Comp Question 2012 – NRSA proposal

For female mammals, reproduction is an energetically expensive function and many aspects of energy balance regulation are altered to meet the energy demands of pregnancy and lactation (Augustine, Ladyman, & Grattan, 2008; Roberts & Coward, 1984). A rich literature, which for the most part stems from work with laboratory female rodents, provides compelling evidence that the changes involve both metabolic as well as behavioral adaptations and engage central as well as peripheral neural and neuroendocrine systems (García et al., 2003; Ladyman, Sapsford, & Grattan, 2011). A lot of attention has been directed to the role of ovarian and pituitary hormones, as well as those of peptides of peripheral and hypothalamic origin in the orchestration of these responses to energy demands; the hormonal profile of female mammals and its impact on central peptidergic systems change in remarkable ways as animals go through pregnancy and lactation.

For this question, assume that the principal investigator (PI) of your research group just returned from a sabbatical doing field work in the rain forest of Brazil. She has brought to the lab a small rodent species that shares many endocrine and behavioral features with laboratory rodents, such as mice and rats. What is remarkable about these animals is that they are strongly monogamous and both males and females take care of the pups until weaning. During the females' pregnancy both sexes cooperate in the building of nests and in the hoarding of food. Preliminary data from your PI's sabbatical work show convincingly that during the pregnancy and lactation of their mates, the males of a breeding pair display many changes in feeding and energy balance that resemble those displayed by pregnant and lactating female laboratory rats. Prominent among these changes in energy balance is a salient hyperphagia and a partitioning of fuels in favor of storage, rather than immediate utilization.

Based on the preliminary behavioral and metabolic data already available about the males of this species (i.e., their hyperphagia and conservative metabolism), your task is to develop a research plan to elucidate the mechanisms responsible for these phenomena in males, with attention to, e.g., hypothalamic neuropeptides (see Brogan, Grove, & Smith, 2000), and to do so by generating and testing hypotheses presented as a research plan typical of NRSA pre-doctoral proposals. Your plan should be informed by the extensive literature about reproduction and energy balance in female rats and the pronounced sex differences present in mammals.

Structure your answer as follows:

1. Prepare a Specific Aims page that identifies the hypotheses to be initially tested. Note that you may have different aims for the different hypotheses or, alternatively your first aim may involve an experiment that could potentially differentiate among competing hypotheses, with the other aims further challenging the hypotheses or hypothesis that survives the initial challenge. Limit this section to 2-3 specific aims. The Specific Aims page is single-spaced; all the other pages of the body of the proposal are double-spaced; the page limit is 12 pages not counting the reference section. Please number your pages.

2. Provide a background section explaining the significance of your research and how your approach is innovative.
3. Provide a detailed description of the experiments that you propose in order to test the hypotheses presented under each specific aim. There is no prescribed or preferred level of analysis for this section. The particular experiments may involve molecular, cellular, system or organismic levels of analysis. What is important is that the experiments proposed serve to differentiate among the alternative hypotheses you present. In this section you should have enough details about the experimental design and proposed treatment of the data to make it possible for reviewers to evaluate the merit of what you want to measure and how you will interpret possible outcomes. For each experiment identify the predicted outcome from each hypothesis, and how the different outcomes may allow you to falsify or support each hypotheses.
4. Include a reference section with full bibliographical information for each paper cited in your proposal. Below is a reading list to get you started but ultimately, it is expected that you will read and discuss more than these five.

Reading list

Augustine, R. A., Ladyman, S. R., & Grattan, D. R. (2008). From feeding one to feeding many: hormone-induced changes in bodyweight homeostasis during pregnancy. *The Journal of Physiology*, *586*(2), 387–397.

Brogan, R. S., Grove, K. L., & Smith, M. S. (2000). Differential regulation of leptin receptor but not orexin in the hypothalamus of the lactating rat. *Journal of Neuroendocrinology*, *12*(11), 1077–1086.

García, M. C., López, M., Gualillo, O., Seoane, L. M., Diéguez, C., & Señarís, R. M. (2003). Hypothalamic levels of NPY, MCH, and prepro-orexin mRNA during pregnancy and lactation in the rat: role of prolactin. *FASEB Journal*, *17*(11), 1392–1400.

Ladyman, S. R., Sapsford, T. J., & Grattan, D. R. (2011). Loss of acute satiety response to cholecystokinin in pregnant rats. *Journal of Neuroendocrinology*, *23*(11), 1091–1098.

Roberts, S. B., & Coward, W. A. (1984). Lactation increases the efficiency of energy utilization in rats. *The Journal of Nutrition*, *114*(12), 2193–2200.

Characterizing the positive energy state of reproductive male *Rattus monogamouse*

Specific Aims

According to the center for disease control, Americans pay $150 billion, annually, on treatment of obesity related illnesses. Given the exorbitant costs, research on mechanisms underlying weight gain is vital. Hyperphagia, weight gain, and leptin (Lep) insensitivity, all associated with obesity in humans, are present in pregnant laboratory rodents. Unfortunately, analyses in this model are hindered by complications of gestational and placental hormones, and other commonly used models are complicated by obesity-related health problems. Male *Rattus monogamouse* (*Rm*) provides a reversible model of hyperphagia and weight gain devoid of any of these considerations, as they show hyperphagia and weight gain during their partner's gestation and lactation.

Among the first tasks in studying this novel model is characterizing the external causes and internal mechanisms of hyperphagia in reproductive male *Rm*. Two potential external factors may cause the males' reproductive hyperphagia: 1) the partner's pregnancy may cause the male to enter a positive energy state to support paternal care upon parturition, and 2) the female partner's hyperphagia and increased BW may cause the male to likewise increase his food hoarding, intake, and storage. Many internal mechanisms may underlie this hyperphagic state, and two will be tested here: 1) central Leptin insensitivity, reduced responsivity to the anorexigenic hormone secreted by peripheral fat cells, may permit the continued hyperphagia during a positive energy state, and 2) a shift in hypothalamic neuropeptide synthesis in favor of orexigenic rather than anorexigenic factors may contribute to reproductive hyperphagia. I hypothesize that perceptible changes in pregnant and lactating female *Rm*s cause Lep insensitivity and changes in hypothalamic neuropeptide synthesis to promote hyperphagia and weight gain in pairbonded male *Rm*s.

Specific Aim 1. Determine contributions of partner's reproductive state, partner's bodyweight, and Leptin insensitivity. Although I hypothesize that it is the female's pregnancy that brings about changes in her partner's metabolic state, it is alternatively possible that he changes his feeding behaviors to match that of his partner. In humans, if a man's spouse gains enough weight to reach levels of obesity, he is more than 35% more likely to become obese himself [1]. I will pairhouse males with either another male, a fertile female, or an infertile overweight female to test these competing hypotheses. In addition, half of the males from each group will be injected with exogenous Lep to test for central Lep insensitivity, which could contribute to hyperphagia in a positive energy state as it does in pregnant female rats [2] [3] [4]. I predict that males paired with fertile females will gain more weight, more body fat, and be less responsive to Lep than either of the other groups.

Specific Aim 2. Compare hypothalamic neuropeptide synthesis in reproductive and nonreproductive *Rm* males. In presence or absence of Lep insensitivity, altered hypothalamic synthesis of orexigenic and anorexigenic neuropeptides can promote hyperphagia. I will measure mRNA levels of orexigenic neuropeptides: neuropeptide Y (NPY) and Agouti related protein (AgRP) as well as proopiomelanocortin peptides (POMC) which is cleaved into the anorexigenic neuropeptide α-melanocortin stimulating hormone (αMSH) in males with pregnant partners and males housed with same-sex conspecifics. I predict that males with pregnant partners will have higher levels of NPY and AgRP mRNA and lower levels of POMC mRNA within distinct hypothalamic nuclei compared to males housed another male. Alternatively, I may see the opposite expression pattern suggesting that hypothalamic neuropeptide synthesis is reacting to the positive energy state in order to restore homeostatic balance by decreasing feeding behavior.

In completing these non-interdependent Aims, I will begin test the contributions of partner's reproductive and energy states, central Lep insensitivity, and hypothalamic neuropeptide synthesis in the continued hyperphagia seen in reproductive males of a monogamouse, biparental species. These four factors may contribute independently or in conjunction with each other to allow or promote hyperphagia during a positive energy state. These experiments have great translational value for obesity in humans.

Significance

Hyperphagia, obesity, and Leptin. Hyperphagia, or increased food intake, can lead to elevations in body weight (BW), body fat (BF), and if sustained without compensatory energy output, can result in obesity and obesity-related illnesses. Many mechanisms contribute to homeostatic maintenance of BW and BF. For example, adipocytes secrete Leptin which acts *peripherally* to increase breakdown and decrease synthesis of fats and *centrally* to 1) reduce synthesis of orexigenic agents that stimulate feeding behaviors and 2) stimulate synthesis of anorexigenic agents that inhibit feeding behaviors. Deficits in these homeostatic mechanisms can arise during persistant states of hyperphagia. For example, most obese humans are leptin-resistant [5] as are many obesity models used in laboratories [6, 7]. Given the prevalence and incurred costs of obesity-related illnesses, any insights into potential mechanisms of hyperphagia and leptin-resistance are well within the mission of NIH to gain and apply knowledge to reduce the negative physical, financial, and social consequences of illness.

Table 1. Common Abbreviations

Hypothalamic Subregions	Orexigenic agents	Other
PVN: paraventricular nucleus DMH: dorsomedial hypothalmus ARC: arcuate nucleus SON: supraoptic nucleus VMH: ventromedial hypothalamus	NPY: neuropeptide Y AgRP: agouti-related protein Anorexigenic agents POMC: proopiomelanocortin peptides α-MSH: α-melanocortin stimulating hormone, POMC cleavage product Lep: leptin	BW: body weight BF: body fat FI: food intake *Rm*: *Rattus monogamouse* OVX: ovariectomized Preg: pregnant

Energy maintenance during pregnancy and lactation. Pregnancy and lactation are both characterized by hyperphagia, but differ in energetic balance (Table 2). Pregnancy is characterized by a positive energy balance: energy intake exceeds metabolic expenditures resulting in increased storage of fuels, primarily as fat. These deposits are necessary to support successful pregnancy and lactation because after parturition, the dam shifts to a negative energy balance: her energy demands exceed her caloric intake. The need is so far in excess that the mobilization and utilization of fats stored during pregnancy do not make up the difference, leading some researchers to infer that lactating animals actually have increased energy efficiency [8]. Given that hyperphagia is constant across gestation and lactation while energy state reverses in the two reproductive states, similarities

in the metabolic pathways of pregnant and lactating animals may promote hyperphagia while differences in the same pathways may be responding to or contributing to a shift in energy balance toward storage rather than utilization of fuels.

Concurrent with their positive energy state, pregnant females have high peripheral Lep that drops to virgin levels [9] or lower [10] during lactation. Hi levels of Lep would usually act to decrease food intake, but reduced efficiency in Lep transport across the blood brain barrier during pregnancy [3], contributes to the *central* Lep insensitivity that permits continued hyperphagia during pregnancy.

We recently established the first laboratory colony of *Rattus monogamouse (Rm)*, a monogamous and biparental species that shares similar endocrine and behavioral parameters with rats and mice. Interestingly, male *Rm*s show pronounced hyperphagia and a positive energy state characterized by increased BW, BF, and Lep during their partners' gestation and lactation. Thus, reproductive *Rm*'s show striking similarities to pregnant female rodents in terms many metabolic parameters.

Table 2: Peripheral measures of energetic balance

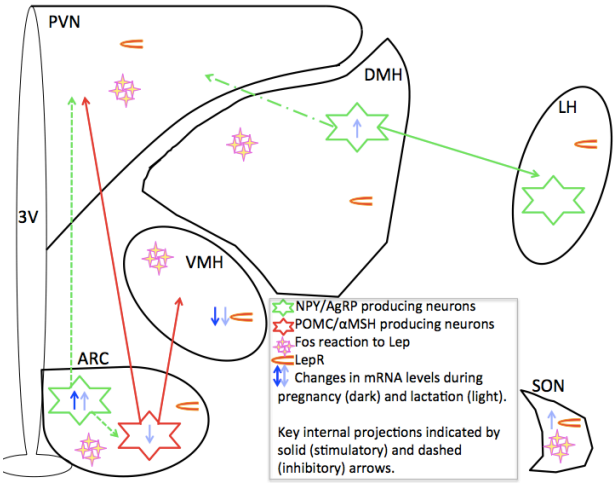
	BW	BF	Plasma Lep	Energy State
Pregnant Rat	High, inc	High, inc	High	Positive
Lactating Rat	High, dec	High, dec	Low - Norm	Negative
Male *Rm* SameSex housed	Norm, ns	Norm, ns	Norm	Neutral
Male *Rm* PregPartner	High, inc	High, inc	High	Positive

<u>Gestational central Leptin insensitivity and hypothalamic neuropeptides.</u> Without Lep insensitivity, Lep would counteract the positive energy state of pregnancy by inhibiting feeding and stimulating energy utilization. In addition to decreased Lep transport across the blood brain barrier discussed earlier, pregnant females show decreased sensitivity to Lep within the hypothalamus. LepR expression is reduced therein during late pregnancy due to reduced RNA transcription [2] [4] [11] [3] and possibly receptor trafficking [12]. Of the several isoforms of LepR, LepRb is particularly important with regard to regulation of FI and BW [13]. Throughout this proposal LepR refers specifically to this isoform.

There is an extensive literature demonstrating the role of the hypothalamus in metabolic pathways. Pharmacological manipulations within the hypothalamus that can have robust effects on feeding [14], and subregions within the hypothalamus that respond to changes in food availability [9]

and Lep [15] [16] [17] include paraventricular nucleus (PVN), dorsomedial hypothalamus (DMH), ventromedial hypothalamus (VMH), arcuate nuecleus (ARC), and supraoptic nucleus (SON). Further, hypothalamic Lep sensitivity and neuropeoptide synthesis change during pregnancy and lactation (Figure 1).

Figure 1: Hypothalamic nuclei



Hypothalamic neuropeptides that affect feeding include orexigenic agents: NPY and AgRP and anorexigenic agent: POMC and its cleavage product, αMSH. Melanocortin receptors are strongly associated with energy intake: the agonist, α-MSH, inhibits feeding behaviors, while the antagonist, AgRP, increases feeding behaviors [14].

Lactating rodents show increased NPY and AgRP mRNA and decreased POMC/αMSH mRNA in the ARC and increased NPY mRNA in the DMH [9, 10], which could all support hyperphagia. Activation of LepR sets off an intracellular cascade involving transcription factors that regulate transcription of several proteins relevant to energy homeostasis including increased POMC transcription and decreased NPY and AgRP transcription [17, 18]. Lep-insensitive mid- and late-pregnant animals show an impaired intracellular response to Lep [4] [3], reducing Lep's effects on transcription of these neuropeptides. In deed, pregnant rodents show increased expression of NPY in the ARC [9]. Lep resistance during pregnancy is also accompanied by insensitivity to downstream effects: ICV infusion of αMSH fails to suppress eating in pregnant females although this effect is robust in virgins [19].

Like pregnant females, reproductive males are in a positive energy state but continue to participate in hyperphagia throughout their partner's pregnancy and lactation. Thus, I *hypothesize* that pairbonded males of the novel monogamous rodent species, Rattus monogamouse, develop a leptin resistance and show altered hypothalamic neuropeptide synthesis during their partners' gestation and

lactation to support their conservative metabolism. Lep insensitivity is not necessary for hyperphagia to exist, however. For example, lactating females show hyperphagia in absence of Lep insensitivity [20]. Altered neuropeptide synthesis can induce or support hyperphagia in conjunction with or in absence of Lep insensitivity [14] [19] [9, 20].

Reproductive Investment. Males of most species do not gain weight in anticipation of the birth of their offspring because, for many males, the energetic burden of reproduction is complete at conception. Males in monogamous, biparental species incur a much higher cost of reproduction than males of other species, however, and male *Rm*'s are not alone in their weight gain during their partners' pregnancies. This pattern is seen in several biparental primate species including cotton top tamarins and common marmosets [21] [22] and some men [23]. Where male *Rm*'s differ from these biparental primates is their continued positive energy state during their partners' lactation.

Whereas the primates discussed above utilize the energy from their fat stores by caring for offspring, *Rm*'s continue to gain weight and store excess energy as fat throughout their partners' lactation. This divergence from other systems may serve to refute the hypothesis that reproductive weight gain in male *Rm* is an adaptation to support paternal care. Instead, the hyperphagia and positive energy state may reflect a response to the partner's behavioral changes: the pairbonded male may simply increase his food hoarding and intake to match that of his partner.

Social networking and obesity. Perhaps surprisingly, social relationships may be a mode of transmission in the obesity epidemic. Social, but not geographic, proximity to obese individuals confers increased risk of obesity [1]. Notably, if an individual's spouse becomes obese, his or her likelihood of becoming obese increases by almost 40%. One important consideration is that weight gain of social partners was not experimentally manipulated, and thus a causal role cannot be concluded from this association. Social influences are known to affect feeding behaviors in humans, however: simulated ostracism or social rejection causes increased intake of fattening foods in adolescents [24] and adults [25]. This finding, together with the fact that fat stores are not used in

child rearing, suggest that reproductive male Rm's shift toward a positive energy state may be in response to his partner's weight gain rather than an internally regulated metabolic adaptation.

Innovation

Although many laboratory models of hyperphagia and Lep insensitivity are already in use, many involve confounding or complicating variables such as the gestational and fetal hormones seen in pregnant mammals and the altered light schedules utilized to study seasonally hyperphagic rodents that are absent in *Rm*. Further, reproductive male Rm lacks the health issues present in obese models and the developmental confounds inherent in transgenic animals. This is the first characterization of a model for continued hyperphagia during a positive energy state without any of these complications and thus provides an excellent research opportunity. Further, to my knowledge, this will be the first experiment involving the effects of experimental manipulation of a female's BW on her pairbonded male's energy state.

Approach

Specific Aim 1. Determine contributions of partner's reproductive state, partner's bodyweight, and Leptin.

Rationale: Lep insensitivity has been observed during positive energy states in female rodents that enter pregnancy [2] [3] [4] as well as male and female seasonal rodents during short-day seasons [26] and in many obese models [7] [6] [26] [5], and is thus a likely mechanism underlying the positive energy state in reproductive male *Rm*s. Lep insensitivity could be conferred by multiple mechanisms including reduced hypothalamic expression of LepR, as seen in pregnant [4] [2] [3] and lactating [27] female rats. Any reduction in LepR expression due to reproductive state would support the hypothesis of Lep insensitivity, but a lack thereof would not falsify the hypothesis because insensitivity could also be conferred by reduced Lep transport across the blood brain barrier as seen in pregnant rats [3] or by reduced effects of second messengers activated by the Lep/LepR system [3] [4]. Therefore intravenous Lep administration will more efficiently confirm or falsify the presence of Lep insensitivity.

We have shown that males with pregnant or lactating partners enter a positive energy state but this could be due to the perceptible changes indicating the female's reproductive state or to the female's increased BW, which is confounded with reproductive state. The female's increased BW may contribute to the male's positive energy state synergistically with or independently of reproductive state. Although the female partner's energy state is negative during lactation when the male continues to express a positive energy state, she continues to show hyperphagia and increased BW (Table 2). When a man's spouse fains weight and becomes obese, his risk of developing obesity increases by nearly 40% [1], suggesting a role of partner's BW in determining and individual's metabolic state. Weight gain and fuel partitioning during pregnancy is necessary for successful pregnancy and lactation, so I will induce weight gain in nonreproductive males' partners to segregate the effects of partner's BW from partner's reproductive state. This manipulation will be easily attained by ovariectomizing females, which prevents reproduction and induces significant weight gain in female rats [28] unless provided with estrogen treatment [29].

Methods: *Subjects and Treatment:* All subjects will be housed in standard laboratory cages and provided with food and water ad libitum in a temperature- and humidity-controlled facility with a 12:12 light:dark schedule with lights on at 07:00. Male subjects and female partners will be anesthetized with ketamine (ip; 90 mg/kg) and xylazine (im; 8 mg/kg). Females will undergo either an ovariectomy (n = 10) or sham surgery (n = 20). Male subjects (n = 30) will receive a permanent guide cannula implanted in the juglar vein. One week later, males will be weighed and assigned to one of three groups in a pseudorandom manner to ensure that all groups have equal starting BW. Males will be housed with either 1) a fertile female (PregPartner), 2) an ovariectomized female (OVXPartner), or 3) another male (SameSex). All subjects will be weighed daily between 09:00-11:00. Pairs will be excluded from analysis if PregPartner females are not pregnant within one week of pairing or if OVX females do not gain significant weight within one week.

PregPartner males will undergo a Leptin challenge 21 days after pregnancy is confirmed by presence of sperm in vaginal smear; OvXPartner and SameSex males will be yolked along with PregPartner males (i.e. one male from each group will always be tested at the same time). I will take a final measure of BW then inject either Lep (100μg in 100μL, PreproTech London, UK) or vehicle via the cannulae (with half of the males in each group receiving Lep), place males in a clean cange alone, and measure FI for 4 hours at which time animals will be sacrificed and adipose tissue removed and weighed for a measure of BF.

Statistical Analyses: Before analysis, I will confirm that BW, BF, and FI are normally distributed. Effects of partner on BW and BF will be analyzed with a one-way ANOVA. Tukey's HSD post-hoc tests will be used to follow up any significant difference. Analysis of effects of Leptin on FI will be analyzed with t-tests (1 for each partner-type). Statistical significance will be indicated by *p*s < 0.05.

Expected and Alternative Outcomes (SA1): For all effects, it is possible that FI, BW, and BF will be affected differentially (e.g. BF increased without effect on BW), which would indicate a significant but perhaps less robust treatment effect. Although this is an important distinction, for clarity, I will discuss measures as single entity when discussing possible outcomes.

Effects of Partner: During this study I will replicate our previous demonstration that male *Rm* subjects increase BW and BF during their partners' gestation and lactation. Confirming that this phenomenon continues in a laboratory setting is imperative to the use of this model. Preliminary data convincingly shows that this is the case. Given my hypothesis that reproductive state, and not partner's BW, affects energy state of male Rattus monogamouse, I expect that final BW and BF will be higher in PregPartner males than OVXPartner or SameSex males. Alternatively, if OVXPartner males have equal BW and BF to PregPartner males, I will conclude that partner's weight gain, and not reproductive state, drive the increase in reproductive male *Rm*s. Finally, if OVXPartner males have intermediate BW and BF (higher than Same-Sex-housed, but lower than PregPartner males) I

will conclude that partner's weight gain affects male Rm energy state, but that reproductive state contributes over and above partner's weight gain.

Effects of Lep: In accordance with my hypothesis that Lep insensitivity contributes to the metabolic changes in reproductive *Rattus monogamouse* males, I predict that IV Lep infusion will reduce FI in nonreproductive (both OVXPartner and Same-Sex-housed) males but will not affect FI of PregPartner males. If Lep does reduce FI of PregPartner males, I will reject my hypothesis that Lep insensitivity contributes to the altered energy state. Because food-intake induced Lep insensitivity, as seen in obese models, develops very slowly, I do not expect to see Lep insensitivity in OVX Partner males even if they gain weight during the experiment. Therefore, I expect Lep to reduce FI in both OVXPartner and SameSex males. If Lep infusions do not affect FI in nonreproductive males, I will not be able to test my hypothesis but this is highly unlikely given the extensive literature on effects of Lep.

Potential Problems and Alternative Approaches: If we do not find a difference between PregPartner males and OVXPartner males it could be due to an inability for the males to differentiate between the states of pregnancy and ovariectomy, which are both characterized by low estradiol and increased BW. This is unlikely, but we will take care to demonstrate that the male does differentiate between the two partners. This could be accomplished in two ways: we could demonstrate that Fos-immunoreactivity is different following exposure to the volatile and nonvolatile components of urine and vaginal secretions collected from females in either state or we could use a learning paradigm to teach males to favor one smell or the other, which would show that the male was able to differentiate the two odors and alter his behavior in response. Males could also fail to bond with ovariectomized females, but this is unlikely given that gonadectomized adults of a similar monogamous, biparental species, *Peromyscus californicus* easily form pairbonds (unpublished observation), and behavioral observations suggest that this is also the case in *Rm*.

Fasting overnight before the Lep challenge may prove necessary in order to increase sensitivity of the test and reduce group differences in endogenous Lep levels. This methodological concern is not

proposed here because it would necessitate either separation from or concurrent food deprivation of the housing partner, which could provide an additional confound (e.g. the pregnant partner may become very distressed by food deprivation which could in turn distress her partner). Finally, this design is limited in that BF is not measured throughout the experiment. BF of OVXPartner males could increase initially then decrease back to normal levels because of homeostatic regulation. If daily BW measures indicate that this may be the case, we could measure BF using MRI scanning throughout a similar experiment in the future. This method was not proposed here because it may be stressful for the animals and the HPA axis is known to interact with metabolic processes.

Specific Aim 2. Compare hypothalamic neuropeptide synthesis in reproductive and nonreproductive *Rm* males.

Rationale: Lep affects hypothalamic neuropeptide synthesis to decrease FI. Lep insensitivity could ameliorate this effect [3], but hypothalamic neuropeptide synthesis could change with or without Lep insensitivity as demonstrated by altered neuropeptide synthesis in both pregnant rats [9] that are Lep insensitive and lactating rats [10] that are not Lep insensitive. Thus, it will be important to test whether hyperphagic Rm males show altered hypothalamic neuropeptide synthesis. In addition, the direction of these shifts (toward synthesis orexigenic or anorexigenic agents) will be useful in determining whether changes reflect underlying causes of hyperphagia or responses to the positive energy state.

Methods: *Subjects and Treatment*: Male subjects will be pairhoused with either their pregnant partner (PregPartner, n=8) or a male conspecific (SameSex, n=8) and provided with food and water ad libitum in a temperature- and humidity-controlled facility with a 12:12 light:dark schedule with lights on at 07:00. Subjects will be weighed at the start of the experiment and immediately before sacrifice to confirm that PregPartner males gained significantly more weight than SameSex males. PregPartner males will be sacrificed 21 days after pregnancy was confirmed by presence of sperm in vaginal smear; SameSex males will be yolked along with PregPartner males (i.e. one same sex male will always be sacrificed at the same time as each PregPartner male). Animals will be sacrificed by

decapitation between 09:00-11:00 to control for circadian effects on neuropeptide synthesis [30], and brains will be rapidly removed and transferred to dry ice for *insitu* hybridization

Insitu Hybridization and Analysis: mRNA levels of orexigenic NPY and AgRP and anorexigenic POMC will be measured in 20μm thick coronal sections. Equivalent sections will be collected from each subject by matching sections to plates in the Rm brain atlas we have compiled. Sections analyzed will contain the DMH, ARC, and LH, which will each be traced for analysis. In situ hybridization will be performed as was done previously [3, 9]. Briefly, I will mount coronal sections onto microscope slides and fix them with 4% paraformaldehyde. I will then dehydrate them in ethanol through four increasing concentrations, then use 3'-end 35S-αdATP labeled antisense oligodeoxynucleotide probes in hybridization solution (10mL/slide; 10^6cpm each probe) to detect NPY, AgRP, and POMC mRNA overnight at 37°C. I will then wash sections 5 times in sodium citrate solution, rinse them in 70% ethanol with 300mM ammonium acetate, and finally air-dry, and incubate them in Hyperfilm β-Max for three days.

Image and Statistical Analyses: Slides will be scanned for autoradiographic signals and analyzed using ImageJ-1.33 h software (NIH). Optical density will be measured within traces of each subregion, and means for each subregion (~10 sections/animal) for each animal will be used for data analysis. Effects of Leptin and partner will be analyzed in separate paired t-tests for each subregion analyzed.

<u>Expected and Alternative Outcomes</u>

Given my central hypothesis that likens reproductive male *Rm* subjects to pregnant female rodents in measures of metabolic maintenance, I predict that compared to SameSex males, PregPartner males will show only one significant effect: increased expression of NPY in the ARC [9]. Alternatively, PregPartner males could show: 1) an increase in the orexigenic and a decrease in the anorexigenic peptides, like pregnant and lactating animals, but in different subregions, or 2) an increase in anorexigenic and decrease in orexigenic peptides. The first alternative would support the hypothesis

that hypothalamic neuropeptide synthesis underlies hyperphagia in reproductive males, and that changes therein reflect an adaptation to store energy perhaps to support paternal investment. The second pattern of results would suggest that neuropeptide synthesis in the hypothalamus is a reaction to rather than a cause of hyperphagia seen in reproductive males, as seems to be the case in developmentally overfed mice [31]. The latter case would suggest that hyperphagia was caused by an external influence (e.g. partner's weight gain, food availability) rather than internal state and that they hypothalamic neuropeptide synthesis represents a homeostatic drive to reinstate a neutral energy state. It is clear, however, that NPY overexpression is sufficient to induce weight gain and extreme adiposity [32], suggesting a bidirectional relationship between hypothalamic neuropeptide synthesis and energy state.

Potential Problems and Alternative Approaches: Although internal mechanisms studied in this proposal are likely candidates for the neural mechanisms underlying hyperphagia in reproductive male *Rm*s, they are by no means exhaustive. There are some indications that pregnant and lactating females are also insensitive to Cholocystokinin [33], another circulating hormone that indicates peripheral energy state. Further, there are many other brain regions that may be involved in these changes including, but not limited to the nucleus of the solitary tract and the mesolimbic dopamine system.

Summary: The experiments proposed here will identify external causes and internal mechanisms underlying the hyperphagia and positive energy state in reproductive male *Rm*s by beginning to test the hypothesis that perceptible changes in pregnant and lactating female *Rm*s cause Lep insensitivity and changes in hypothalamic neuropeptide synthesis to promote hyperphagia and weight gain in pairbonded male *Rm*s.

Works Cited

1. Christakis, N.A. and J.H. Fowler, *The spread of obesity in the large social network over 32 years.* The New England Journal of Medicine, 2007. **357**: p. 370-379.

2. Garcia, M.d.C., et al., *Gestational profile of leptin messenger ribonucleic acid (mRNA) contents in the placenta and adipose tissue in the rat, and regulation of the mRNA levels of the leptin receptor subtypes in the hypothalamus during pregnancy and lactation.* Biology of reproduction, 2000. **62**: p. 698-703.

3. Trujillo, M.L., et al., *Hyperphagia and Central Mechanisms for Leptin Resistance during Pregnancy.* Endocrinology, 2011. **152**(4): p. 1355-1365.

4. Ladyman, S.R., *Suppression of Leptin Receptor Messenger Ribonucleic Acid and Leptin Responsiveness in the Ventromedial Nucleus of the Hypothalamus during Pregnancy in the Rat.* Endocrinology, 2005. **146**(9): p. 3868-3874.

5. Schwartz, M., et al., *Cerebrospinal fluid leptin levels: relationship to plasma levels and to adiposity in humans.* Nat Med, 1996. **2**(5): p. 589-93.

6. El-Haschimi, K., et al., *Two defects contribute to hypothalamic leptin resistance in mice with diet-induced obesity.* J. Clin. Invest., 2000. **105**: p. 1827-1832.

7. Hileman, S., et al., *Characterization of short isoforms of the leptin receptor in rat cerebral microvessels and brain uptake of leptin in mouse models of obesity.* Endocrinology, 2002. **143**(3): p. 775-783.

8. Roberts, S.E. and W.A. Coward, *Lactation increases the efficiency of energy utilizatoin in rats.* Journal of Nutrition, 1984. **114**: p. 2193-2200.

9. Garcia, M.C., *Hypothalamic levels of NPY, MCH, and prepro-orexin mRNA during pregnancy and lactation in the rat: role of prolactin.* The FASEB Journal, 2003. **17**(11): p. 1392-1400.

10. Smith, M.S. and K.L. Grove, *Intregration of regulation of reproductive function and energy balance: lactation as a model.* Frontiers in Neuroendocrinology, 2002. **23**: p. 225-256.

11. Ladyman, S.R., R.A. Augustine, and D.R. Grattan, *Hormone interactions regulating energy balance during pregnancy.* Journal of Neuroendocrinology, 2010: p. no-no.

12. Mitchell, S.E., et al., *Leptin receptor gene expression and number in the brain are regulated by leptin level and nutritional status.* The Journal of Physiology, 2009. **587**(14): p. 3573-3585.

13. Bates, S.H. and M.G. Myers, *The role of leptin receptor signaling in feeding and neuroendocrine function.* Endocrinology and Metabolism, 2003. **14**(10): p. 447-452.

14. Ludwig, D.S., et al., *Melanin-concentrating hormone: a functional melanocortin antagonist in the hypothalamus.* American Journal of Physiology Endocrinology and Metabolism, 1998. **247**: p. E627-E633.

15. Elmquist, J.K., et al., *Leptin activates neurons in the ventrobasal hypothalamus and brainstem.* Endocrinology, 1997. **138**(2): p. 839-842.

16. Elmquist, J.K., et al., *Leptin activates distinct projections form the dorsomedial and ventromedial hypthalamic nuclei.* Proc. Natl. Acad. Sci., 1998. **95**: p. 741-746.

17. Elias, C.F., et al., *Leptin differentially regulates NPY and POMC neurons projecting to the lateral hypothalamic area.* Neuron, 1999. **23**: p. 775-786.

18. Bates, S.H. and M.G. Myers, *The role of leptin receptor signaling in feeding and neuroendocrine function.* Trends in Endocrinology & Metabolism, 2003. **14**(10): p. 447-452.

19. Ladyman, S.R., et al., *Loss of Hypothalamic Response to Leptin During Pregnancy Associated with Development of Melanocortin Resistance.* Journal of Neuroendocrinology, 2009. **21**(5): p. 449-456.

20. Cui, J.G., G.B. Tang, and D.H. Wang, *Hypothalamic neuropeptides, not leptin sensitivity, contributes to the hyperphagia in lactating Brandt's voles, Lasiopodomys brandtii.* Journal of Experimental Biology, 2011. **214**(13): p. 2242-2247.

21. Ziegler, T.E., et al., *Pregnancy weight gain: marmoset and tamarin dads show it too.* Biology Letters, 2006. **2**(2): p. 181-183.

22. Rodriguez, S.M.S., et al., *Body weight increase in expectant males and helpers of cotton-top tamarin (Saguinus oedipus): A symptom of the Couvade Syndrome?* Psicothema, 2008. **20**(4): p. 825-829.

23. Longobucco, D.C. and M.S. Freston, *Relation of somatic symptoms to degree of paternal-role preparation of first-time expectant fathers.* Clinical Studies, 1989: p. 482-488.

24. Salvy, S.-J., et al., *Impact of simulated ostracism on overweight and normal-weight youths' motivation to eat and food intake.* Appetite, 2011. **56**(1): p. 39-45.

25. Baumeister, R.F., et al., *Social Exclusion Impairs Self-Regulation.* Journal of Personality and Social Psychology, 2005. **88**(4): p. 589-604.

26. Tups, A., *Physiological Models of Leptin Resistance.* Journal of Neuroendocrinology, 2009. **21**(11): p. 961-971.

27. Brogan, R.S., K.L. Grove, and M.S. Smith, *Differeintial regulation of leptin receptor but not orexin in the hypothalamus of the lactating rat.* Neuroendocrinology, 2000. **12**: p. 1077-1086.

28. Klump, K.L., et al., *The effects of ovariectomy on binge eating proneness in adult female rats.* Hormones and Behavior, 2011. **59**(4): p. 585-593.

29. Seidlová-Wuttke, D., et al., *Effects of estrogen receptor alpha- and beta-selective substances in the metaphysis of the tibia and on serum parameters of bone and fat tissue metabolism of ovariectomized rats.* Bone, 2008. **43**(5): p. 849-855.

30. Stutz, A.M., et al., *Circadian expression of genes regulating food intake.* Obesity, 2007. **15**(3): p. 607-615.

31. Ferretti, S., et al., *Developmental overfeeding alters hypothalamic neuropeptide mRNA levels and response to a high-fat diet in adult mice.* Peptides, 2011. **32**(7): p. 1371-1383.

32. Ferreira, R., et al., *Neuropeptide Y inhibits interleukin-1β-induced phagocytosis by microglial cells.* Journal of Neuroinflammation, 2011. **8**(1): p. 169.

33. Ladyman, S.R., T.J. Sapsford, and D.R. Grattan, *Loss of Acute Satiety Response to Cholecystokinin in Pregnant Rats.* Journal of Neuroendocrinology, 2011. **23**(11): p. 1091-1098.

Student # 6

Grade: Pass

The applicant presents a comprehensive plan to explore the mechanisms responsible for changes in body weight and food intake seen in males of a recently discovered bi-parental rodent species. The application is well-written and the narrative reflects a good understanding of the current knowledge about the regulation of energy balance, which involves both central and peripheral mechanisms. The investigator also describes comparative work, including human studies, which serves to identify differences and similarities between the new animal model and what has been described in other bi-parental mammals. A very intriguing aspect of the approach is the idea that a signal or signals from the pregnant/lactating females triggers changes in the brain of males that then result in an increase in food intake and body weight. The test of this idea is part of a "mixed" Aim 1 that includes manipulations of the stimulus features of the partners as well as the assessment of the males' sensitivity/responsiveness to leptin. Because of this lack of focus aim one fails to describe a complete and systematic test of the "social hypothesis" of the applicant. The applicant should consider developing a more aggressive test of this very interesting hypothesis. In spite of this apparent weakness, the work on leptin and the approach described under Aim 2 reflect a good understanding of the neurobiology of energy balance and reproduction. The research plan is logical and the applicant considers alternative outcomes and interpretations. Overall the work should help develop a new model for understanding the multiple causes of obesity, particularly those associated with social influences on energy balance.

Minor things:

- Good: the translational perspective at the end of the specific aims page!
- Several typos ('in deed', 'juglar') that should be avoided in a grant proposal.