

# Top-down Approaches to the Study of Natural Variation in Complex Physiological Pathways Using the White-footed Mouse (*Peromyscus leucopus*) as a Model

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## Abstract

Variation in complex physiological pathways has important effects on human function and medical treatment. Complex pathways involve cells at multiple locations, which serve different functions regulated by many genes and include complex neuroendocrine pathways that regulate physiological function. One of two competing hypotheses regarding the effects of selection on complex pathways predicts that variability should be common within complex pathways. If this hypothesis is correct, then we should expect wide variation in neuroendocrine function to be typical within natural populations. To test this hypothesis, a complex neuroendocrine pathway that regulates photoperiod-dependent changes in fertility in a natural population of white-footed mice (*Peromyscus leucopus*) was used to test for natural genetic variability in multiple components of the pathway. After testing only six elements in the photoperiod pathway in *P. leucopus*, genetic variation in the following four of these elements was evident: the circadian clock, melatonin receptor abundance or affinity, sensitivity of the reproductive axis to steroid negative feedback, and gonadotropin-releasing hormone neuronal activity. If this result can be extended to humans, the prediction would be that significant variation at multiple loci in complex neuroendocrine pathways is common among humans, and that variation would exist even in human populations from a common genetic background. This finding could only be drawn from an “exotic” animal model derived from a natural source population, confirming the continuing importance of non traditional models alongside the standard laboratory species.

**Key Words:** evolutionary physiology; neuroendocrine evolution; nonresponder; photoperiod; reproduction; seasonal breeding

## Introduction

Human brains differ, and those differences define who we are and affect our health. Variation between individuals in brain physiology and behavior exists in complex form in neural and neuroendocrine pathways in the

brain. These complex neural and neuroendocrine pathways are vital in regulating behavior and physiological function. Among humans, natural variation in these pathways arises from the environment and our genes. Some of the genetic variation exists in the form of deleterious alleles, which cause physiological pathologies recognized as genetic diseases. However, some unknown fraction of the genetic variation, possibly a very large fraction, is not categorized as genetic disease. These alleles either are neutral in effect or are favored in at least some individuals at some times. Some of the alleles that contribute to complex neuroendocrine regulatory pathways may have epistatic effects. They may be deleterious in combination with particular alleles at other loci or favorable in combination with alternative alleles at those loci.

These effects of normal levels of variation in the brain have important health consequences (Bittner and Friedman 2000). Individual variation creates differences in the way two individuals respond to the same environmental influences, causing environments that are benign for some to be harmful to others. Individual variation also underlies differences that cause one person to react well to a particular drug and another to react adversely, creating problems that range from merely irritating to life threatening. In economic terms, pharmaceutical development and medical treatment may be affected more by some consequences of individual variation—the need to minimize the risk of adverse responses by a small fraction of individuals—than it is by the average benefit from a particular drug or treatment.

We still know virtually nothing about the underlying neuroendocrine bases of natural individual variation, except in the cases of a few genetic diseases. Substantial resources have been committed to understanding individual variation underlying such things as tissue typing and genetic disease subtypes (e.g., muscular dystrophy), with obvious benefits; however, we know little about individual variation in brain structure and function. As a result, we have a very poor sense of how to deal with variation in the brain as a public health issue.

Major efforts are under way to identify genetic variation that contributes to health and disease. Genome projects use microarray methods and genetic markers to identify and study genetic variation in humans and model organisms (Cheung and Spielman 2002). These approaches attempt to correlate variability in small groups of genes with specific states of health or disease (Mackay 2001). These gene-to-phenotype studies can be termed “bottom-up” approaches,

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in that they attempt to start with genetic variation and connect it to health states.

Bottom-up approaches are likely to be highly successful in identifying single alleles or small groups of alleles that contribute to a particular state of health or response to a health treatment. However, this approach has limitations (Flint and Mott 2001). First, understanding a genetic cause does not necessarily facilitate the identification of the physiological events that actually contribute to a particular health state. Especially for alleles that have pleiotropic effects, identifying which of many possible physiological effects actually contributes to the health state will be challenging. Second, if alleles at a large number of genetic loci with epistatic effects contribute to a particular health state, the mathematical and statistical problems of identifying genes and physiological states that contribute to health will be considerable (Glazier et al. 2002; Mackay 2001). Finally, even when these methods succeed at identifying individuals at risk, the actual development of effective treatments is likely to require an understanding of the consequences of physiological variation. Because of these limits, complementary methods following “top-down” designs (or “physiology to gene”; see Feder et al. 2000) are likely to be an important and necessary addition to bottom-up approaches in understanding the significance of natural genetic variation.

In a top-down approach, the particular health state (or phenotype) is defined, along with the sources of physiological or neuroendocrine variation that contribute to that state. Because the top-down approach begins with the identification of specific physiological or neuroendocrine sources of variation, which may later be tied to genes with either pleiotropic or epistatic effects, this approach can ultimately produce an understanding of genetic variation at both a physiological and genetic level. Top-down approaches are likely to be most useful in studying variation in particularly complex pathways, including neuroendocrine regulatory pathways in the brain. Top-down and bottom-up methods provide complementary approaches to describe and understand variation.

How much variation is to be expected in physiological pathways? Two competing hypotheses central to evolutionary physiology (see Garland 1998, 2002; Taylor and Weibel 1981) make predictions about the extent to which complex physiological pathways are likely variable. Complex pathways generally involve cells at multiple locations, are affected by many genes, and often serve more than one function. One hypothesis theorizes that natural selection operates to produce complex pathways that function optimally (Lindstedt and Jones 1987), or at least with a good match, between demands of the environment and each component of the pathway (e.g., the “symmorphosis” hypothesis, Taylor et al. 1981; Weibel 1998; Weibel et al. 1998). One reasonable prediction from various forms of the “optimality” hypothesis is either that levels of variability in elements of a pathway will be low or that the effects of such variation will be neutral because nonoptimal alleles will be continu-

ally removed by selection. A competing, “adequacy” hypothesis is that although natural selection does act to increase fitness, it produces systems that function adequately and rarely, if ever, optimally (Bartholemew 1987; Garland 2002; Lindstedt and Jones 1987). Potential forces inhibiting optimization are many and include the presence of gene interactions or selection pressures that vary over the long periods of time needed for the evolution of complex pathways. According to the adequacy hypothesis, natural selection will act strongly only to eliminate highly deleterious alleles, producing systems that function adequately but not optimally. Thus, selection on most alleles is so weak that it allows the persistence of alleles that are only slightly deleterious, on average, or alleles that can be either advantageous or deleterious, depending on gene interactions. The result should be complex pathways that function adequately at any given time but rarely optimally (Garland 2002; Lindstedt and Jones 1987). A reasonable prediction from the adequacy hypothesis is that high levels of important genetic and phenotypic variation should exist within complex pathways. If optimal function is rare and due to chance combinations of alleles, then natural populations will contain wide variance in performance. Furthermore, a single species may evolve multiple different solutions for a particular physiological pathway in response to similar selection pressures (Garland 2002).

The answers to questions about these hypotheses have important implications for medicine as well as evolutionary and functional biology. If genetic variation is common in complex physiological pathways, and if multiple physiological solutions exist within a single species, then high levels of variation in human populations will result in decreased effectiveness of medical treatments for many individuals.

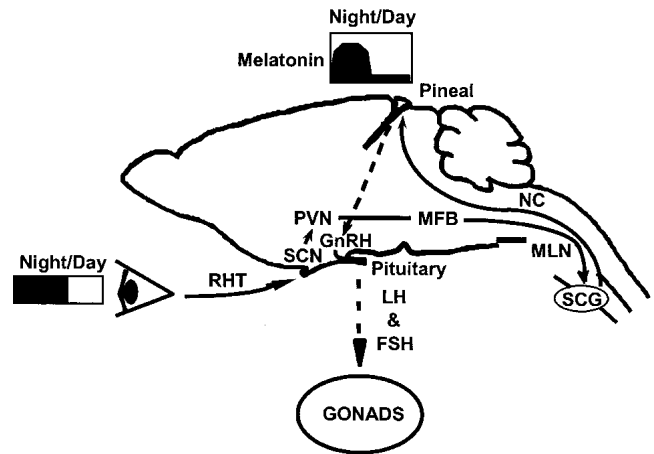
The goal of this article is to discuss ways in which top-down approaches using nontraditional animal models can complement the equally essential molecular-level, bottom-up approaches to understanding variation. For a full understanding of the nature and importance of physiological variation, both approaches are necessary. A brief description of how to design top-down approaches to study natural variation appears below.

Traditional laboratory animal models lack natural variation or are too expensive to study. Laboratory rat and mouse strains are moderately to highly inbred. Outbred strains of rats and mice, which may have higher levels of variation than inbred strains, have usually been subject to large amounts of laboratory selection and genetic drift and are not likely to be representative of the variation present in natural populations. Some of our mammalian models (e.g., some primates or domestic carnivores) come from natural or highly outbred populations. However, because studies of individual variation often require large sample sizes, with a minimum of 20 to 30 individuals per test even for traits that are highly variable, these large animals would be much too expensive to use for basic research on individual variation. At our current stage of understanding of natural variation,

an ideal model for studies of natural variation is a small mammal that is cheap and easy to maintain in the laboratory, is easily obtained from natural populations, and has neural or neuroendocrine circuits that are already known to be variable in a function that is biologically important.

In many ways, the wild relatives of laboratory mice and laboratory rats are good candidates for the study of variation (Guenet and Bonhomme 2003), especially because the rat and mouse genome projects make it increasingly simple to identify specific allelic variation. For the work described in this paper, wild populations of laboratory mice or rats were not chosen, primarily because of difficulties in assessing natural variation in these species and partly because of the specific pathway chosen for study. *Rattus norvegicus* and *Mus musculus* (or their close relatives) are commensals of humans that have been transported unintentionally all over the world by humans. Because of those movements and subsequent hybridization, many populations may even contain genes from multiple subspecies or species. In addition, these populations have been subject to sudden changes in selection induced by changes in human actions (e.g., crop and household management practices or exposure to rodenticides and many other anthropogenic chemicals) and to bottlenecks and other causes of rapid genetic drift. Thus, truly natural populations of wild relatives of laboratory rats and mice may be impossible to identify. Of course, because interbreeding among modern human populations has been highly variable, one can suggest that fully understanding human variation may require study of both relatively undisturbed natural populations and highly mixed populations, possibly including populations of wild laboratory mice and rats.

The study of natural variation in a complex brain pathway requires a model system that meets important preconditions for this kind of study. The pathway that regulates reproduction according to photoperiod (Figure 1), using a natural population of one nontraditional model organism, the white-footed mouse (*Peromyscus leucopus*), fulfills this criterion. White-footed mice are easy to obtain from relatively natural populations in woodlands over much of North America. The neuroendocrine pathway is long, complex, intensely studied, and relatively well known (Ebling and Cronin 2000; Goldman 2001; Prendergast et al. 2002). There exist natural populations of this species and of closely related species that are known to contain high levels of variation in this pathway (reviewed by Prendergast et al. 2001), and it is known that some of this variation is genetic in origin (Desjardins et al. 1986; Heideman and Bronson 1991; Heideman et al. 1999a; Wichman and Lynch 1991). Indeed, other researchers (Blank 1992; Horton and Rowsemitt 1992) have addressed related questions on variability in species of *Peromyscus* and other nontraditional species, some of which are cited below. An understanding of the neuroendocrine basis and the underlying genetics of variation in these species can be used to develop a model for natural individual variation in the brain.



**Figure 1** Schematic view of the pathway through which photoperiodic information regulates reproduction in mammals. FSH, follicle stimulating hormone; GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone; MFB, medial forebrain bundle; MLN, mediolateral nuclei of upper thoracic spinal cord; NC, nervi conarii; PVN, hypothalamic paraventricular nuclei; RHT, retinohypothalamic tract; SCG, superior cervical ganglia; SCN, suprachiasmatic nuclei.

## Neuroendocrine Pathway of Reproductive Photoresponsiveness

Daylength exerts effects on the mammalian reproductive system via a complex pathway (see Figure 1) (Ebling and Cronin 2000; Goldman 2001; Turek and Van Cauter 1994). The neuroendocrine signal for light is passed from retinal photoreceptors through the retinohypothalamic tract to the suprachiasmatic nuclei (SCN<sup>1</sup>). The SCN are a major component of the daily biological clock (or “circadian” clock), and a functional circadian clock is necessary for assessment of daylength (Goldman 2001). From the SCN, neuronal signals are passed to the paraventricular nuclei (PVN<sup>1</sup>) of the hypothalamus, to the superior cervical ganglia, and then to the pineal gland via adrenergic neurons of the sympathetic nervous system (Prendergast et al. 2002). The pineal gland releases melatonin only at night, which provides a general physiological signal for night (Bartness et al. 1993; Goldman 2001; Prendergast et al. 2002). In *P. leucopus*, melatonin receptors are particularly abundant in the pars tuberalis (PT<sup>1</sup>) of the pituitary, SCN, medial preoptic area

<sup>1</sup>Abbreviations used in this article: BNST, bed nucleus of the stria terminalis; CM, control mice; DMH, dorsomedial hypothalamic area; GnRH, gonadotropin-releasing hormone; IMEL, iodomelatonin; LD, long daylengths; LH, luteinizing hormone; ME, median eminence; MPOA, medial preoptic area; NMDA, N-methyl-D-aspartate; NRM, nonresponsive mice; OVX + B, ovariectomized mice given a blank silastic implant; OVX + E, ovariectomized mice given silastic implants with estradiol; PT, pars tuberalis; PVN, paraventricular nuclei; PVNt, paraventricular nucleus of the thalamus; RM, responsive mice; SCN, suprachiasmatic nuclei; SD, short daylengths.

(MPOA<sup>1</sup>), bed nucleus of the stria terminalis (BNST<sup>1</sup>), and dorsomedial hypothalamic area (DMH<sup>1</sup>) of the hypothalamus, as well as in the paraventricular nucleus of the thalamus (PVNt<sup>1</sup>) (Heideman et al. 1999b; Weaver et al. 1990).

The duration of the rise in circulating melatonin is generated by the circadian system, which may also be necessary to assess the melatonin signal (Goldman 2001; Prendergast et al. 2002), including the SCN and possibly other elements of the circadian system, to provide the neuroendocrine signal for daylength. Melatonin acts indirectly (Ebling and Cronin 2000; Morgan et al. 1994) through this system to modify the secretion or release of the gonadotropin-releasing hormone (GnRH<sup>1</sup>), the master regulatory hormone of reproduction. GnRH is released at the median eminence (ME<sup>1</sup>) into the pituitary portal system for transport to the gonadotropes of the anterior pituitary gland. In the anterior pituitary, GnRH regulates secretion of luteinizing hormone (LH<sup>1</sup>) and follicle-stimulating hormone, which stimulate sex steroid production and gametogenesis, respectively.

Reproductive status depends largely on the level and pattern of GnRH secretion, although there is also evidence for actions of melatonin at the level of the anterior pituitary (Everett 1994; Kordon et al. 1994; Page 1994; Sagrillo et al. 1996; Vanecek 1988; Vanecek and Watanabe 1999). Melatonin also appears to act on lactotrophs to regulate seasonal changes in prolactin that mediate some of the nonreproductive effects of photoperiod (Lincoln 1999; Wittkowski et al. 1999). In most (and perhaps all) photoresponsive species, the melatonin signal is both necessary and sufficient for reproductive and nonreproductive responses (Bartness et al. 1993; Turek and Van Cauter 1994). Melatonin acts in some way, possibly at multiple sites (Freeman and Zucker 2001; Goldman 2001) and possibly directly on GnRH neurons (Roy and Balsham 2002), to modify the release of GnRH from a diffuse population of hypothalamic neurons. Many of these GnRH neurons project to the ME, where GnRH is released into the pituitary portal system in a pulsatile fashion to be carried directly to the gonadotropes of the anterior pituitary. The many inputs that control fertility are thought to be integrated largely at the level of GnRH neurons.

Nonreproductive effects of melatonin and this pathway include the induction of altered food intake, body weight, coat color, coat density, nesting behavior, and immune system function (Blank 1992; Boon and Daan 1997; Heideman et al. 1998; Lynch et al. 1989; Nelson and Drazen 1999; Nelson et al. 1998). It is known that reproductive and nonreproductive effects of photoperiod can occur independently (Duncan et al. 1985; Goldman 2001) and that reproductively nonphotoresponsive animals may still retain nonreproductive responses to photoperiod (Blank 1992; Goldman 2001; Prendergast et al. 2002).

The general neuroendocrine pathway described above is characteristic of humans, and various elements play roles in many human health problems (Arendt et al. 1987; Palm et al. 1991; Petrie et al. 1989) and may (Arendt 1999; Reiter 1998; Roenneberg and Aschoff 1990; Weaver et al. 1993; Wehr 1997) or may not (Bronson 1995; Prendergast et al.

2002) modify human reproductive physiology and behavior. In humans, individual variation in this pathway has medical relevance in at least one mental disorder (seasonal affective disorder), and the underlying biological rhythms play important roles in many aspects of human behavior. Individual variation has been identified as one of two particularly important clinical research topics in the study of human rhythm disorders (Wehr 1997).

## Natural History and Husbandry

To study natural genetic variation in this complex pathway, mice from a natural wild population of *P. leucopus*, the white-footed mouse, have been used in our laboratory.<sup>2</sup> *P. leucopus* are abundant in woodland habitats from the most southeastern region of Canada, most of the eastern United States not including Florida and parts of neighboring states, west as far as the Great Plains states and Arizona, and south into eastern Mexico as far as the Yucatan Peninsula (Jones and Birney 1988). *P. leucopus* are nocturnal omnivores that feed on nuts and seeds, berries, fruits, small invertebrates (Jones and Birney 1988), and fungal fruiting bodies. These mice forage in bushes and small trees as well as on the forest floor over home ranges up to 1 hectare in area (Jones and Birney 1988). Nests are built under rocks and logs or in hollows or burrows. In some areas of the country, access to wild populations is facilitated because mice will use nest boxes buried in the ground or in trees, although some populations use nest boxes only rarely at particular times (Terman 1996). Body mass of nonobese adults is approximately 15 to 25 g.

*P. leucopus* can be readily kept in captivity, using procedures that are also suitable for keeping wild species of *Mus* and other species of *Peromyscus*. Mice are held in laboratory mouse cages, either individually housed or in single-sex groups, and are given standard mouse food and water ad lib. Because some individuals attempt to bite during handling, we routinely handle mice using soft cotton gardening gloves, usually only on the hand used to capture and hold the mouse. Mice either can be immobilized in the soft cotton glove, held firmly by the loose skin of the neck, or can be held by the tail. *P. leucopus* are agile and are good jumpers, therefore we change cages and handle mice in light plastic bins that are a minimum of 15 inches tall, approximately 15 to 20 inches wide, and 20 to 40 inches long. With a few hours of instruction and a few days of practice, most people become adept at handling this species.

In our colony, potential breeders are paired together repeatedly if they successfully produce young. However, the potential breeders are given a new mate if no litter is produced within about 3 mo, and they are culled if not

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<sup>2</sup>In this article, subsequent discussions of the author's studies refer to work with colleagues in their laboratory at the College of William and Mary Department of Biology.

successful with the new partner. Successful breeders generally produce a litter approximately every 1 to 2 mo. Most breeding pairs may be held together continuously, but some males can be aggressive toward their own young and may require separation from a litter. Disturbance within 48 hr after parturition sometimes results in a female abandoning her litter. Litter size ranges from one to seven, averaging about four to five. The estrous cycle is about 4 to 5 days, and gestation is about 22 to 30 days (longest in those females simultaneously lactating and gestating a new litter). Females often have a postpartum estrus within 1 day of parturition. Pups are weaned at 21 to 23 days. We provide a high-fat breeder mouse chow and cotton nesting material to breeder pairs because it appears to improve reproductive success slightly. Many authors have published husbandry methods suitable for species of *Peromyscus*, including *P. leucopus* (see review by Joyner et al. 1998). Species of *Peromyscus* can carry some potentially harmful infections, and a number of reports include information on biohazards associated with *P. leucopus* (e.g., hantavirus; Jackson 1997).

In our experience, some populations of *Peromyscus*, even of the same species, will breed much more readily in the laboratory than others. Of the 70 mice we captured to found our colony; 48 bred successfully and established our breeding colony (Heideman et al. 1999a). In subsequent generations with this colony, only about 50% of breeding pairs are successful. Most populations with which I am familiar have had a higher level of breeding success, but some have been even less successful at breeding in the laboratory. Much additional information on *P. leucopus* and related species can be found in general reviews (Dewey and Dawson 2001; Joyner et al. 1998; King 1968; Kirkland and Layne 1989).

## Identifying Genetically Based Variation in Response to Photoperiod

Offspring of our wild-caught mice were variable in response to short daylengths (SD<sup>1</sup>; L8:D16) typical of mid-winter. First generation offspring raised in SD were strongly suppressed in reproductive development, intermediate in degree of reproductive suppression, or only weakly suppressed in reproductive development. Artificial selection was used to increase the proportions of alleles for extreme phenotypes of reproductive responses to photoperiod in each of two selected lines (Heideman et al. 1999a). In an effort to avoid inbreeding and inadvertent loss of genetic variation, sib-sib matings were not allowed, and we attempted to establish 20 to 50 successful breeding pairs for each line in each generation. The first three generations of selection produced one line, responsive mice (RM<sup>1</sup>), which comprised primarily individuals that were strongly photoperiod responsive and remained reproductively immature when raised in SD. A second line, nonresponsive mice (NRM<sup>1</sup>), comprised primarily individuals that were rela-

tively photoperiod nonresponsive and became reproductively mature despite being raised in SD. A third, unselected line, control mice (CM<sup>1</sup>), comprised mice that in SD have phenotypes in the proportions found in the original population (Heideman et al. 1999a). After five additional generations of selection, the two selected lines now produce mostly mice that have the extreme photoperiod responsive phenotype (RM line) or photoperiod nonresponsive phenotype (NRM line). These phenotypes were present in the original population and are still present in the CM line. The pattern of responses to selection suggests that the difference between lines is caused by multiple loci (Heideman et al. 1999a).

## Potential Sites of Individual Variation in the Photoneuroendocrine Pathway

Individual genetic variation in the source population potentially occurs at single or multiple points in the photoneuroendocrine pathway, and at anatomical locations that occur early or late in the pathway (see Figure 1). As a partial list of possibilities, variation might be present in any combination of many components, including the following: in the SCN (in circadian function); in the neurons projecting from the SCN to the pineal gland; in the secretion of melatonin from the pineal; in the location or abundance of melatonin receptors; in neurotransmitter systems that might mediate the effects of melatonin; in the sensitivity to melatonin of the sex-steroid negative feedback system; in the response of GnRH neurons to the melatonin signal; in the response to melatonin of neurons regulating nonreproductive responses to melatonin; or in the response to “gating” signals, such as food intake that may modify photoperiod responses (reviewed by Majoy and Heideman 2000).

## Circadian, or “Biological Clock,” Variation

Variations in circadian function have been shown to affect photoresponsiveness in some rodent species by altering the manner in which the circadian system interprets short-day information (Goldman 2001; Prendergast et al. 2002). The circadian system also regulates daily timing of an immense array of events from the level of molecules to behaviors (Dunlap et al. 2004). Two previous studies on outbred populations of white-footed mice (*P. leucopus*) did not find significant differences in circadian-system organization between reproductively responsive and nonresponsive mice (Carlson et al. 1989; Johnston and Zucker 1980). Given the importance of a precisely functioning circadian clock for so many biological systems, this finding for relatively outbred colonies that had been recently derived from wild mice may not be surprising. In contrast, some studies on nonresponsive variants identified within laboratory stocks of rodents have found changes in the circadian system that cause variation in responsiveness to the reproductive effects of short

days (Kliman and Lynch 1992; Loudon et al. 1998; Lynch et al. 1989; Puchalski and Lynch 1986, 1988, 1991, 1994; Shimomura et al. 1997; Stirland et al. 1996a,b). Nevertheless, some variants reported in these laboratory stocks may produce such severe effects on daily rhythms that the alleles involved might be under strong negative selection and not persist in natural populations.

We tested the hypothesis that variation in circadian function might contribute to differences in photoresponsiveness in our lines of RM and NRM. Running-wheel activity patterns of male and female mice were monitored under SD, long daylengths (LD<sup>1</sup>; L16:D8), and constant dark. Although both lines showed evidence of a functional circadian system, NRM displayed a significantly longer mean free-running period in constant darkness than RM (Majoy and Heideman 2000). Other entrainment characteristics (duration of active phase, phase angle of activity) under SD, LD, and constant dark were similar between RM and NRM. To determine whether variation in photoresponsiveness in *P. leucopus* was due to differences in free-running period rather than variation elsewhere in the photoneuroendocrine pathway, downstream from the central circadian clock, we exposed young RM and NRM to constant dark and measured the effect on reproductive organ development (Majoy et al. 2000). If variation in free-running period affects how the circadian system of mice interpreted SD, then both RM and NRM exposed to constant dark should undergo a delay in gonadal development. However, only RM exhibited pubertal delay in constant dark. NRM had large paired testes, paired seminal vesicles, paired ovaries, and uterine weights typical of mice nonresponsive to SD, whereas RM had reproductive organ weights typical of mice responsive to SD (Majoy et al. 2000). These data suggest that although there is variation in the circadian system within this population, variation in photoresponsiveness is not due to differences in how the circadian systems of RM and NRM interpret the day/night cycle.

## Melatonin Secretion

Reproductively nonresponsive *P. leucopus* from a Georgia population had the same daily rhythm of melatonin production as mice from a largely photoresponsive Connecticut population (Lynch et al. 1982). Photoresponsive and non-responsive deer mice (*Peromyscus maniculatus*) within a single variable population have been shown to exhibit similar amounts of melatonin in the pineal (Blank et al. 1988) and similar nocturnal rhythms of 6-sulphoxymelatonin (Ruf et al. 1997) but differences in response to melatonin (Blank and Freeman 1991). These findings suggest that variation in responsiveness is due not to differences in melatonin secretion but rather to the response of the reproductive system to melatonin.

We have not tested directly for differences in melatonin secretion or sensitivity between RM and NRM. Other evidence from our laboratory indicates that NRM are likely to

have a functional pineal that is able to produce a physiologically usable signal in both long and short photoperiod. Most importantly, NRM that were subjected to a mild food-restriction treatment underwent inhibition of the reproductive system in SD, but not in LD (S. Joiner, R. Oum, and P.D.H., in preparation). This evidence that NRM can respond to SD indicates that mice in that line retain the ability to measure photoperiod, but they express the difference only under specific stressful conditions such as reduced food availability. However, because we have not compared melatonin secretion patterns between lines directly, it is possible that differences in levels or timing of melatonin secretion contribute to differences in photoresponsiveness.

## Melatonin Receptors

A previous comparison of mice from a nonresponsive Georgia population with mice from a largely photoresponsive Connecticut population found that these populations differ in their sensitivity to melatonin. Connecticut mice responded to exogenous administration of melatonin with gonadal involution, whereas Georgia mice were insensitive to the reproductive effects of exogenous melatonin (Carlson et al. 1989; Heath and Lynch 1982). The same two populations were found not to differ in melatonin receptor expression or affinity (Weaver et al. 1990); however, individual variation in this trait might exist in other populations and could contribute to variation in photoresponsiveness.

In our population of *P. leucopus*, we found that RM in SD had lower binding of radiolabeled iodomelatonin (IMEL<sup>1</sup>) in two areas, the MPOA and the BNST, than did NRM in SD (Heideman et al. 1999b). Differences in IMEL binding in the PT, SCN, DMH, and PVNt were not statistically significant (Heideman et al. 1999b). The MPOA has been previously identified as a site of melatonin action on the reproductive system in *P. leucopus* (Glass and Lynch 1982). The MPOA and BNST are important sites of sex-steroid binding in many species (Meisel and Sachs 1994) and are involved in aspects of sexual behavior. These results suggest that genetic variation in melatonin receptor abundance or affinity may contribute to differences in photoresponsiveness.

## Electrical Response of Neurons to Melatonin

In current work (Fetsch et al. 2003), we are testing for differences in electrical activity of hypothalamic neurons to melatonin. This study involves single-unit recordings of extracellular neuronal activity in tissue-slice preparations from RM and NRM mice held in long or short photoperiods. Melatonin might act on multiple populations of hypothalamic neurons involved in many different regulatory functions. In this study, we are distinguishing temperature-sensitive neurons that might mediate either circadian or seasonal changes in temperature regulation from neurons

that are not sensitive to temperature, some of which may mediate responses to functions other than temperature.

Neurons in the anterior hypothalamus that were classed as either temperature sensitive or insensitive were treated with melatonin (1 mM) while temperature was held constant. Preliminary results have indicated that temperature-sensitive and -insensitive neurons may differ in their responses to melatonin, but no effect of photoperiod or of our selected lines has been detected in preliminary analyses (Fetsch et al. 2003).

## N-Methyl-D-Aspartate (NMDA<sup>1</sup>) Receptors

It has been suggested that NMDA-type glutamate receptors may play a role in photoperiodic control of reproduction (Ebling et al. 1995; Urbanski and Ojeda 1990). In a series of experiments, we tested the following two hypotheses: (1) that NMDA-type glutamate receptors influence the timing of puberty in white-footed mice; and (2) that these effects would depend on photoperiod. In several experiments, 4-wk-old mice from the RM and NRM lines were given 4 wk of daily injections of NMDA, the competitive inhibitor MK-801, or a vehicle control. Mice treated with NMDA had significantly smaller testes and seminal vesicles than vehicle-treated controls (A. L. Tatum, J. Bonzo, and P.D.H., in preparation). These data are consistent with studies on other species, which suggest that the NMDA receptor is involved in regulating maturation (Ebling et al. 1995; Urbanski and Ojeda 1990); yet the data differ in that NMDA in our studies consistently inhibited reproductive development and did not stimulate reproductive development. There were no differences between lines or photoperiods in responses to NMDA or MK-801, suggesting that differences in the roles of NMDA receptors do not contribute to genetic variation in photoreponsiveness in these mice.

## Sex Steroid-Negative Feedback

Changes in sensitivity to sex steroid-negative feedback on GnRH and LH secretion have been shown to be involved in causing seasonal changes in reproductive status in ewes (Legan and Karsch 1980), golden hamsters (Turek 1977), and *P. leucopus* (Glass and Dolan 1988). We are testing the hypothesis that individual variation in sensitivity to estrogen-negative feedback governs differences in photoreponsiveness. LH levels were measured in ovariectomized female RM and NRM given either estradiol (OVX + E<sup>1</sup>) or blank (OVX + B<sup>1</sup>) silastic implants and held in either SD or LD. Preliminary findings (K. Schubert, C. Raymond, J. Bowles, and P.D.H., in preparation) were that LH levels in OVX + E RM were significantly lower than in OVX + E NRM in SD and in the transition to LD. In addition, LH levels in OVX + B mice were high in NRM in both photoperiods and for RM mice in LD, but significantly lower for RM mice in SD (K. Schubert, C. Raymond, J. Bowles,

and P.D.H., in preparation). These preliminary results suggest that variation in sensitivity to estrogen-negative feedback and also to steroid-independent regulation of LH secretion both may contribute to variation in photoreponsiveness in the natural population.

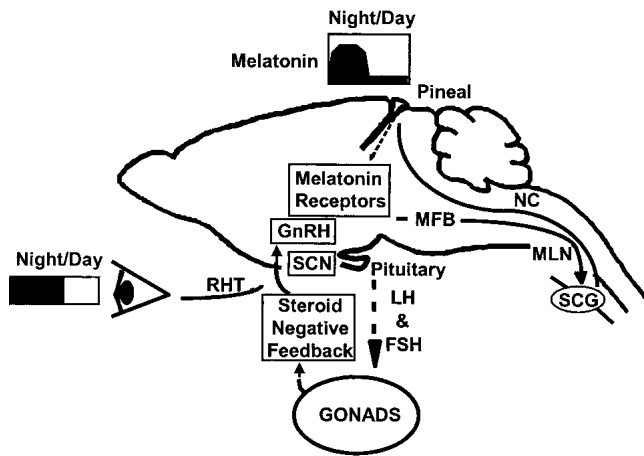
## GnRH Neuronal Responses

Previous experiments conducted on *P. leucopus* (Glass 1986) and *P. maniculatus* (Korytko et al. 1998) indicate that reproductively responsive males inhibited by SD possess greater numbers of immunoreactive GnRH neurons than nonresponsive males. The antibodies used in the immunocytochemistry of these studies, GnRH-BDB (Glass 1986) and LR1-GnRH (Korytko et al. 1995, 1998), are known to bind to an epitope of pro-GnRH. It has been suggested that responsive males sequester prohormone in the neurons that synthesize GnRH but fail to secrete mature peptide when placed in inhibitory photoperiods (Glass and Knotts 1987; Korytko et al. 1997, 1998).

We tested for differences in immunoreactive GnRH neurons of RM and NRM in SD. Mice were castrated and given silastic implants of testosterone to control for differences in steroid-negative feedback. Expression of mature GnRH immunoreactivity in the brain was detected using SMI-41 antibody, which binds specifically to mature GnRH (Tai et al. 1997). RM in SD had significantly lower total numbers of immunoreactive GnRH neurons than did NRM in SD (M. Avigdor, S. Sullivan, and P.D.H., unpublished data). The major difference in the abundance of these neurons was found in the anterior hypothalamus and preoptic areas (M. Avigdor, S. Sullivan, and P.D.H., unpublished data). These data suggest that individual variation in the GnRH neuronal system contributes to variation in photoreponsiveness in *P. leucopus*.

## Summary and Conclusion

After testing six elements in the photoperiod pathway in white-footed mice, we have evidence for intrapopulation genetic variation in four of these elements (Figure 2). If this proportion is representative, then we might expect variation in more than half of the many elements of this complex pathway that have not yet been tested. It is conceivable that the variation we have observed is due to differences at only a single genetic locus, but we suspect that there are multiple loci are involved. It seems likely that variation in circadian characteristics (Majoy and Heideman 2000), sensitivity to steroid-negative feedback (K. Schubert, C. Raymond, J. Bowles, and P.D.H., in preparation), and steroid-independent GnRH neuronal differences (M. Avigdor, S. Sullivan, and P.D.H., unpublished data) are controlled by different loci. Differences in abundance or affinity of melatonin receptors in the hypothalamus (Heideman et al. 1999b) may be due to variation at yet another locus. How-



**Figure 2** Schematic view of the photoperiod pathway showing components that are variable in white-footed mice enclosed in boxes. FSH, follicle stimulating hormone; GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone; MFB, medial forebrain bundle; MLN, mediolateral nuclei of upper thoracic spinal cord; NC, nervi conarii; PVN, hypothalamic paraventricular nuclei; RHT, retinohypothalamic tract; SCG, superior cervical ganglia; SCN, suprachiasmatic nuclei.

ever, because we did not control for sex-steroid levels in our test on melatonin receptors (Heideman et al. 1999b), we cannot reject the possibility that the differences in melatonin receptor expression were due to different levels of circulating sex steroids causing steroid-dependent levels of expression of melatonin receptors in intact RM and NRM.

The major finding of this body of research is that detectable genetic variation is present at multiple sites in a complex neuroendocrine pathway, and that all of this variation is present within a single natural source population of white-footed mice. These results are consistent with the hypothesis that even under strong selection, complex pathways are rarely, if ever, optimized because there are too many gene interactions and because selection pressures vary over the periods of time necessary to eliminate alleles that are not highly deleterious (Garland 1998). If the results we have observed can be extended to humans, the prediction would be that significant variation is present among humans and might be present even in relatively small and isolated human populations. Because of this variability, drug treatments designed to alter the function of this kind of complex pathway might be expected to have different effects on different individuals. For example, a drug designed to act on this pathway by altering steroid or melatonin levels, or by binding to steroid or melatonin receptors, would have the intended effect on some individuals but little or no effect, or even a contrary effect, on others. When we consider complex physiological and brain pathways, an assumption that people from a similar genetic background are likely to have similar physiological responses to the same drug treatment might be wrong.

The significance and amount of genetic variation pres-

ent in natural populations cannot be studied in a conventional laboratory animal model for the following reasons: (1) Evaluation requires study of a wild source population to demonstrate that variation is not a laboratory artifact. Laboratory stocks are likely to hold alleles that may be present only because of relaxed selection and/or genetic drift after domestication. (2) Importantly, the source population must be natural in having persisted under normal conditions for that species, subject to the natural selection pressures induced from nonhuman sources (or at least selection that has not changed frequently and abruptly due to humans), and not holding alleles that have been recently introduced by anthropogenic causes. Within a model species such as the white-footed mouse, wild populations are easy to identify in relatively undisturbed habitats, and they are much more likely to have been subject to purely natural (i.e., nonanthropogenic) selection pressures over the past few hundred generations. Ultimately, it will be important to be able to link natural variation in the function and molecules of complex pathways to the underlying genes controlling that variation. Therefore, genome projects for nontraditional species are particularly important for understanding natural variation in function. As the current *Peromyscus* genome project matures, it should become easier to establish connections between phenotypic physiological variation and specific genetic variation.

The studies described herein also illustrate the importance of considering functional analyses of variation. These selected lines of mice had statistically significant differences in a measure of circadian function that has been shown to be linked to variation in photoresponsiveness in some laboratory stocks of several species (Goldman 2001). However, that difference appears not to be the cause of differences in photoresponsiveness in the natural population of mice.

Within and across human cultures, the diversity of physical traits and behavior are extremely important characteristics of human societies. In clinical health and disease, we recognize increasingly that natural genetic diversity is just as important. Development of an understanding of natural genetic variation will require natural populations and new approaches in biomedical research.

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