive or nonresponsive, respectively. Alternatively, individuals in each line may be variable in the mechanisms that cause them to display a particular phenotype, converging on shared phenotypes via many alternative combinations of genetically variable neuroendocrine traits.

Evidence from other populations and other species

In laboratory populations of Siberian hamsters, phenotypic variation in photoresponsiveness appears

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to be due to differences in melatonin secretion and circadian traits that affect photoperiodic time measurement (Puchalski and Lynch 1986, 1988, 1991; Freeman and Goldman 1997a). In contrast, populations of *P. leucopus* from Georgia and Connecticut that vary in photoresponsiveness did not differ in circadian measures, but instead differed in their reproductive response to short photoperiod or melatonin (Carlson et al. 1989). Similarly, extensive studies by Blank and colleagues (Blank 1992; Korytko et al. 1997; Mintz et al. 2007) on phenotypic variation in photoresponsiveness in the deer mouse, *Peromyscus maniculatus*, have identified variation in the response of the HPG axis to the photoperiodic signal, as in our population. It appears likely, however, that the mechanisms of variation in the HPG axis in our population differs from mechanisms of variation in the HPG axis in the population of *P. maniculatus* studied by Blank and colleagues (see Avigdor et al. 2005; Heideman et al. 2005, 2007).

Many previous studies on rodents have shown that restricted access to food combined with short photoperiod causes greater inhibition of reproduction than does short photoperiod alone (e.g., Desjardins and Lopez 1983; Blank and Desjardins 1985; Nelson et al. 1997; Demas and Nelson 1998; Edmonds et al. 2003). The mechanism proposed for these effects is that a reduction in intake of food causes reduced energetic reserves or poor nutritional condition, which in turn inhibits the reproductive system (Desjardins and Lopez 1983; Nelson et al. 1997; Demas and Nelson 1998; Edmonds et al. 2003). To us, a more interesting question has been whether restricted access to food might interact with, and enhance, photoperiodic inhibition of reproduction even when energetic reserves and body condition were unaffected. The experiments on our NR line described by Reilly et al. (2006) is the only study of which we are aware that shows that restricted access to food acts independently of body mass and energetic status to induce reproductive suppression by SD. Reilly et al. (2006) is also the only study to suggest that there is intrapopulational genetic variation in the norm of reaction to food intake, as NR and R mice differ in their response to *ad libitum* food in SD.

Age-related decreases in reproductive photoresponsiveness have been reported in multiple previous studies (Johnston and Zucker 1979; Donham et al. 1989; Freeman and Goldman 1997b; Edmonds and Stetson 2001), as well as in our population (Broussard et al. 2009), and may be common in temperate-zone rodents. Gorman and Zucker (1997) found that exposure to the longest photoperiods of summer subsequently reduced reproductive photoresponsiveness in Siberian hamsters, a form of phenotypic plasticity that their data suggest could be a cause of age-related reductions in photoresponsiveness. In addition to an age-related reduction in photoresponsiveness, our population contains genetic variation in the norm of reaction for these age-related changes, as there is a variable genetic basis to this trait in our population (Broussard et al. 2009).

A general conclusion from these comparisons is that reproductively photoresponsive and reproductively nonphotoresponsive phenotypes in different populations and species may be based on different forms of underlying physiological variation. It remains to be tested whether these multiple physiological mechanisms for similar phenotypes are equivalent and selectively neutral. Alternatively, the underlying physiological variation in each population may be due to differences in reproductive phenotypes that are not detected readily in the laboratory, or may be due to selection on correlated traits that favors specific genotypic mechanisms for variation in photoresponsiveness.

Model for genetic variation in winter reproduction

We propose a conceptual model (Fig. 2) for photoresponsiveness in our population of *P. leucopus* that is consistent with our results. The model is speculative, but it provides a framework for further thinking and testing of hypotheses about the causes of genetic variation in neuroendocrine systems.

The model is based on the hypothesis that variation in timing arises from variation in (1) GnRH neurons or (2) inputs to GnRH neurons. GnRH neurons must communicate with each other in a network to release synchronous pulses of GnRH (Moenter et al. 2003; Veldhuis et al. 2008). GnRH pulsing at a sufficient frequency and amplitude induces LH pulses and release of FSH sufficient to support reproduction. Below some threshold, frequency and amplitude of GnRH pulses are too low to support reproduction, and the reproductive axis becomes inactive, while above that threshold, the reproductive axis is active. The GnRH neuronal system and the cells that secrete LH have the capacity to produce far higher levels of GnRH and LH, respectively. However, the fact that individuals can be exquisitely sensitive to small inhibitory inputs indicates that it is not the maximal secretory capacity of these cells that is critical to either maintain or halt reproduction, but rather the net effect of

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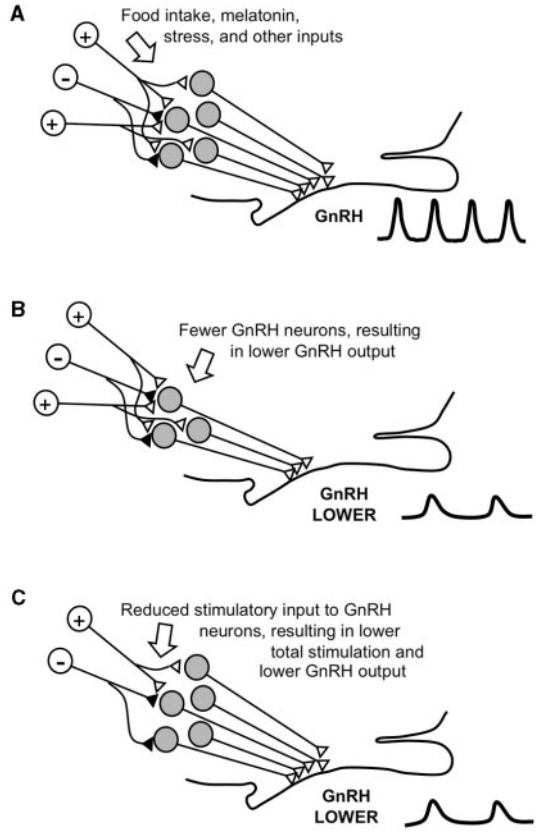

Fig. 2 A conceptual model of genetic neuroendocrine variation leading to variation in function of the reproductive axis. Each panel shows a sagittal view of the base of the hypothalamus with GnRH neurons projecting to the median eminence. (A) GnRH neurons (gray) receive multiple stimulatory inputs (open arrows and open axon terminals) and inhibitory inputs (dark arrows and dark axon terminals) from neurons and hormones. GnRH pulse output, and therefore the function of the HPG axis, depends upon the summed stimulatory and inhibitory input to GnRH neurons. (B) Individuals with fewer GnRH neurons, each receiving the same summed stimulatory and inhibitory input as in (A), secrete lower total GnRH. The result is lower amplitude and/or lower frequency of GnRH pulses. In this case, the reduction in GnRH neurons is predicted to cause an individual to be less likely to maintain reproductive function under any conditions; conversely, an increase in number of GnRH neurons is predicted to cause an individual to be more likely to maintain reproductive function under any conditions. (C) Alternatively, individuals with a reduction in neural stimulatory input (open axon terminals) to GnRH neurons will have reduced secretion of GnRH from each GnRH neuron experiencing reduced stimulation. The result is lower amplitude and/or lower frequency of pulses of GnRH. In this case, changes in stimulatory or inhibitory inputs could result in reproductive adjustments that are specific to a particular environmental or physiological signal such as photoperiod or nutrition, respectively.

stimulatory and inhibitory inputs. GnRH neurons receive multiple stimulatory and inhibitory inputs (Moenter et al. 2003; Veldhuis et al. 2008) arising from photoperiod, nutrition, stress, and other factors. These stimulatory and inhibitory inputs sum to induce an overall level of activity of GnRH neurons within the network.

First, a possible source for variation in timing is in the number (or activity) of GnRH neurons. If there are fewer neurons (Fig. 2B), then a particular sum of stimulatory inputs might no longer be sufficient to support reproduction. The effect is to raise the threshold of stimulation necessary to support fertility because each remaining GnRH neuron would need to secrete more GnRH in order to support reproduction. Again, while GnRH neurons have the capacity to secrete far more GnRH, if the same input per GnRH neuron reaches fewer neurons in total, then GnRH secretion will decline proportionately, and GnRH pulse amplitude and/or frequency will be reduced. Thus, in our Responsive mice with fewer IR-GnRH neurons, short photoperiod suppresses GnRH secretion below the threshold. If there are fewer neurons, then a particular sum of stimulatory inputs might no longer be sufficient to support reproduction. In Nonresponsive mice, with more IR-GnRH neurons, lower stimulatory inputs acting on the greater number of GnRH neurons could maintain secretion above the lower threshold adequate to support fertility. Thus, a difference in number of neurons could create a difference between reproductive responses of R and NR mice to SD without requiring any difference in the inputs to GnRH neurons.

While the suggestion in this model is that number of GnRH neurons is critical, an equally reasonable proposal is that differences between R and NR mice is due to genetic differences in level of activity of the same number of GnRH neurons. In this case the same stimulatory input to the same total number of GnRH neurons in R and NR mice would stimulate small pulses in R mice, having neurons that were less active, and larger pulses in NR mice, having neurons that were more active. In either case, the observation of differences in IR-GnRH neurons between lines suggests possible differences in total output of GnRH. A novel prediction from this hypothesis is that the R line should be more sensitive to reproductive inhibition from any source.

Second, a possible source for variation in timing is in the strength of stimulatory and inhibitory inputs to GnRH neurons (Fig. 2C). For example, greater or weaker inputs to neurons that mediate negative feedback of sex-steroids on GnRH neurons during SD would make the HPG axis vary in responsiveness (Glass and Dolan 1988; Malpaux et al. 2001). Similarly, greater or weaker inputs from neurons that assess melatonin, nutrition, stress, or other

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factors would make the HPG axis vary in responsiveness. Interactions are plausible here. For example, increased intake of food by NR mice might provide nutritional cues that increase stimulatory inputs to GnRH neurons. The result would be that, in any photoperiod, the HPG axis of an NR mouse would receive greater stimulatory input than would an R mouse. In LD, both R and NR mice might be fully fertile. In SD, both would be reproductively suppressed to some degree by photoperiod. However, the greater stimulatory input would allow an NR mouse to maintain fertility despite partial reproductive suppression by SD. Similarly, changes in melatonin receptor expression or binding in areas that regulate reproduction could make NR mice less sensitive to full reproductive inhibition by SD.

Finally, the hypotheses above are not mutually exclusive. All of these forms of genetic variation could contribute to variation in sensitivity of the HPG axis to SD. Furthermore, other mechanisms are possible, including variation in sensitivity or activity of cells secreting or responding to other hormones in the HPG axis, including LH and FSH. Further testing is needed to characterize variation as well as to contrast these alternatives.

Evolutionary constraint versus evolutionary potential

An important question in the microevolution of reproductive timing is whether seasonal switches evolve such that hormone secretion/receptor/response/clearance systems evolve in a coordinated fashion (the ''evolutionary constraint'' hypothesis) (Hau 2007) or whether there is independent genetic variation, such that hormone secretion, receptor expression, cellular responses, and hormone clearance systems evolve independently (the ''evolutionary potential'' hypothesis) (Hau 2007) (for a related discussion, see McGlothlin and Ketterson 2008). Assessing these hypotheses requires simultaneous measurement of two or more sources of variation. Our data on testis size and number of IR-GnRH neurons suggests that genetic variation in these two traits is independent in our population, as there was no correlation between these traits in our NR and R lines (Avigdor et al. 2005) or in our unselected C line (Heideman et al. 2007). Overall, our data suggest (but do not yet show) genetic variation at multiple loci that are potentially evolving independently, consistent with the evolutionary potential hypothesis.

It is important to note that the evolutionary constraint and evolutionary potential hypotheses are not easy to distinguish. Consider a phenotypic trait that has a very narrow observed reaction norm in a population. The nature of the underlying neuroendocrine cause of the narrow reaction norm may be quite variable, illustrated by two examples. First, a population may be genetically nearly uniform for the number of hormone-secreting cells, the number of target cells, the number of receptors on each cell, and the response to activation by receptors. Mutations that affect any of these traits would alter the phenotype, and loci affecting each single trait could have independent and additive effects on the phenotype. This scenario is consistent with predictions of the evolutionary potential hypothesis. Alternatively, a population may have a single phenotypically plastic control point that limits phenotypes to a narrow range. During development, a tuning process would make adjustments until a specific phenotypic outcome was reached. For example, an individual with a genetically low number of hormone-secreting cells, a genetically low number of target cells, a genetically low number of receptors on each cell, and a genetically low response to activation of receptors could appear phenotypically identical to an individual who was high for all four attributes simply by having a tuning process during development that upregulated secretion of chemical message until a particular endpoint was reached. In this latter example, only mutations in the tuning process would affect the phenotype. This latter system would evolve in a coordinated fashion based on single control points, consistent with the evolutionary constraint hypothesis. In the examples above, simultaneous measurement of all relevant variable neuroendocrine traits might be necessary to distinguish between the hypotheses.

Evolution of optimal pathways and the symmorphosis hypothesis

One of the two major competing hypotheses in evolutionary physiology is that selection on complex physiological pathways will eliminate deleterious genetic variation to provide a pathway that functions in some optimal fashion, the symmorphosis hypothesis (Lindstedt and Jones 1987; Weibel et al. 1998). The alternative hypothesis is that selection on different functions of the multiple genes controlling such complex pathways may be too weak or too variable to produce a single optimal result, resulting in sustained high levels of variation (Bartholemew 1987; Lindstedt and Jones 1987). The evidence for variation in GnRH neurons (Avigdor et al. 2005), binding of IMEL (Heideman et al. 1999b), intake of food

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(Heideman et al. 2005), and phenotypic plasticity in response to food (Reilly et al. 2006) and aging (Broussard et al. 2009) suggests that the population we study has not reached some single optimal reaction norm of phenotypes. This is consistent with the hypothesis that complex physiological pathways may not reach optimal function in response to natural selection even if the possibility of optimal function exists. Furthermore, there may be life-history tradeoffs that prevent evolution of a single optimal life-history strategy (Heideman et al. 2005). If these results extend to other vertebrates, then substantial genetic variation in hormonal traits may also be common in humans and other species. High levels of natural genetic variation in life-history traits may enable rapid microevolutionary change, and might have contributed to macroevolutionary trends in the evolution of the vertebrate neuroendocrine system.

Broader conclusions

In summary, *P. leucopus*, as well as other species of vertebrates, exhibit intrapopulational variation in life-history traits related to reproduction. The photo-neuroendocrine pathway studied here is one of the few for which the physiological traits that produce variation in life history are being identified systematically and tested for phenotypic and genetic variation within single populations. The implication from the data to date is that genetic variation in this, and possibly other, neuroendocrine pathways can be high, and is likely to be due to multiple neuroendocrine causes. Additional phenotypic variation is created by phenotypic plasticity that is itself genetically variable (Reilly et al. 2006; Broussard et al. 2009). More studies making these connections are necessary in order to link variation in life-history traits to neuroendocrine variation and ultimately to the genes that contribute to variation in life history in natural populations (Lessells 2008; McGlothlin and Ketterson 2008; Williams 2008).

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