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Genetic Variation in Food Intake and GnRH Neurons in Female White-footed Mice (Peromyscus leucopus)

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Abstract

During the short photoperiod of winter, some short-lived rodents repress reproduction and other non-essential functions, while others continue to reproduce. The capacity to reproduce during short photoperiod or to repress reproduction is genetically variable, signaling a potential microevolutionary lifehistory tradeoff. Previous results linked this variation to the location and abundance of immunoreactive (IR) gonadotropin releasing hormone (GnRH) neurons in male white-footed mice (*Peromyscus leucopus*). Independently, previous results also found differences in food intake in males linked to genetic variation in the same population. In this study we tested for the relationship between food intake and the number of IR-GnRH neurons in females. The experiment used three selected lines of *P. leucopus* derived from a wild population. The three lines were, respectively, reproductively inhibited in short photoperiod (R line), reproductive in short photoperiod and therefore non-responsive to photoperiod (NR), and a control line not deliberately under selection. We performed immunostaining and counted the number of IR-GnRH neurons in one anterior and three preoptic brain sections. We tested for variation in the number of GnRH neurons in female *P. leucopus* and compared food intake, reproductive development and the interaction of all three. We found a significant difference in food intake among lines, with the NR mice eating 20 percent more than the R mice. The difference in food intake is consistent with a microevolutionary lifehistory tradeoff, similar to previous findings in males, but smaller in magnitude. There was no significant difference in the number of IR-GnRH neurons among lines. There was no correlation between the number of IR-

GnRH neurons and the other measures. This suggests either a need for additional testing for females or that there is an actual difference in the number of IR-GnRH neurons between males and females.

Introduction

Genetic variation within species controls the placement and abundance of neurons in the brain, which in turn contributes to the evolution of brain function and behavioral output (Avigdor et al., 2005). Neural circuitry is involved in the regulation of both reproductive physiology and behavior, so neuronal content should also affect life history strategies. The photoneuroendocrine pathway is a neuroendocrine pathway that controls the impact of photoperiod changes on winter reproduction and other winter adaptations (Figure 1). During the winter months, small mammals, such as *Phodopus sungorus* (Siberian hamsters), *Peromyscus maniculatus* (the deer mouse) and *Peromyscus leucopus*, face increased metabolic demands in the colder temperatures and increased risk of predation due to limited food sources and the need for extended foraging periods. Many species in temperate zones use the photoperiod to conserve energy and other resources by eliminating or curtailing non-essential functions. Winter changes in the length of daylight are the signal that inhibits gonadal function. Winter inhibition of gonadal function occurs through a cascade of neuroendocrine responses along the hypothalamic-pituitary-gonadal axis. These are regulated by hypothalamic neurons that secrete gonadotropin releasing hormone (GnRH).

GnRH hormone is a decapeptide produced by specialized neurons that secrete pulses of hormone intermittently from the median eminence of the basal hypothalamus. GnRH travels to the pituitary to signal the synthesis and secretion of the pituitary gonadotropins: LH and FSH.

(Sisk and Foster, 2004). Two modes of GnRH release regulate the estrous cycle, the pulse and the surge mode. The pulse mode of secretion is responsible for follicular development and steroidogenesis. The surge mode of secretion is responsible for only the induction of LH surges, which induces ovulation. The combination of these two modes of GnRH secretion is responsible for triggering the physiological events of the reproductive cycle, such as the onset of puberty and the estrous cycle (Ohkura et. al., 2009). Evidence from various species suggests that GnRH synthesis rises during development to reach adult rates prior to puberty; puberty is not due to a sudden increase in the rate of production of GnRH (Clarke and Pompolo, 2005). The secretion of GnRH is under the influence of many peripheral factors, including gonadal hormones as well as metabolic and environmental cues such as the shortened day length of the winter months (Fernandez et al., 2006).
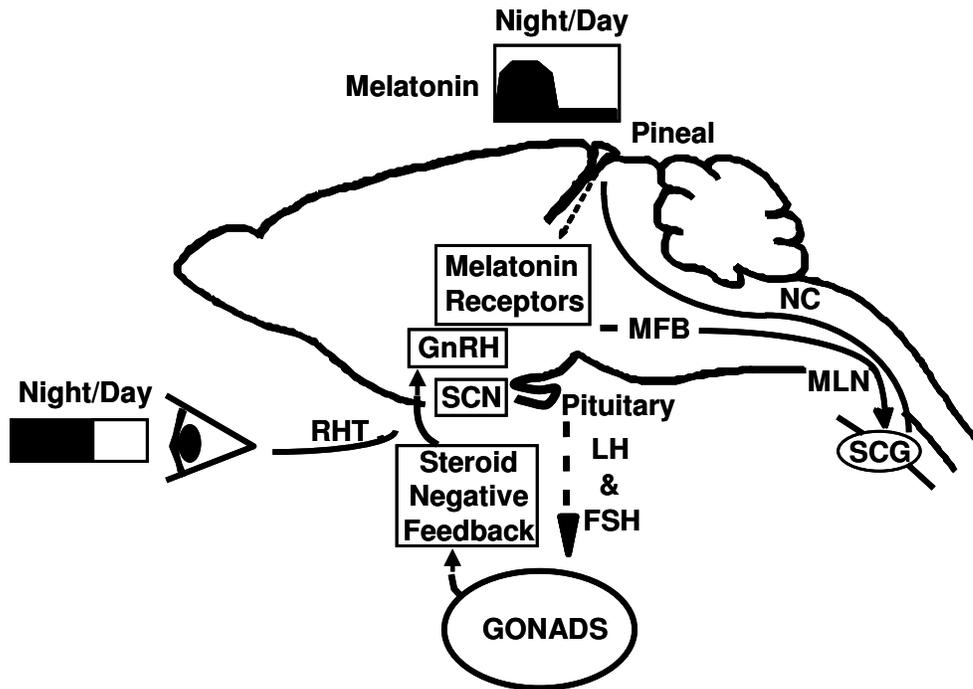
Figure 1

Figure 1: Schematic of the photoneuroendocrine pathway. The light signal is received through the eye, passed through the retinohypothalamic tract (RHT) to the suprachiasmatic nuclei (SCN). The neuronal signals are passed to the paraventricular nuclei (PVN) of the hypothalamus to the superior cervical ganglia (SCG) and then the adrenergic neurons of the sympathetic nervous system take the information to the pineal gland. The pineal gland releases melatonin at night. Melatonin acts indirectly through an unknown mechanism to modify the secretion of gonadotropin releasing hormone (GnRH). GnRH regulates both lutenizing hormone (LH) and follicle stimulating hormone (FSH). Figure from Heideman (2004).

There is genetic variation for winter suppression of reproduction (Prendergast et al., 2001). Winter suppression of reproduction can increase fitness because it minimizes nonessential energy expenditures and limits exposure to predators. Alternatively, winter suppression of reproduction might instead decrease fitness because individuals might die without reproducing. Therefore, attempting to delay reproduction until spring might cause even lower fitness than reproducing in a harsh winter. In both sexes, selection for winter reproduction increases the costs of reproduction through increased exposure to predators while foraging for food and searching for mates, as well as the cost of producing gametes and young. If chances of survival through a

long harsh winter period are low, then reproducing in what might be considered unfavorable circumstances could confer a selective advantage. However, while selection for winter reproduction may increase fitness, it will also increase the need for food, which could in turn increase mortality, indicating a potential for microevolutionary lifehistory tradeoffs (Heideman et al., 2005). Related physiological trade-offs can occur when there is a reallocation of resource intake among competing needs. These physiological trade-offs are considered microevolutionary trade-offs when there is genetic variation within a population for two or more traits, and when a change in one trait increases fitness, but results in a change in the other trait that decreases fitness (Heideman et al., 2005; Stearns 1992).

In *P. leucopus,* the response to day length in the wild varies greatly on an individual basis, with some individuals responding strongly to short photoperiod showing gonadal regression, while others are capable of reproducing throughout the year (Bronson and Heideman, 1994; Heideman et al., 1999a). This suggests that there is genetically based variability in the reproductive photoneuroendocrine pathway. Previous experiments by Avigdor et al. (2005) found that selection on life history traits in male *P. leucopus* may alter specific neuronal traits in a population, such as the abundance and density of GnRH immunoreactive (IR) neurons. The number of IR-GnRH neurons was positively correlated with testes size in short photoperiod. The secretion or release of GnRH is controlled by melatonin, which acts indirectly through an unknown mechanism via the photoneuroendocrine system to (Heideman, 2004). Previous studies in males indicated a significant difference in food intake between the lines. Female *P. leucopus* face many of the same costs of winter reproduction, such as exposure to predators and increased metabolic demands requiring higher rates of food intake. In addition, because females need to

support offspring through gestation and lactation, the potential costs and the amount of food necessary are higher than the demands on males.

We tested for genetic variation in the number of IR-GnRH neurons by comparing females from two artificially selected lines and one control line of wild derived *P. leucopus*. The lines were selected by their response to short day photoperiods. Mice that stop reproducing in response to short photoperiods are considered responsive (R), while the individuals that continue to reproduce throughout the winter months are considered non-responsive (NR). Based on the previous studies in males, we predicted a higher number of IR-GnRH neurons in the NR line compared to the R or control (C) line. We also predicted higher food intake in the NR line. Given the potentially higher metabolic costs for the NR mice in winter, we predicted that levels of food intake would be significantly higher in the NR mice. Finally, based on previous results in males (Heideman et al. 1999a) we predicted that body mass would not differ among lines. In this study we hypothesized that higher demand for food during short photoperiod reproduction would require higher food intake. Therefore, we predicted a positive correlation between the number of IR-GnRH neurons and food intake.

Materials and Methods

This project was begun by Jessica Robertson (JR) with assistance from TF Mahoney (TFM) in the Spring of 2006. For our initial data collection JR collected food intake data and TFM sectioned the brains. Starting in the summer of 2007 TFM took over the project completely. The majority of the data on food intake were taken by TFM. Perfusions were performed by JR for the earlier mice and later by Dr. Julian Pittman. TFM performed the

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immunocytochemistry and performed all neuron counts; some neuron counts conducted independently by Dr. Paul D. Heideman as a check for accuracy.

Animals

Mice used for this experiment came from the Population and Endocrinology laboratory in Williamsburg, VA. The source of this population was a single wild population of *P. leucopus* sampled in 1995 in Williamsburg, VA (latitude 37° 16' N). The founders were raised in long day (LD) (16 h light, 8 h dark), to generate a stock population for selection experiments. Artificial selection was used to create two selected lines for either reproductive inhibition (R) in short day (SD) (8 h light, 16 h dark) or lack of reproductive inhibition in SD (NR). The criterion for assigning mice to a line was based on gonadal development. Mice classified as NR had gonadal development in SD that was within typical ranges for summer reproductive organs, while R mice in SD had gonadal development that would typically be classified as infertile during the summer months. The control line was not subject to deliberate selection, and served as a control for unintentional selection or domestication. Mice in the control line displayed a range of phenotypes, mimicking the natural population (Heideman, 1999a). Each line had 20-50 breeding pairs per generation, with no mating between sibling pairs.

Experiments were performed using female mice from the F_8- F_{14} generations. We collected data on food intake and gonad size in a sample of 72 (the food intake sample, FIS). For this experiment, our total sample (FIS) included a total of 72 mice, 27 from the NR line, 23 from the R line and 22 from the C line. For a subset of this sample, the neuron count sub-sample (NCSS; N=34, 7 NR, 12 R and 15 C mice), we performed immunocytochemical staining to determine the number of IR-GnRH neurons present. We selected non-sibling females raised in

SD at 40- 60 days to measure the food intake over a two-week period. To measure food intake, mice were given a weighed quantity of food in a clean cage and the amount of food was reweighed at weekly intervals. Food in the cage was measured at the beginning, middle, and end of the two-week period to calculate an average daily food intake.

Perfusions and Fixation

Mice were anesthetized with an overdose of isoflurane (Abbot Laboratories, North Chicago, IL) and allowed to enter respiratory arrest before the perfusion was started. Perfusions were performed by Dr. Julian Pittman and Ms. Jessica Robertson. The technique for the perfusions followed that of Avigdor et al. (2005). At 70 ± 10 days, mice were weighed and anesthetized with isoflurane (Abbot Laboratories, North Chicago, IL) and perfused with 4% paraformaldehyde (Fisher Scientific, Fair Lawn, NJ) and saturated picric acid (Sigma-Aldrich, Philadelphia, USA) in Phosphate Buffered Saline (PBS: Na_2HPO_4 0.18%, KH_2PO_4 0.03%, NaCl 1%, KCl 0.025%). After perfusion, the mass and diameter of the uterus and the mass and length of the ovaries were taken as a measure of gonadal and reproductive development. The brains were removed, post-fixed in Zamboni's fixative (4% paraformaldehyde, saturated picric acid, 10N NaOH) sectioned and stored in a -20° freezer until immunocytochemical staining was performed for IR-GnRH neurons. All brains were sectioned within seven days of perfusion. Frozen coronal sections were cut at 30 μm on a freezing sliding microtome and placed in four vials of antifreeze (37.5% sucrose, 37.5% ethylene glycol, and 10g Polyvinylpyrrolidone-40 in 500 mL 0.02 M Tris-buffered saline), each containing every fourth section. The brains were stored at -20°C.

Immunocytochemistry

   The number of IR-GnRH neurons was determined using immunocytochemistry (ICC). Mature immunoreactive GnRH neurons were detected in the brain using a single-labeled avidin-biotin-complex (ABC) method. Brain slices were rinsed six times over a thirty-minute period in (4°-6° C) 0.02M tris-buffered saline (TBS), (Tris base 0.75%, NaCl 1.095%) followed by a rinse in 1% sodium borohydrate for 25-30 minutes. The brain slices were then rinsed 3 times in cold TBS with ten minutes for each rinse, to remove the sodium borohydrate. The tissue was then incubated overnight in the primary antibody, SMI-41 monoclonal antibody (Sternberger Monoclonals, Lutherville, MA) at a dilution of 1:20,000 in PBS with 0.25% lambda-carrageenan (Sigma-Aldrich, Philadelphia, USA), 1% bovine serum albumin (Sigma-Aldrich, Philadelphia, USA) and 0.3% Trition X-100 in TBS at a pH of 7.8. SMI-41 is a mouse monoclonal IgG1 antibody that binds only to mature GnRH hormone, as it is reactive with five amino acid sites at the C-terminus tail of the GnRH peptide and the amidation site (Avigdor et al., 2005). The following day the tissue was rinsed 6 times for ten minutes in TBS, and incubated in the secondary antibody of biotinylated horse anti-mouse IgG 1:500 in TBS with 0.25% lambda-carrageenan (Sigma-Aldrich, Philadelphia, USA), 1% bovine serum albumin (Sigma-Aldrich, Philadelphia, USA) and 0.3% Trition X-100 for an hour at room temperature. After rinsing three times in TBS the sections were then placed in the ABC complex (Vector Labs, California, USA), for 60-75 minutes. The ABC technique stains by employing unlabeled primary antibody, followed by biotinylated secondary antibody and then a preformed avidin and biotinylated horseradish peroxidase macromolecular complex. Tissue was rinsed three times for ten minutes in TBS, and then placed in a 1 mL solution of diaminobenzidine with hydrogen peroxide (Sigma-

Aldrich, Philadelphia, USA). The diaminobenzidine and hydrogen peroxide produce an insoluble colored reaction product at binding sites of the primary antibody (Figure 2). The reaction was allowed to proceed for 11-12 minutes, and then the sections were rinsed in TBS 6 times for ten minutes each. The stained tissue was then mounted on gelatin coated slides, air dried, dehydrated in xylene and then coverslipped with Permount (Fisher, Pittsburg, USA). A total of 12 independent ICC runs were carried out over a period of 12 months.
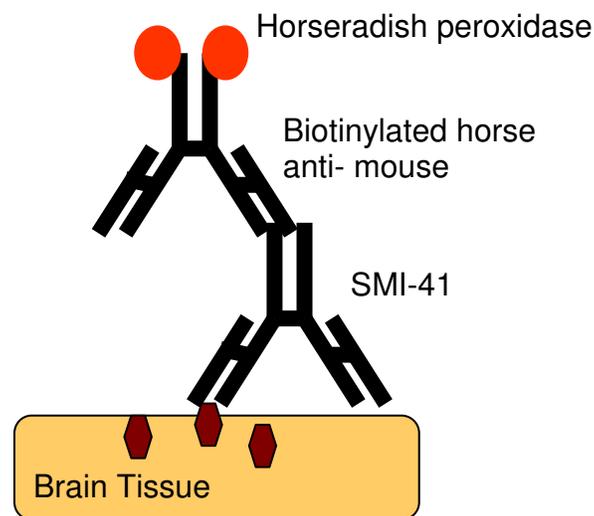

Figure 2: Schematic of the mechanism-of-action of the ABC complex

Neuron Assessment

The number and location of mature GnRH-secreting neurons were determined blind with respect to treatment and line using an Olympus CH2 compound microscope. The quality of staining was assessed independently of neuron counts, based on the quality of the brain tissue and the level of staining. Those with evidence of poor perfusion and poor staining were discarded. We stained a total of 49 individuals, of which 15 were discarded due to tissue damage at time of perfusion or, after ICC, due to poor quality staining, leaving a total of 34 in the NCSS.

Brain areas were estimated using a stereotaxic coordinate atlas of the rat brain (Paxinos and Watson, 1986). We estimated neuron number by counting neurons in four representative areas as indicated by Avigdor et al. (2005) looking at plates 17, 18, 19 and 22 (Paxinos and Watson, 1986). Plate 17 is in the anterior hypothalamic region, which includes brain structures posterior to the fusion of the two halves of the corpus callosum, but anterior to the preoptic hypothalamic region (Paxinos and Watson, 1986). The remaining plates are in the preoptic hypothalamic region, which is rostrally limited by the medial preoptic area and parts of the lateral hypothalamus (Avigdor et al 2005).

Statistical Analysis

Statistical analysis of the data was performed using Statview SE + Graphics software (Abacus Concepts, Berkeley, CA) on a Macintosh and JMP on a PC. We compared the body weight, average daily food intake, number of IR-GnRH neurons and ovary and uterine mass among selected lines using a one-way ANOVA with line as the factor. ANOVAs compare group means by analyzing comparisons of variance estimates. We also performed ANCOVA analysis for the relationship between food intake and body mass. We used $P < 0.05$ as the level of significance throughout. We also performed Pearson's correlation analyses, on the FIS and NCSS to test for relationships between body weight, food intake, ovary mass, uterine mass and number of IR-GnRH neurons. These included tests for the correlation of body mass with food intake, ovary mass, and uterine mass. Because the variances for uterine mass were not equal among lines, before analysis we log transformed uterine mass. We also tested for correlations between neuron counts and average daily food intake, uterine mass, and ovary mass.

Results

Food Intake

In the full FIS, there were significant differences in food intake among lines (N= 72, F=4.38; P= 0.0161; Figure 3A) with higher food intake in the NR than R and C lines. In the smaller NCSS sample, there was no statistically significant difference among lines (N=29, F= 0.050, P=0.9518; Figure 3B).
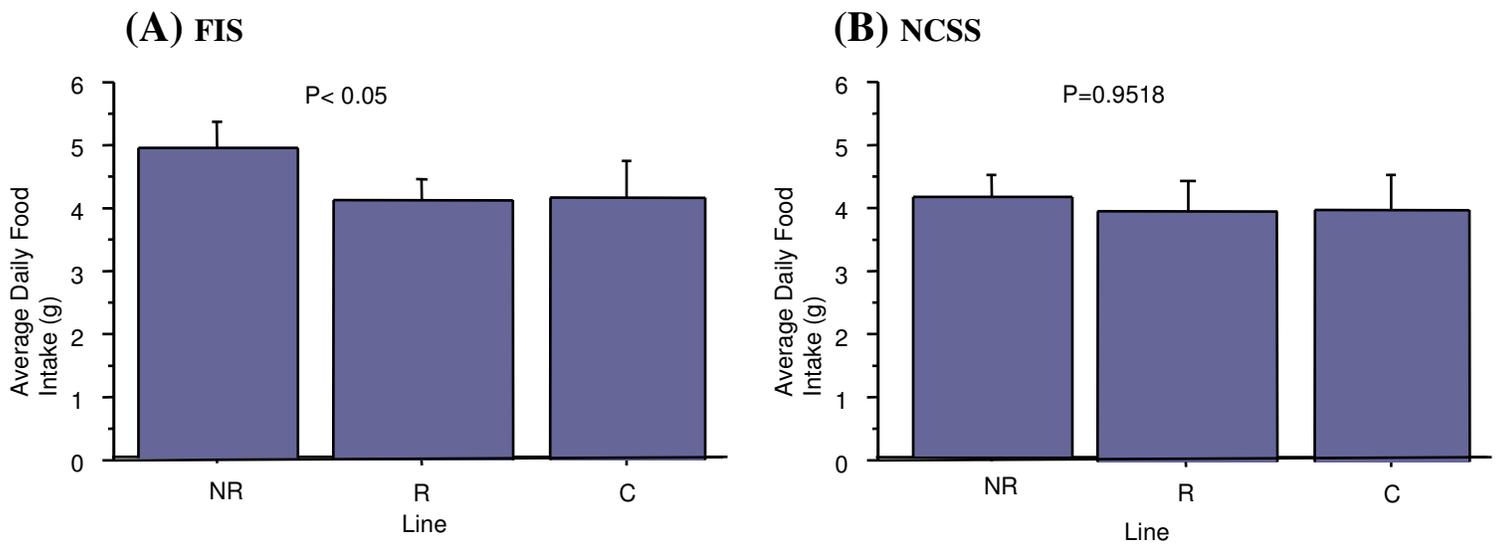
(A) FIS

(B) NCSS

Figure 3: Average daily food intake over a two week period by line: control [C] reproductively inhibited [R] and non-responsive [NR]. (A) Food intake sample (FIS) (*n= 72*). (B) Neuron count sub-sample (NCSS) (*n=29*). Bars indicate the 95% confidence intervals.

In the full FIS there was no significant difference in the body mass among lines (n= 52; F=.809; P=.4512; Figure 4A). In the NCSS there was no significant difference in body mass among lines (n= 30, F=.074; P=.9289; Figure 4B).
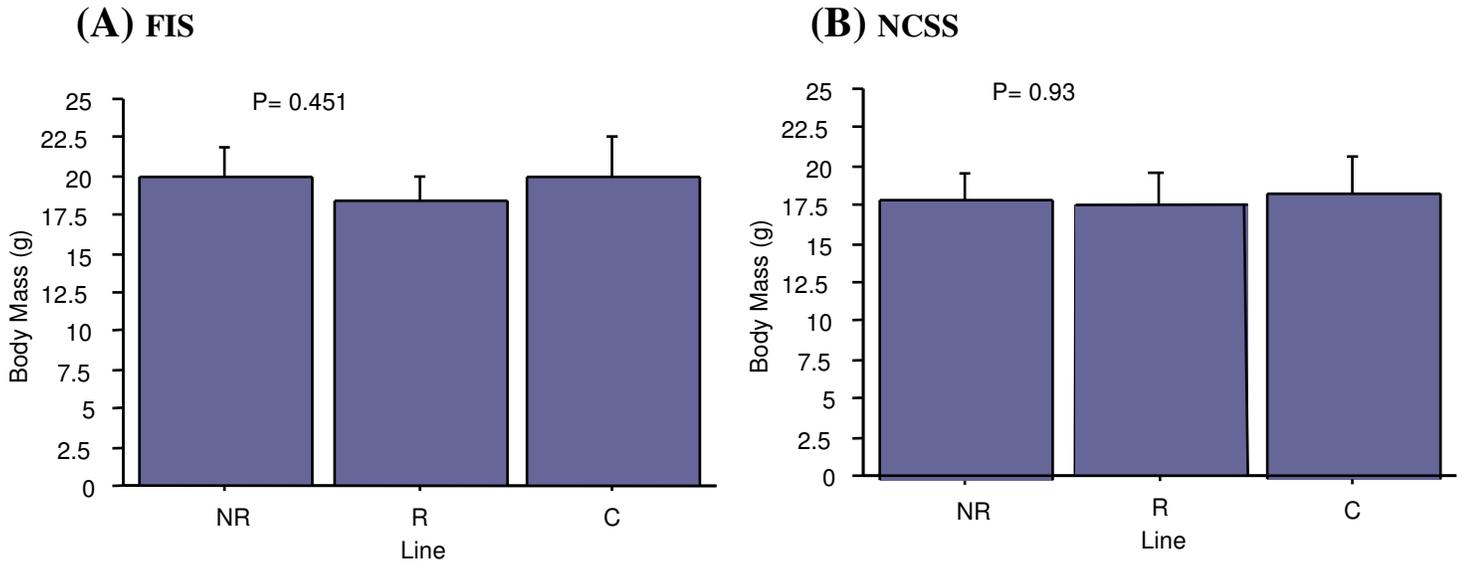
(A) FIS

(B) NCSS

Figure 4: Body mass illustrated by line. (A) ANOVA analysis FIS, control (C) *n=21*, non-responsive [NR] *n=16* and reproductively inhibited [R] *n=15*. (B) ANOVA of NCSS, [C] *n=15*, [NR] *n=6* and (R) *n=9*. Bars indicate the 95% confidence intervals.

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In neither the full FIS (R^2=0.085; P=0.0345; Figure 5A) nor the NCSS correlated (R^2= .055; P=.271; Figure 5B) was there a correlation between body mass and average food intake.
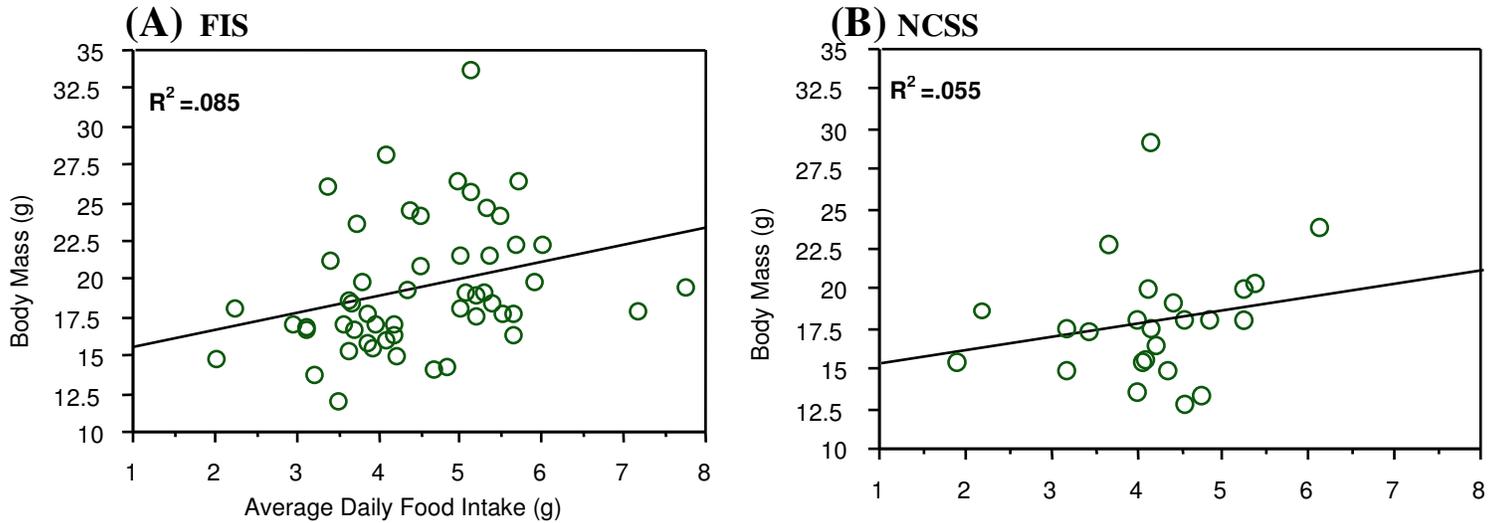

Figure 5: The relationship between body mass and food intake in (A) the food intake sample (FIS) and in (B) the neuron count sub-sample, NCSS.

Reproduction

We examined ovary and uterine mass as indicators of reproductive status. Ovary mass is an indicator of sexual development and potential ovulatory cycling. Uterine mass is sensitive to sex steroid levels and can serve as an indicator of recent average steroid levels. We found a statistically significant difference among lines in ovary weight (F= 18.298; P< 0.0001; Figure 6A). The NR line had ovaries of a higher mass than either the C or R lines. We found a significant difference among the lines in uterine mass (F=36.7; P< 0.0001; Figure 6B) with the uterine mass of NR mice higher than both the C and R lines. In this analysis, the C line's uterine mass was significantly below the R line. There was a significant correlation between the log of

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uterine and ovary mass ($R^2 = 0.646$; P< 0.0001; Figure 6C). The uterine data was log transformed because of unequal variances of the uterine masses.
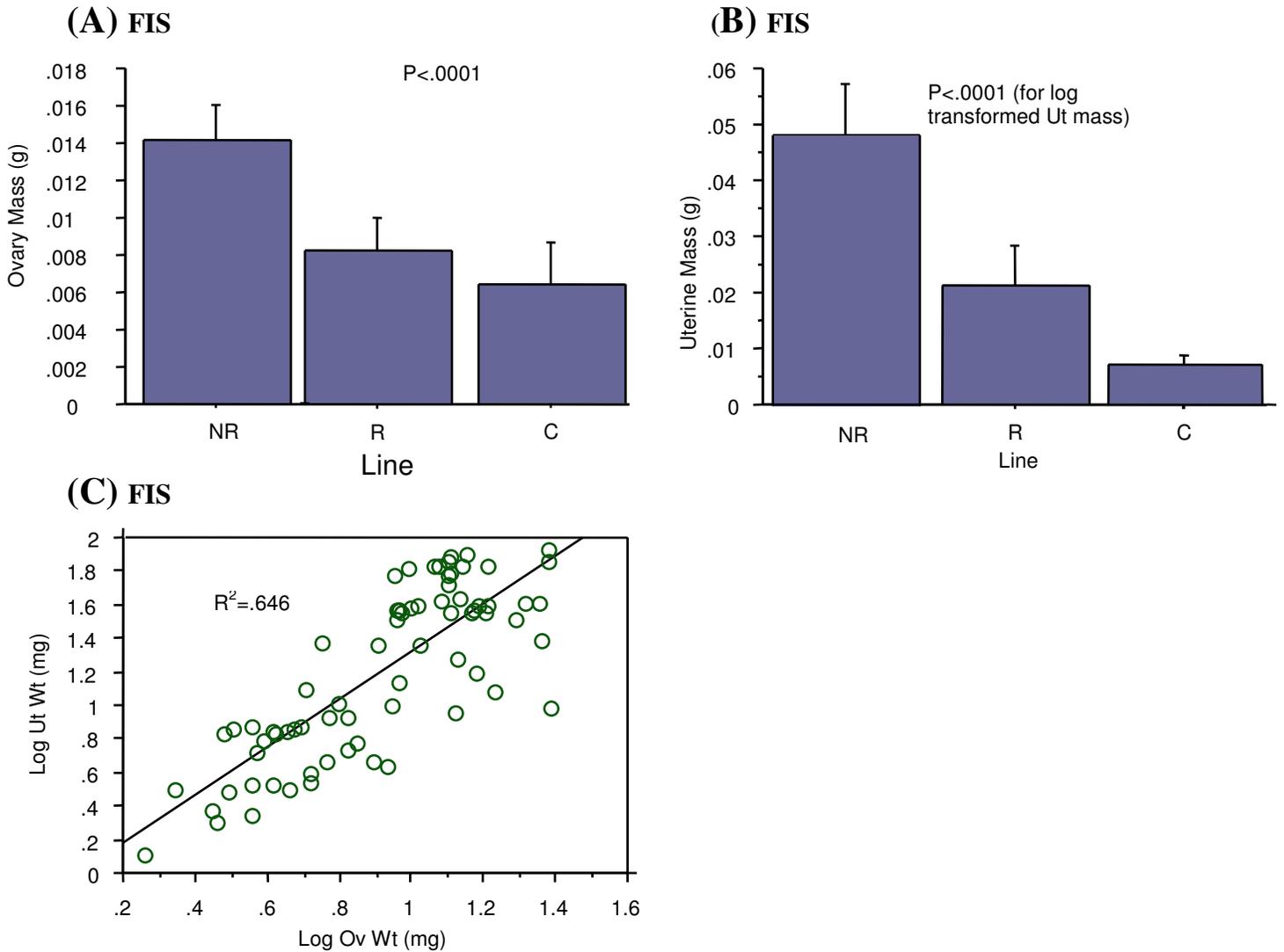
(A) FIS

(B) FIS

(C) FIS

Figure 6: Ovary and uterine mass by line. (A) Ovary weight by line for the FIS (B) Uterine weight by line for FIS (C) Correlation of the log of uterine (ut) mass in mg on the log of ovary (ov) mass in mg. Bars indicate a 95% confidence interval.

Data on the ovary and uterine mass of the NCSS were also analyzed by lines. Ovary mass differed significantly among lines (F= 8.396; P=0.0012; Figure 7A). The ovary mass was higher in NR mice, compared with that of R and C mice. Log-transformed uterine mass differed

significantly among lines (F=18.492; P<.0001; Figure 7B), with higher values for NR mice than

R and C mice. Log transformed uterine mass was significantly correlated with ovary mass ($R^2=$

0.595; P<0.0001; Figure 7D).

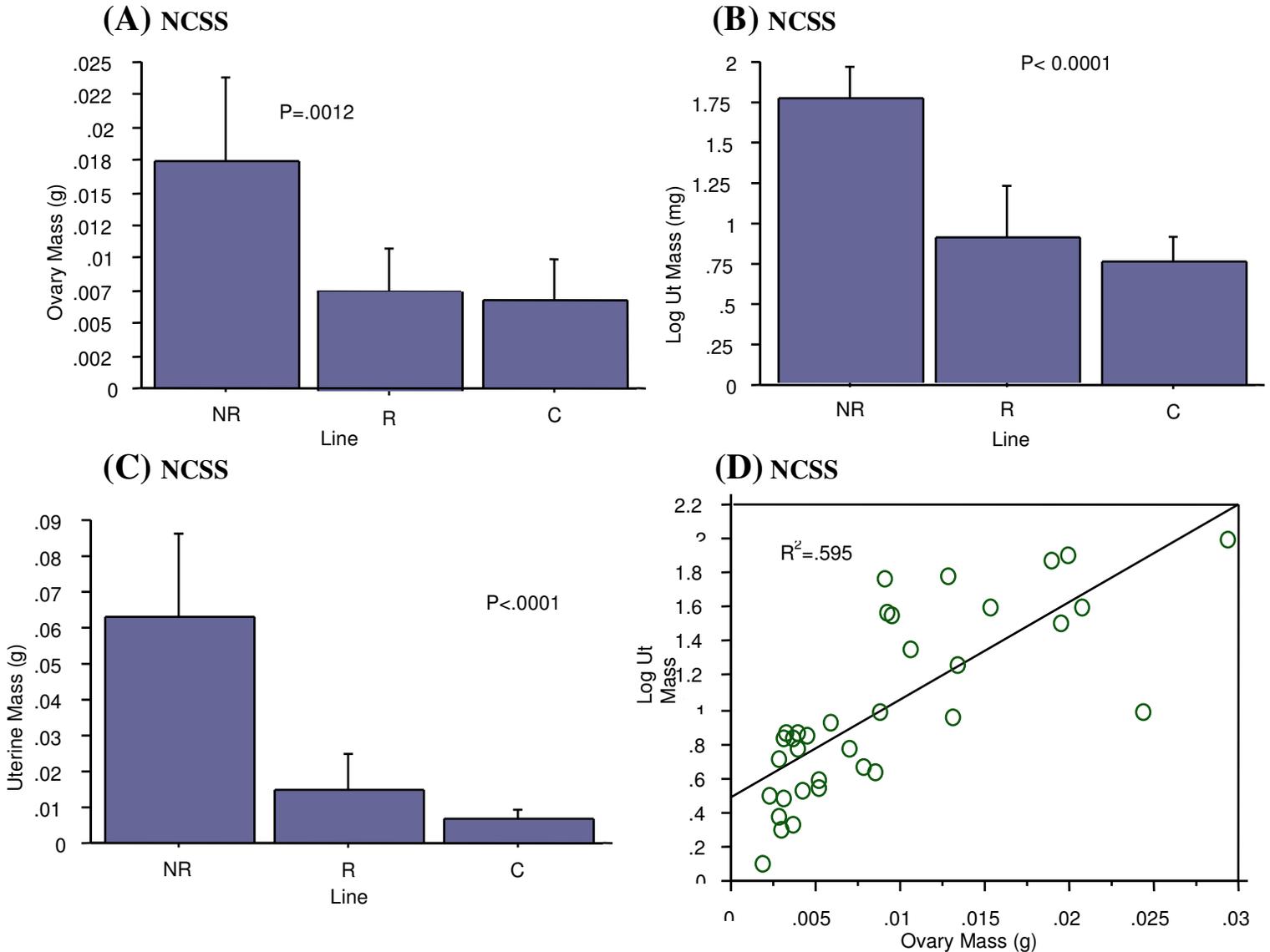Figure 6: Uterine and ovary data by line for the neuron count sub-sample (NCSS). (A) The ovary weight by line for NCSS, the non-responsive [NR] *n=7*, reproductively inhibited [R] *n=12* and control [C] *n=16*. (B) Log transformed uterine (ut) weight by line for the neuron count sample (C) Raw data of uterine weight by line. (D) A regression of the log of uterine mass versus ovary mass. Bars indicate a 95% confidence interval.

Thresholds can be drawn using ovary and uterine mass to identify reproductively capable and reproductively suppressed mice. For females that have not yet gone through puberty, this threshold is an ovary mass ≤ 0.005 g and uterine mass< 0.008 g (Figure 8, grey boxes). From previous studies, an ovary mass of 0.005 g is equivalent to an ovary length of 2 mm, which is the upper limit threshold of complete reproductive inhibition (P.D. Heideman, unpublished data; Avigdor et al., 2005). In reproductively mature mice, ovary and uterine mass vary greatly at different stages of the 4-5 day estrous cycle.
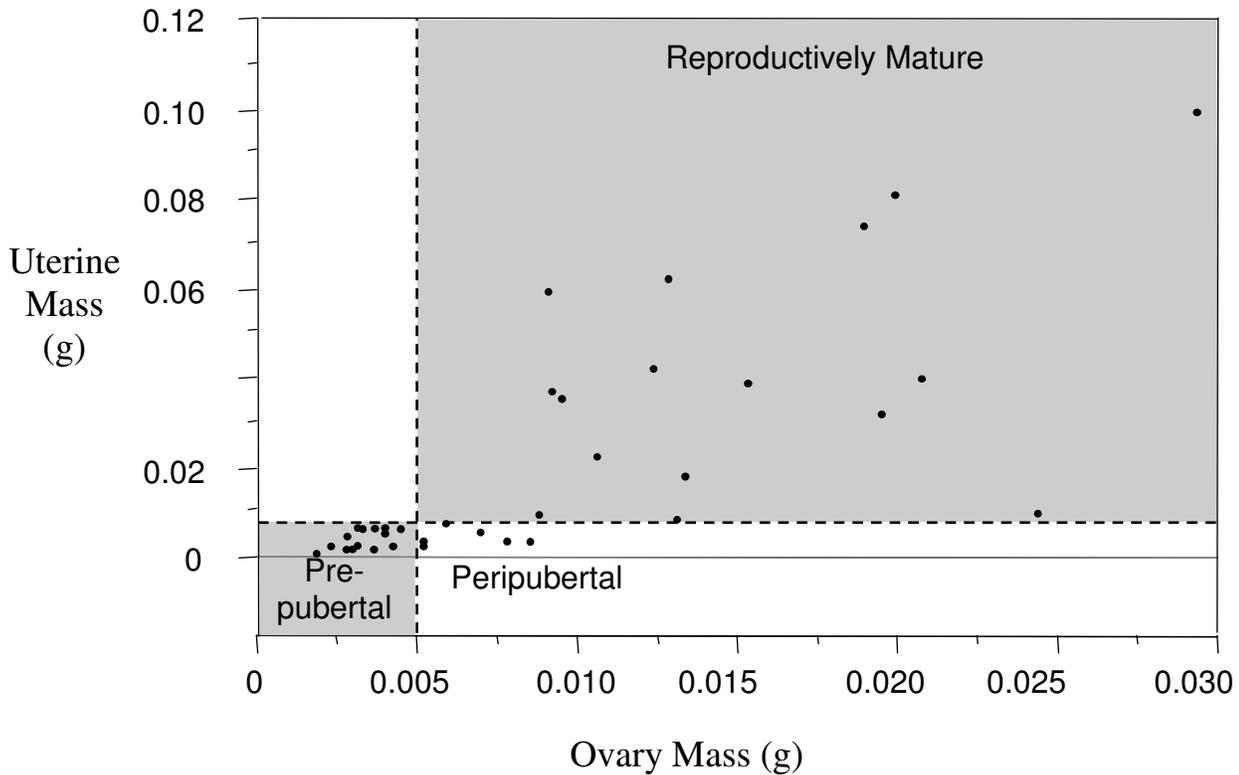

Figure 8: Ovary and uterine mass of prepubertal, peripubertal, and reproductively mature mice. Peripubertal mice are in the process of completing puberty, and reproductive mice are in different stages of estrous cycling.

A representative section showing GnRH staining is shown in Figure 9.

Figure 9: Representative staining for GnRH neurons in a brain section, in the region of plate 17 of Paxinos and Watson (1986). The arrows point to GnRH neuron cell bodies. The third ventricle is visible in the bottom center of the image, and part of the anterior commissure in the upper right.

To compare the immunoreactive GnRH neuron content among selected lines, we counted neurons in the brain sections corresponding to plates 17, 18, 19 and 22 (Paxinos and Watson, 1986) for 34 individuals in the NCSS. There were no significant differences among lines in average number of IR-GnRH neurons per section (F= 1.385; P=.2653; Figure 10). We also compared the lines for number of neurons in each of the sections and found no significant differences (P> 0.1 for each; data not shown because results were similar to the summary data).

Figure 10: Immunoreactive gonadotropin releasing hormone (IR-GnRH) neuron count by line. Bars indicate 95% confidence intervals.

There were also no significant differences in average number of IR-GnRH neurons per section in relation to stage of maturity (F= 0.272; P= 0.7635; Figure 11).
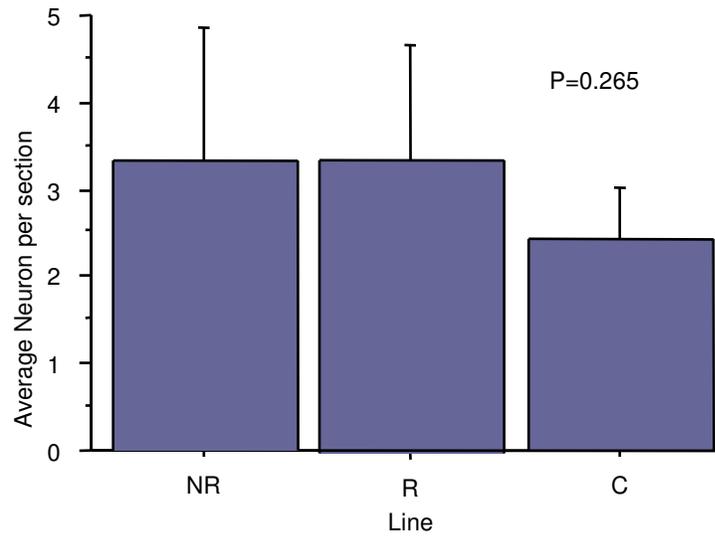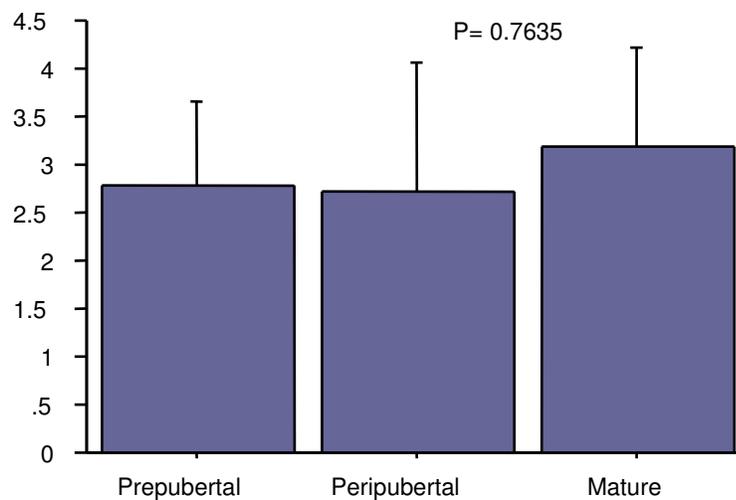

Figure 11: Immunoreactive Gonadotropin releasing hormone (IR-GnRH) neuron count by maturity level. Bars indicate 95% confidence intervals.

Food Intake and Reproduction

There were significant differences among lines in uterine mass, ovary mass, and in food intake. We tested for correlations between ovary mass or uterine mass and food intake. We also tested for correlations between body mass and uterine or ovary mass. There was no correlation of average daily food intake with ovary mass in the NCSS (R^2=.005; P=0.723; Figure 12A). There was also no correlation between ovary mass and body mass (R^2=.009; P=0.619; Figure 12B). Likewise, there was no correlation between log transformed uterine mass and food intake (R^2=7.2 x 10^{-5}; P=0.965; Figure 12C) or log transformed uterine mass and body weight (R^2=.006; P=0.677; Figure 12D). Finally we tested whether food intake was related to stage of reproductive maturity (prepubertal, peripubertal, or mature) and found no significant effect (F= 0.288; P= 0.7510).
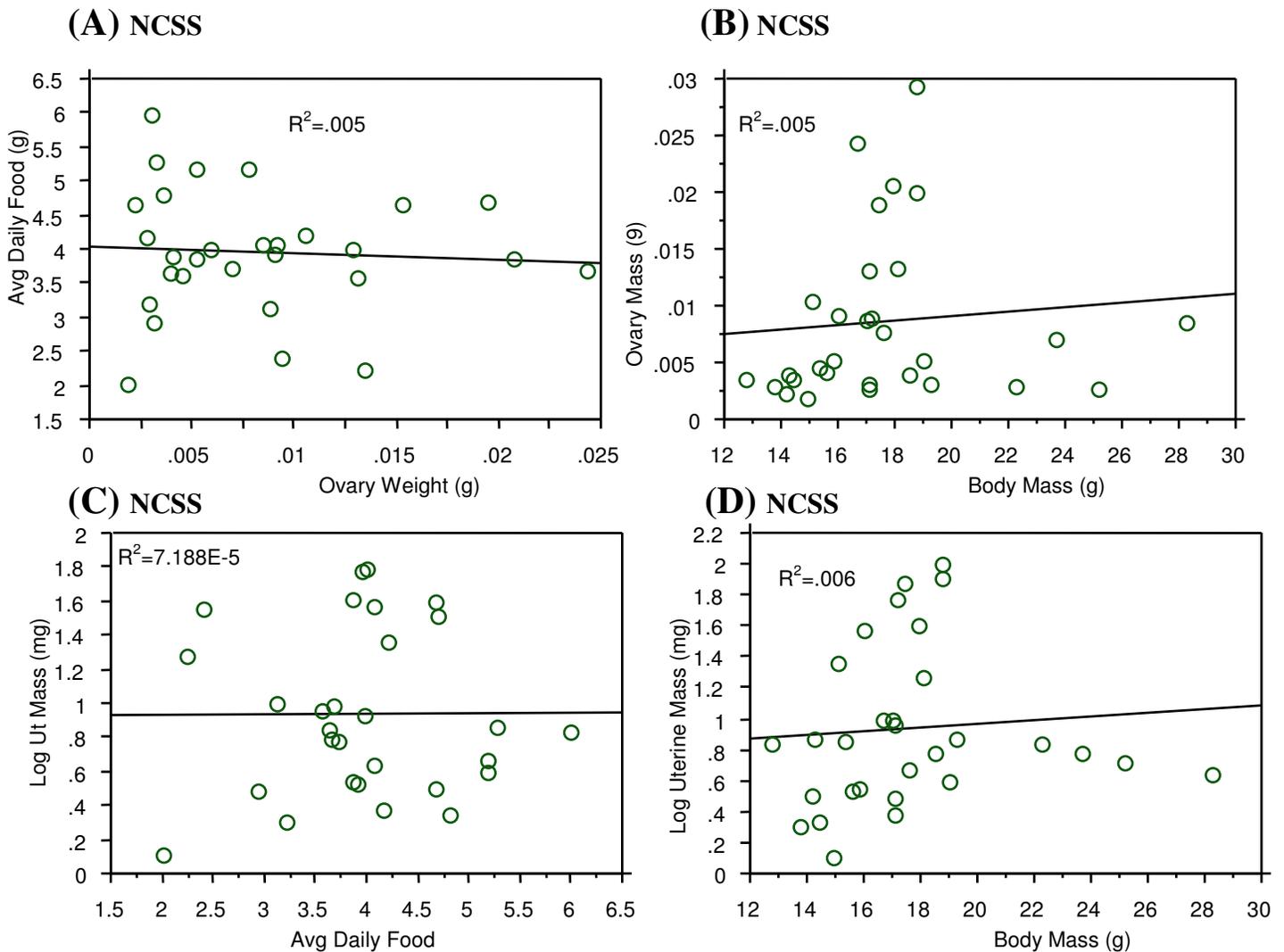
Figure 12: Ovary and uterine mass of the neuron count subsample (NCSS) against average daily food intake and body mass (A) Ovary mass vs. food intake (B) Ovary mass and body mass (C) Log of uterine mass and food intake. (D) Log transformed uterine mass and body mass.

Food Intake, Reproduction and Neuron Counts

We tested for correlations of the average number of IR-GnRH neurons per section with the average daily food intake, uterine mass and the ovary mass in the NCSS. There was no significant correlation for any of these (P>0.05 for all of these, data not shown).

Discussion

General Summary

We hypothesized that females reproductively capable in short day would have a higher

food intake to support the demands of gestation and lactation, and predicted that NR mice would

have a higher food intake than R or C over the two-week period. The results support this

hypothesis, although the difference among lines was smaller than that observed in males

(Heideman et al., 2005). We hypothesized that the number of IR-GnRH neurons was linked to

reproductive behavior in SD and predicted that NR mice would have a higher number of IR-

GnRH neurons than either R or C mice. The results do not support this hypothesis, though

further testing is needed. The total number of IR-GnRH neurons found is one half to one third

the number found in males (Avigdor et al., 2005). Further tested is needed to determine if this

difference between the sexes is due to actual physiological differences or some flaw in the

staining procedure.

Food Intake

In the FCS (N=72) average daily food intake differed significantly among lines (Figure

2A), but in the smaller NCSS (N=29) the average daily food intake did not differ significantly

(Figure 2B). In the FCS, the C and R mice had very similar average daily food intake, with

overlapping confidence intervals. Past research by our lab has indicated that food intake differs

among selected lines in males, with the C line falling intermediate to the R line and NR line for

food intake (Heideman et al., 2005). In males, differences among lines were higher, with R at 3.3

g/day and NR at 4.8g/day (Heideman et al., 2005). The overlap of food intake of R and C

females may indicate a sex-specific difference in food intake or may indicate that the C and R

lines have drifted towards a shared food intake phenotype. The lack of significant difference among groups in the smaller NCSS may be due to sampling error due to the smaller sample sizes. The lack of a relationship between stage of reproductive maturity and food intake indicates that food intake varies according to photoperiod responsiveness, not levels of sex steroids.

There was no significant difference in body mass among lines in either the FIS or the NCSS (Figure 3A and B). There was no correlation between body mass and average daily food intake for the FIS or the NCSS (Figure 4 A and B). This is consistent with previous studies in males (Heideman et al., 2005; Reilly et al., 2006).

Differences in food intake may indicate different life history strategies (Heideman et al. 2005). During times when fewer resources are available, such as winter, animals may need to adjust their behavior or physiology to maximize survival and lifetime reproductive success. One strategy is to limit non-essential activities like reproduction, minimizing energetic costs to lower the amount of food they need to sustain themselves (Yoshimura, 2006; Mintz et al., 2007). Many mammals that breed seasonally in temperate regions exhibit increased testicular size during the breeding season and decreases during the non-breeding season (Yoshimura, 2006). Previous research in *Peromyscus maniculatus* and *Oryzomys palustris* (the marsh rice rat) showed that food intake and photoperiod interacted to determine whether an animal was reproductively capable (Nelson et al. 1992; Edmonds et al., 2003), which is also the case in our population (Reilly et al., 2006). In some short-lived mammals, such as *P. leucopus*, it can be advantageous in some circumstances to accept the cost of foraging for more food with the potential fitness gain that year round reproduction can provide. These life history strategies may be represented by our NR and R lines, and one of the potential tradeoffs seems illustrated in patterns of food intake that appear to be linked to patterns of reproduction. This may imply a genetically-based physiological

link between winter reproduction and food intake in this population of *P. leucopus*. As winter variable reproduction is widespread across small rodents in the temperate zones (Nelson, 1987), and if genetic variation in food intake is correlated with genetic winter reproduction, then this may indicate a microevolutionary lifehistory tradeoff that affects a variety of species.

It is interesting that the difference in food intake across female lines in this present study was smaller than that found in males, perhaps indicating a sex-specific difference. In males the NR mice had a 50% higher food intake than the R mice (Heideman et al. 2005), while the females NR mice had a food intake 20% higher than the R mice. This may indicate that the difference in energy requirements for maintenance of winter reproduction is larger in males than females. In males, it has been hypothesized that there is genetic variation in the tendency to seek mates and levels of activity (Heideman et al. 2005). In male *Peromyscus,* it may be that one key aspect of breeding that varies genetically is to be more active and seek mates. With large testes, there are higher levels of circulating testosterone potentially stimulating increased levels of activity. It may be that increased activity causes the more active mice to have higher food intake, thus creating a link between reproduction and fertility. Males do not differ in food intake between SD and LD, suggesting that the phenomenon is not testosterone dependent (Heideman et al. 2005).

Reproductively active males engage in high levels of scent marking and searching for females in estrous. In non-pregnant females, fertility does not necessarily cause a large increase in activity; when present, activity increases may be brief (Cushing, 1985). Therefore, reproductively active females increase activity only on the night of estrous, on that one night out of four or five when females are sexually receptive and mate (Cushing, 1985). This might explain why the difference among lines for females is smaller than for males. Thus a non-

pregnant, non-lactating and ovulating female may need little or no increase in food intake above

the level of a reproductively suppressed female. If an ovulating mouse mates and becomes

pregnant, however, the cost of pregnancy and lactation forces large increases in metabolic

demands, which causes large increases in food intake. Both female sheep and hamsters suppress

reproduction when not enough food sources available to support the energy intensive investment

of pregnancy, even in the presence of stimulatory photoperiods (Wade and Jones, 2004).

Therefore, the smaller difference in food intake between NR and R female mice in this study

relative to males (Heideman et al., 1999a) could be due to the low costs of ovulation alone.

Because the female C mice were as strongly inhibited reproductively as the R females, it is

consistent that their food intake is similar. There is evidence of a life history tradeoff between the

increase of fitness due to the potential to reproduce and the decrease in fitness due to increased

food intake, but it suggests a lower cost for this life history tradeoff in females relative to males.

Reproduction

Ovary and uterine mass varied among the selected lines and were significantly correlated

(Figures 5C, 6C, 6D). Both the ovary and uterine mass were greater in the NR mice, which is

consistent with previous results (Heideman et al. 1999a; Reilly et al., 2006). Uterine mass is

highly affected by levels of sex steroids, and so uterine mass is related to the amount of

circulating estrogen and progesterone (Brenner and Slayden, 1994). Ovary size indicates whether

a mouse is reproductively active, as well as the size of the follicles or corpora lutea at that point

in the mouse's cycle. Large ovary and uterine masses are typical of sexually mature and

reproductively active mice. An unexpected result was that the control line, which was intended to

control for unintentional selection. In this study, the control line had lower uterine and ovary

mass than the R line. This difference could be due to sampling error or drift, because the C line

has been intermediate in other studies (Heideman et al., 1999a). For the NCSS we did not obtain

our goal sample size, so this lowered sample size can be viewed as a type of sampling error.

Female mice are either reproductively capable or not. Therefore, females either have tiny

ovary and uterine masses or large ovary and uterine masses, with few falling between. Only a

few females are intermediate, and thus presumably undergoing puberty. After puberty, ovary and

uterine mass vary according to the estrous cycle. Thus, most of the variation in size of female

sex organs above the threshold for reproduction is due to mice in different stages of the estrous

cycle. Analysis of food intake according to reproductive state, mature, peripubertal, or

prepubertal, revealed no correlation between the two factors. This indicates that differences in

food intake are probably a genetic difference among lines, rather than the effect of sex steroids or

sexual maturity.

IR- GnRH Content

The number of IR-GnRH neurons was not significantly different among lines. Avigdor et

al., (2005) found that male NR *P. leucopus* had a larger complement of GnRH releasing cells in

both SD and LD, consistent with the hypothesis that NR have a higher number of GnRH

neurons. This may result in greater production of GnRH in general, so that even if NR mice

experience some suppression during short day, NR mice are still capable of reproduction

(Avigdor et al., 2005).). Previous results by Korytko et al. (1995) indicate that the related species

P. maniculatus exhibits a shift in the number of IR-GnRH neurons between mice in SD and

animals in LD. Comparing reproductively non-responsive males, reproductively responsive mice

in natural and artificial conditions, there was a difference in total number of IR-GnRH neurons

(Korytko et. al., 1995, Korytko et. al., 1998). Studies on unselected female mice from a different

location showed a correlation between photoresponsiveness and number and location of GnRH neurons (Glass, 1986). This suggests that females from our selected lines should vary in a similar way to males.

However, analysis of our 34 NCSS sample did not yield any statistically significant differences in either the average number of neurons per section or in individual sections of brain examined. Previous studies have suggested that differences between the selected lines are not distributed evenly across brain regions. However, the four sections we examined were the sections with the highest number of neurons in the male sample from Avigdor et al., (2005). A study counting the number of GnRH neurons in female mink (*Mustela vison*) found more GnRH neurons in summer months than winter months (Toumi et. al., 1992), a result that seems to coincide with the hypothesis that more GnRH neurons are detectable when reproduction occurs.

Another difference between this study and Avigdor et al. (2005) is the average number of neurons we found. Avigdor et al. (2005) found numbers of IR-GnRH neurons equivalent to 9.5 neurons per section in NR and 5.8 in R mice. In our sample, we found a mean of 3.3 neurons per section in both the NR and R, approximately a third to a half the number of IR neurons. It is possible that female mice simply have fewer IR-GnRH neurons. However, given previous results in other species, this seems unlikely. A recent study found similar numbers of GnRH neurons in males and females of a mutant mouse strain, although they found more neurons were needed in females to support puberty (Herbison et. al., 2008). A study comparing the number of GnRH neurons in fetal lambs found comparable numbers of GnRH neurons in both males and females (Wood et. al., 1992). However another study in halfspotted goby (*Asterropteryx semipunctata*) found a sex specific difference in the number of GnRH neurons (Maruska et. al., 2007). Examining feedback loops involving GnRH, Boehm et. al. (2005) found that some areas of

neurons and connections exhibited sexual dimorphism. Further testing for potential variation in immunocytochemistry method that could have caused differences in staining is an important next step.

Future Directions

Our finding that males and females appear to differ in IR-GnRH neuron counts makes further study necessary. At this point it is unclear whether the differences in results from the Avigdor et al. (2005) are due to a difference in staining technique, or a physiological difference in female mice. On average, staining of female mice in this study yielded one third to one half the numbers of neurons found in a typical male mouse of each respective line in Avigdor et al. (2005). Despite troubleshooting and numerous attempts to improve the method, staining levels have not changed. The first step in distinguishing between these possibilities is applying the same immunocytochemical staining techniques to male brains; this should allow us to determine if it is a difference in technique, a flaw in the staining protocol or an actual physiological difference between males and females. One option is running ICC on brains from the same mice from the Avigdor et al. study from 2005, which have been preserved at -20° C and should not have experienced significant degradation. Another option is collecting food intake data on a new set of males, and testing whether we obtain similar staining from the previous study. Perhaps females simply have fewer stainable IR-GnRH neurons, or perhaps the staining technique differs in some way from previous papers (Avigdor et al., 2005; Heideman et al., 2007). Another potential reason for the inconclusive results is the relatively small sample size with neuron counts, particularly the number from the NR. To clarify these results we also intend to do more trials collecting food intake data on mice from every line.

Another direction to pursue in evaluating the sex-specific distinctions of maintaining winter reproduction might be investigating the effect of sex steroids, such as estrogen and progesterone. To ensure that any differences in IR-GnRH neuronal complements in females between selected lines are due to responsiveness to photoperiod and not the impact of short-term sex steroids, we could perform ovariectomies in order to control the dose of estrogen, progesterone and other sex steroids. However, previous studies in ewes have observed the same seasonal changes in GnRH in ovariectomized and intact animals with implants sustaining constant levels of estradiol (Lehman et al., 1997; Robinson et al., 1984). This suggests that seasonal changes are due to photoperiod responsiveness and not levels of endogenous sex steroids.

Reproductively active males pay a cost by searching more and eating more whether they mate or not, which makes males more vulnerable to predation (Heideman et al., 2005). Females may pay significant reproductive costs only if they become pregnant. If a female mouse becomes pregnant, the cost of reproduction is much greater than for males. This suggests it would be interesting to know if costs of reproduction, such as food intake, are different in SD and LD, unlike the case in males. This would be evidence of a life history tradeoff, but it suggests it is not the same life history tradeoff as in males.

Mammals can have highly variable life histories of reproduction within species (Edmonds et al., 2003; Avigdor et al. 2005; Bronson, 1985). The information available on the photoendocrine pathway indicates that genetic and phenotypic variation within *P. leucopus* is due to multiple neuroendocrine causes. More studies are needed to clearly link life history variations and neuroendocrine variation in wild populations. Studies of this sort examining genetic variation and underlying physiology are important for explaining aspects of brain

function. Understanding variation within a population has important implications for ecology, evolutionary biology and medicine. Predicting the variation within a natural population of mammals in response to selection pressures may help explain brain function and individual variation, with potential applications to the human medical and veterinary fields. Normal variation within the brain and its consequences can have serious implications for human health. For example, individual variation underlies the small subset of the population that responds adversely to a particular drug (Heideman, 2004). Another long-term application of exploring the mechanisms responsible for seasonal reproduction and variation in these mechanisms may help develop new methods for the regulation of fertility in humans and animals.

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