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.

The mammalian body is programmed to maintain proper energy balance via storage or use of metabolic fuels, which is essential for the health, survival, and reproductive success of the individual. When this balance is tilted in one direction, such as favoring storage of metabolic fuels, many human health issues result such as obesity and eventually diabetes (reviewed in [1]). Although a disrupted balance between energy storage and expenditure is associated with negative health effects, in some instances it can be beneficial to increase one's storage of energy and overall food intake. In the case of pregnancy, increased food intake (hyperphagia) and energy storage are essential for the development of the fetus and to prepare for the high metabolic needs associated with lactation [2, 3]; hyperphagia is also associated with changes in both the periphery and central nervous system. Recently, our lab has discovered a new example of hyperphagia, but in the case of males. The Brazil rat (*Brazil rattus*) is a highly monogamous and biparetnal species. During the female's pregnancy, both the male and female contribute to nest building and hoarding food. What is even more intriguing is that during the pregnancy and lactation of their mates, males display many of the same changes in energy balance and food intake as those displayed by pregnant and lactating females; notably, males display hyperphagia and storage of fuels rather than immediate utilization. The mechanisms contributing to these changes in energy balance in males are unknown. One mechanism that may contribute to changes in energy balance in male Brazil rats is via changes in the leptin signaling pathway. Leptin is an adiposity signal released from fat cells and acts within the perpheripy, as well as the central nervous system to decrease food intake [4]; leptin exerts may of its effects on neuropeptidergic cell populations of the hypothalamus (reviewed in [5]). Alterations in the leptin signaling pathway at the level of the hypothalamus occur in pregnant and lactating animals (reviewed in [6, 7]), so it is likely that some of the same changes are occurring in male Brazil rats. ***The following research proposal will test hypotheses related to the mechanisms contributing to hyperphagia and conservative metabolism in male Brazil rats. The overarching hypothesis of this research proposal is that changes within the central nervous system, specifically neuropeptide neuronal cell populations of the hypothalamus, alter aspects of leptin sensitivity through leptin signaling pathways.***

Specific Aim 1: Are male Brazil rats leptin resistant and are changes in leptin sensitivity mediated by the levels of leptin receptors within specific regions of the hypothalamus? For the first part of Aim 1, I will verify that serum leptin levels are increased in male Brazil rats paired with pregnant partners via a radioimmunoassay kit. Leptin levels are high in mid- to late pregnancy and food intake is increased at this time as well; this phenomenon has been interpreted as a sign of leptin resistance [2, 3, 8-10]. Leptin resistance does not occur in early pregnancy nor during lactation [9]. Thus, if male Brazil rats have high leptin levels associated with their hyperphagia it may indicate that these males are in a leptin resistant state, like pregnant animals. For the second part of Aim 1, I will test the hypothesis that reduced leptin sensitivity in male Brazil rats is due to changes at the receptor level. To test this hypothesis, I will perform *in situ* hybridization to measure leptin receptor mRNA levels in areas of the hypothalamus involved in the regulation of food intake, such as the supraoptic nucleus (SON), arcuate nucleus (Arc), dorsomedial hypothalamus (DMH), ventromedial hypothalamus (VMH), and parventricular nucleus (PVN).

Specific Aim 2: Is leptin sensitivity in male Brazil rats mediated by reduced transduction of the leptin signal? Here I will test the hypothesis that reduced leptin sensitivity in male Brazil rats is due to reduced leptin signaling at the molecular level within cell populations of the hypothalamus. I will measure levels of pSTAT3, a molecule within the leptin intracellular signaling cascade and used as a marker for leptin-induced signaling, [11] and SOCS3, an inhibitor of the leptin signaling pathway, to determine whether reduced sensitivity to leptin is mediated via changes in intracellular signaling.

Overall, the proposed experiments will enhance our knowledge of the mechanisms contributing to hyperphagia and conservative metabolism in male Brazil rats. This work will help human health by probing how specific changes in the leptin signaling pathway can contribute to hyperphagia in males.

Significance

Two-thirds of adults over the age of 20 are considered to be overweight or obese in the United States [12] and many of these individuals develop other complications such as Type 2 diabetes, hypertension, and heart disease [13]. Since such a high percentage of population is living with obesity and other metabolic complications, understanding the mechanisms that regulate food intake is essential in providing treatment to such individuals. Although changes in energy balance can have the aforementioned negative health consequences, some situations require changes in energy balance to support food intake when it may in fact be beneficial for the individual. Pregnancy and lactation are two periods in which increased food intake are essential for not only healthy development of the fetus, but also for meeting the high energy demands of lactation [14]. Understanding how the body is altered to promote hyperphagia in women during pregnany and lactation is essential. In addition to understanding the mechanisms associated with changes in energy balance in general, this proposal seeks to understand how this process occurs in males. Less is known about the interactions between reproduction and food intake in males and this proposal seeks to elucidate such mechanisms.

Background

Energy balance: Effects of leptin signaling in the hypothalamus

The control of food intake and metabolic fuel usage occurs both within the periphery and the central nervous system (CNS). Chemicals released from adipose tissue, the pancreas, and the gastrointestinal tract serve as cues to maintain the proper balance of stored and available fuels. One important adiposity signal that serves to reduce food intake is leptin ([4], reviewed in [5]). While there are many sites within the body that contain receptors for leptin both within the periphery and CNS, the hypothalamus contains many discrete nuclei that contain leptin receptors and help regulate energy homeostasis [15-18] (see Fig 1). Specifically, neuropeptides of the arcuate nucleus (Arc) serve to regulate energy homeostasis via leptin signaling. The Arc is composed of neuropeptide Y

(NPY)/agouti-related peptide (AgRP) neurons that promote food intake and pro-opiomelancortin (POMC, the precursor for alpha-melanocyte-stimulating hormone, αMSH) neurons that reduce food intake. Interestingly, leptin acts on both of these neuronal populations by inhibiting NPY/AgRP neurons (NPY mRNA is reduced) and by stimulating POMC neurons (POMC mRNA is increased) leading to an overall decrease in food intake (reviewed in [5, 7]). NPY/AgRP and POMC neurons of the Arc project to the paraventricular nucleus (PVN, decreases food intake), lateral hypothalamus (LH, increases food intake), and ventromedial hypothalamus (VMH, decreases food intake) (reviewed in [5]). In addition to the NPY/AgRP and POMC neurons of the Arc, leptin receptors are also located in the VMH, dorsomedial hypothalamus (DMH), and supraoptic nucleus (SON), other areas involved in the regulation of food intake [15, 17, 18]. Thus, leptin-leptin receptor signaling can occur at many sites within the hypothalamus to effectively reduce food intake.

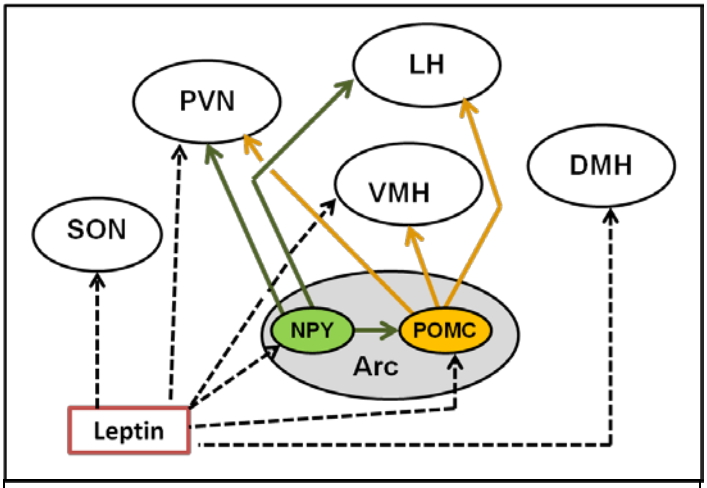

Fig 1. Areas of the hypothalamus involved in food intake and that contain leptin receptors (indicated by the black dotted lines); NPY/ArRP and POMC projections of the arcuate are shown. Modified from [5, 6, 7]

Leptin signaling within the hypothalamus occurs via many intracellular transduction pathways (see Fig 2 for a representation of one). One such pathway is the janus kinase-signal transducer and activator of transcription (JAK/STAT3) pathway. When leptin binds to its receptor, the STAT3 molecule is phosphorylated by JAK; the phosphorylated STAT3 (pSTAT3) forms a dimer and initiates gene transcription within the nucleus of the cell (reviewed in [6, 7]). When leptin signaling occurs, protein inhibitors of the JAK/STAT3 pathway are induced, such as suppressors of cytokine signaling 3 (SOCS3); SOCS3 inhibits leptin signaling via inhibition of the JAK/STAT3 pathway [19, 20]. Changes in the levels of molecules within this leptin transduction pathway may reduce the sensitivity of leptin and ultimately lead to leptin resistance, which occurs in some obese individuals and in females during mid- to late pregnancy [9, 19, 20].

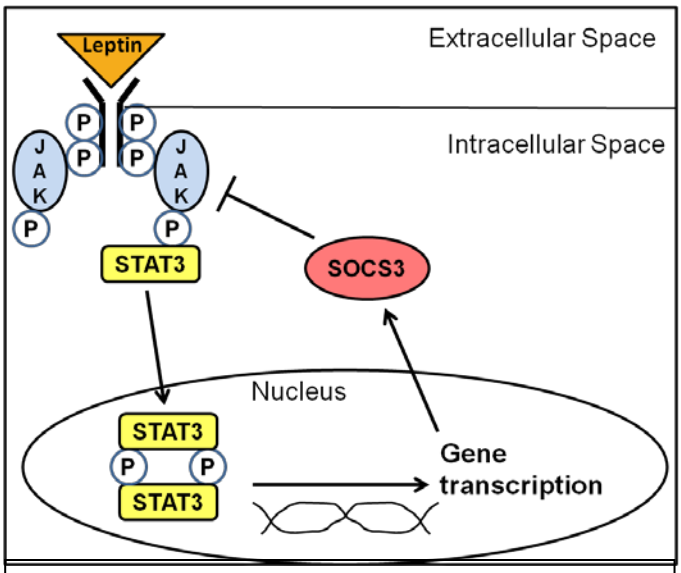

Fig 2. One of leptin's intracellular signaling pathways, JAK/STAT3. Modified from [6, 7].

Energy balance: Pregnancy and lactation

Pregnancy and lactation are associated with high nutritional demand, essential for producing healthy offspring and meeting the energy needs of the mother. During pregnancy, metabolic fuels must be partitioned in such a way to support energy storage rather than expenditure, so energy is available for lactation. Thus, both pregnancy and lactation are characterized by states of hyperphagia [3, 8-10, 14]. Another defining feature of mid- to late pregnancy is high levels of leptin in the plasma, even though increased food intake in maintained over the course of pregnancy [2, 3, 8-10]. This finding is intriguing, since leptin normally suppresses food intake. Thus, mid- to late pregnancy is associated with a state of leptin resistance [2, 3, 8-10]. In addition to leptin resistance, other satiety signals (cholecystokinin, CCK) and neuropeptides (αMSH) that normally inhibit food intake fail to do so during pregnancy; the brain also becomes less responsive to CCK and αMSH [21, 22]. Reduced sensitivity to leptin and other satiety signals suggests that changes within the signaling pathways of these signals is contributing to increased food intake during pregnancy. Such changes could occur at the receptor level or in downstream signaling.

Changes both at the receptor level and within the leptin intracellular signaling pathway occur during pregnancy and, in some instances, during lactation. Levels of leptin receptor mRNA are reduced in the VMH during pregnancy [23] and lactation [24], while leptin receptor mRNA is actually increased in the SON during lactation[24]. Although levels of leptin receptor mRNA are not direct measures of leptin receptor protein, changes in mRNA levels does suggest that local regulation of the production of the leptin receptor is occurring within specific hypothalamic areas. Additionally, changes within the leptin signaling pathway, JAK/STAT3, occur during pregnancy. During mid- and late pregnancy, leptin fails to increase pSTAT3 levels via phosphorylation of STAT3 in the hypothalamus

[10] indicating that intracellular leptin signaling is less efficient. When looking at specific regions of the hypothalamus, only the Arc and VMH display differences in the phosphorylation of STAT3 induced by leptin during pregnancy; there is a decrease in leptin-induced levels of pSTAT3 in the Arc [9] and the number of cells expressing leptin-induced pSTAT3 is reduced in the VMH [23]. When levels of SOCS3, an inhibitor of the JAK/STAT3 pathway, are measured in mid- to late pregnancy there is an increase in SOCS3 both with and without leptin injections [8, 10], suggesting that this inhibiting molecule is upregulated under normal conditions in pregnant animals. Downstream effects of leptin signaling, such as NPY mRNA production are also altered during pregnancy and lactation. In the Arc, NPY mRNA is increased both during lactation and pregnancy [25]. The fact that NPY mRNA is increased in pregnant animals is interesting, since leptin levels are high and leptin normally inhibits NPY transcription (reviewed in [5]), which further illustrates changes within the leptin signaling pathway that occur during pregnancy.

Energy balance: Sex differences and hormonal influences

Pregnancy is also associated with dramatic changes in hormone levles. During pregnancy, estradiol levels are decreased, while levels of progesterone, prolactin, and leptin are increased (see [6]). The leptin resistance that develops during pregnancy is likely to be mediated through changes in the levels of these circulating hormones. For example, estradiol has been shown to inhibit food intake via peripheral and central mechanisms (reviewed in [26]). Estradiol implants targeted at the VMH decrease food intake in female rats [27], as do estradiol implants targeted at the PVN [28], suggesting that estradiol has central effects on regulating food intake in the brain. Another likely candidate for the development of leptin resistance is prolactin. Daily pulses of prolactin occur in early pregnancy, while a sustained increase in prolactin occurs late in pregnancy; prolactin, as well as the hormones secreted from the placenta, such as lactogen, are believed to contribute to leptin resistance in mid- to late pregnancy ([29], for review [6]). Both intracerebroventricular injections of higher doses of prolactin [30] and twice daily injections into the PVN increase food intake in females [31], but to a

greater extent with the twice daily injections, suggesting that changes in not only the levels of prolactin, but the pattern of secretion during pregnancy may contribute to hyperphagia in pregnant animals.

The hormonal influences that may be mediating hyperphagia in our male Brazil rats may be through testosterone converted to estradiol or through prolactin. Peripheral injections effects of testosterone on food intake are believe to occur via aromatization of testosterone to estradiol [32]. In addition, injections of testosterone administered centrally into the VMH decreases food intake, while 5α-dihydrotestosterone (DHTP, a non-aromatizable androgen) has no effect [33]. In addition, estradiol injections into VMH decrease food intake even further than the testosterone injections [33]. These findings suggest that the aromatization of testosterone to estradiol is likely to be contributing the reduced food intake in these males, both in the periphery and in the central nervous system. However, dramatic fluctuations in hormones levels does not occur in males as it does in females during the course the estrus cycle or during pregnancy. It could be possible that overall hormone levels are altered in our Brazil rats paired with pregnant partners. In male marmosets, prolactin levels are significantly increased when males are in captive groups with females with infants, however, these higher prolactin levels could be associated with physical contact, since prolactin levels were higher on days that males were carry infants [34]. However, changes in prolactin levels could be contributing the hyperphagia in our males, but this is speculative at this time point. Additionally, overall changes in testosterone, such as decrease in testosterone due to pairing with pregnant females could be contributing to hyperphagia in our male Brazil rats, but again this is a speculation at this time point. Overall, less is known about interactions between hormones and hyperphagia in males and essentially nothing is known about the hormonal influences mediating hyperphagia in male Brazil rats. Although this proposal will not be focusing on the hormonal effects of hyperphagia in male Brazil rats, future work will be directed at this issue. This proposal serves to provide initial data on the possible mechanisms mediating hyperphagia in male Brazil rats with special attention to the role of neuropeptide neuronal populations in the hypothalamus.

Innovation

This proposal is innovative because very little is known about the neuroendocrine control of positive energy balance in males. How the central nervous system is able to change its responsiveness to signals regarding energy balance to promote beneficial aspects of behavior such food hoarding and nest building in male Brazil rats is interesting. What is even more intriguing is that males of this species are undergoing the same metabolic changes as their female partners *without* experiencing the dramatic alterations in hormone levels associated with pregnancy and lactation. Thus, males in this monogamous species provide an excellent model for studying the mechanisms mediating changes in energy homeostasis and how these changes lead to increased food intake and conservative metabolism to promote potential beneficial aspects of behavior.

Approach

Specific Aim 1: Are male Brazil rats leptin resistant and are changes in leptin sensitivity mediated by the levels of leptin receptors within specific regions of the hypothalamus?

Rationale: The first part of Aim 1 will determine whether serum leptin levels are altered in males paired with pregnant mates. Serum leptin levels will be measured via a radioimmunoassay to determine whether changes in energy balance in these males may be similar to females in mid- or late pregnancy when serum leptin levels are high [2, 3, 8-10], or whether the observed changes in energy metabolism are similar to females in early pregnancy or lactation when leptin serum levels are lower or at normal levels [2, 3, 9]. The second part of Aim 1 will test the hypothesis that reduced leptin sensitivity in male Brazil rats is due to changes at the receptor level. To test this hypothesis, I will perform *in situ* hybridization to measure leptin receptor mRNA levels in areas of the hypothalamus involved in the regulation of food intake, such as the SON, Arc, VMH, DMH, and PVN.

Methods: Adult males housed with a pregnant mate or a bother littermate will be used in this study. I will use males paired with a brother littermate as my control condition to demonstrate that differences I find are due to males housed with pregnant mates and not simply due to being housed

with a conspecific. Animals will be housed under a 12:12 LD cycle with free access to food and water. For the first part of Aim 1, I measure serum leptin levels (n=8) via a multi-species leptin radioimmunoassay (RIA) kit (^{125}I-Mouse Leptin, Catalog # XL-85K, Millipore, Billerica, MA, USA). The inter-assay and intra-assay variations are < 8.7% and < 3.6%, respectively. The standard curve of the assay kit are 1-50ng/mL. For the second part of Aim 1, animals will be sacrificed via rapid decapitation (n=8), brains will be removed, immediately frozen on dry ice, and then stored at -80ºC until sectioning (20µm). The *in situ* hybridization method will be similar to [24]. Briefly, brain sections will be dehydrated, delipidated, rehydrated, air dried, hybridized with 100 µm of probe (593-bp cRNA of the long form of the leptin receptor and transcribed with ^{33}P-UTP, NEN Life Sciences, Boston, MA, USA), coverslipped, and incubated in moist chamber at 50-55ºC overnight. The slides will then go through stringency washes in SSC, in RNase A, and then 0.5 x SSC at 60ºC. Slides will then be dehydrated, and exposed to β–max film for 48hrs. Finally, slides will be in dipped in NTB-2 emulsion, exposed for 22 days at 4ºC, and developed.

Quantification: The standard curve and sample values for the leptin RIA will be calculated via Sigma Plot (Systat Software Inc., San Jose, CA, USA). Leptin standards will be used to create a calibration curve to calculate the unknown samples. For the *in situ* hybridization, silver granules will be analyzed using Optimus Imaging software. A structured outline of each brain region (SON, Arc, VMH, DMH, and PVN) will be created and used for all brain sections. Estimates for each brain area will be determined by the area of silver grains marked within each structured outline.

Expected Results and Predictions: I predict that leptin levels in males paired with pregnant mates will be elevated compared to males paired with brother littermates. Male Brazil rats exhibit hyperphagia when paired with pregnant mates, but their energy demands do not seem to be significantly higher than that before they exhibit hyperphagia. Thus, I predict that these males will have higher leptin levels and may be in a state of leptin resistance similar to pregnant females [2, 3, 8-10]. I also predict that leptin receptor mRNA will be reduced in the VMH of males paired with pregnant mates, since this occurs both in pregnant and lactating rats displaying hyperphagia [23, 24];

I predict that leptin receptor mRNA will not be altered in other areas of the hypothalamus, as found with pregnant females [23]

Alternative Results and Hypotheses: Males paired with pregnant mates may have lower or normal levels of leptin compared to those paired with brother littermates. This would indicate that these males are not in a leptin resistant state. However, this does not rule out the possibility that local regulation of leptin signaling is occurring within the central nervous system, since lactating females have lower to normal levels of leptin, but show changes in leptin receptor mRNA in the VMH [24] and higher NPY mRNA levels in the Arc [25]. It is possible that males paired with pregnant females will show an increase in leptin receptor mRNA potentially within the SON, as found in lactating rats [24]. This too would be interesting as it would indicate that males of this species are showing local changes in leptin receptor mRNA within specific regions of the hypothalamus. Another result may be that males paired with pregnant females do not show any changes in leptin receptor mRNA. This result would not rule out the possibility of local leptin receptor mRNA regulation within specific neuronal populations of the hypothalamus, such as NPY/AgRP versus POMC neurons in the Arc. Future studies could look at these specific neuronal populations to see if leptin mRNA levels are altered. Overall, lack of finding any difference in leptin receptor mRNA could indicate that changes in leptin sensitivity are mediated via changes in leptin signaling downstream of the receptor and this hypothesis will be tested in Aim 2.

Specific Aim 2: Is leptin sensitivity in male Brazil rats mediated by reduced transduction of the leptin signal?

Rationale: Here I will test the hypothesis that reduced leptin sensitivity in male Brazil rats is due to reduced leptin signaling transduction within cell populations of the hypothalamus. If there are no changes in leptin receptor mRNA between males paired with pregnant mates and those paired with brother littermates, as determined by Aim 1, leptin sensitivity may still be reduced due to changes in intracellular signaling pathways. In this Aim, I will measure levels of pSTAT3, a molecule

within the leptin intracellular signaling cascade and used as a marker for leptin-induced signaling [11]. Leptin signaling through the activation of pSTAT3 is required for increased POMC expression within the hypothalamus to essential reduce food intake[35] and levels of leptin-induced pSTAT3 do not change in the Arc between vehicle treated and leptin treated pregnant animals [9]. Additionally, I will measure levels of SOCS3, a molecule transcribed after intracellular leptin signaling and essential in inhibiting the JAK/STAT pathway [19, 20]; SOCS3 serves as intracellular feedback mechanism to the JAK/STAT pathway. Changes in either pSTAT3 or SOCS3 will help determine whether reduced sensitivity to leptin is mediated via changes in intracellular signaling.

Methods: Animals in this study will be housed in a 12:12 LD cycle with free access to food and water. Males will either be housed with a pregnant mate or brother littermate. A total of 16 animals per group will undergo stereotaxic surgery to implant intracerebroventricular cannula, similar to Ladyman & Grattan (2004). Prior to surgery, I will anesthetize the animals with isoflurane gas, shave their heads, inject a local anesthetic (Lidocaine) and cover their eyes with liquid tears. A 23-guage cannula will be implanted with coordinates of 0.0 anterior and 1.3 mm lateral to bregma with 3 mm injection depth below the dura. The cannula will be fixed to the skull with autoclips and cranioplastic cement, while an antiseptic will be applied to the wound. The open end of the cannula will be sealed with a plastic cap until time of injection. Following surgery, animals will housed singly for 24hrs of recovery. Following this recovery period, all animals will be housed again with their pregnant mate or brother littermate for 6 more days. At this time, food will be removed 1hr before the onset of the dark phase for 16hrs of fasting, so endogenous leptin concentrations will be reduced. After the 16hrs of fasting, injections of 2µl of leptin (diluted with artificial cerebrospinal fluid, aCSF, 2µg/µl; PeproTech) or vehicle solution (aCSF with no leptin) will be administered using 2-µl Hamilton syringe (n=8/group/treatment for a total of 32 animals). Thirty minutes after injection, animals will decapitated and brains will be immediately frozen on dry ice and stored at -80ºC.

The western blot procedure will be similar to [9]. Briefly, brains will be cut 300µm thick on a cryostat followed cutting micropunches 500µm in diameter with a micropunch needle (for coordinates

of hypothalamic areas, see Table 1). Tissue will be place in Tris-HCL (pH 6.8), sodium dodecyl sulfate (SDS), and Complete protease inhibitor (Roche Diagnostics, Mannhein, Germany). Samples will then be sonicated and stored at -80ºC. Next, loading buffer containing 2-β mercaptoethanol will be added to the samples, the samples will be boiled for 2 minutes, and loaded on a 7.5% SDS-PAGE gel and electrotransfered to nitrocellulose membrane. Membranes will be incubated with blocking solution for 1hr followed by an incubation in with the pSTAT3 (1:1000, Cell Signaling Technology, Inc., Beverly, CA, USA) or SOCS3 antibody (1:1000, Santa Cruz Biotechnology, CA, USA). Membranes will then be incubated with for 1hr in goat antirabbit peroxidase-conjuaged IgG (1:5000; Santa Cruz Biotechnology, CA, USA). Immunobands will be visualized by the chemiluminescence method (Amersham Life Sciences, Piscataway, NJ). Next, membranes will be washed in stripping buffer to remove excess antibodies and reprobed with the STAT3-specific or α-tubulin antibodies (Santa Cruz Biolotechnology), for pSTAT3 and SOCS3, respectively, following the same procedures as described above.

Table 1. Micropunches of hypothalamic areas

Brain region	Coordinates relative to Bregma			# of punches/brain
Supraoptic nucleus	-1	-1.3	-1.6	6
Arcuate nucleus	-2.5	-2.8	-3.1	3
Ventromedial hypothalamus	-2.5	-2.8	-3.1	12
Dorsomedial hypothalamus	-2.5	-2.8	-3.1	6
Paraventricular nucleus	-1.9	-2.2		4

**Size of punches will be 500µm; Similar to[23]

Quantification: Activation of STAT3 will be measured as a ratio of pSTAT3 signal intensity levels over STAT3 signal intensity levels. The SOCS3 results will be normalized using the α-tubulin levels. Signal intensities will be quantified using ImageJ software (National Institutes of Health, Bethesda, MD).

Expected Results and Predictions: I predict that leptin-induced pSTAT3 levels will not change in the Arc of males paired with pregnant mates, while males paired with brother littermates will have increased pSTAT3 levels after leptin administration. The basis for this prediction is that if leptin signaling is reduced in males paired with pregnant mates, then the phosphorylation of STAT3 will not

occur when leptin is present. Leptin-induced pSTAT3 levels did not change in the Arc of pregnant females after an injection of leptin [9], so if males paired with pregnant mates are in a similar metabolic state, I predict this will occur in them as well. I also predict that baseline SOCS3 levels will be higher and not change after leptin injections in males paired with pregnant mates, while males paired with brother littermates will have lower baseline levels of SOCS3 with SOCS3 levels increasing after leptin injection. It will be interesting to see which areas of the hypothalamus may have higher baseline levels of SOCS3 in males paired with pregnant mates, since previous studies in pregnant animals have examined the hypothalamus as a whole and not discrete areas [8, 10]. However, I would predict that the Arc will most likely show increased SOCS3 levels in males paired with female mates. This prediction is based on the premise that pSTAT3 is reduced in the Arc, so it may be that increased levels of SOCS3 are inhibiting the JAK/STAT3 pathway and preventing the phosphorylation of STAT3.

Alternative Results and Hypotheses: Leptin-induced pSTAT3 may not be different in males paired with pregnant mates versus those paired with brother littermates in any areas of the hypothalamus. This finding would indicate that this portion of the leptin intracellular signaling pathway may not be altered during states of hyperphagia in males of this species. Baseline SOCS3 levels may not be different between males paired with pregnant mates versus those paired with brother littermates. These alternative results would indicate that this portion of the leptin signaling pathway is not altered in areas of the hypothalamus of these males. However, leptin signaling transduction occurs via multiple signal pathways and not all of these pathways are being investigated in the current proposal. Future research could study another component of the leptin signaling pathway or could focus on brain sites downstream of first order neuronal signaling effects of leptin. Alternatively, leptin's actions in the hypothalamus may not be mediating hyperphagia in male Brazil rats, or leptin may not be a key player at all. Further investigation of other satiety signals or the actions of such signals in other areas of the brain or periphery would be warranted in this novel species.

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Student #7

Grade: Pass

The applicant proposes as an explanatory hypothesis that the hyperphagia and obesity of parental males of a novel rodent species stem from a central leptin insensitivity. The rationale for this hypothesis flows from a review of the data for pregnant female rats; these animals in fact become leptin resistant and that change contributes to their typical behavioral and metabolic profiles. A minor weakness of the proposal is that there is no discussion of how the endocrine antecedents of the leptin resistance may have both differences and similarities across the sexes. A review of the literature on hormonal changes in males of other mammalian bi-parental species would have been pertinent here. The experimental approach is presented in a logical fashion and possible explanations for alternative outcomes are carefully discussed and linked to the next proposed step (e.g., how possible negative results from Aim 1 connect with the plan for Aim 2). The applicant is very careful and attentive to details when describing the different techniques used for each of the studies. Lamentably, the same attention is not devoted to the proposed treatment of the data. After reading the proposal one is left completely in the dark about statistical methods. Additional minor weaknesses include the lack of attention to circadian effects and a narrow focus on hypothalamic mechanisms. The incorporation of the work of Grill and colleagues (see Grill HJ, 2010, *Frontiers in Neuroendocrinology* 31: 61 – 78 for a review) could expand the approach to include other brain sites, particularly the nucleus of the solitary tract.

Minor things:

- I suggest wording the specific aims differently: Instead of asking a question, make a statement that you are going 'to determine whether...'.
- Good use of illustrations.
- Several grammatical errors (e.g., singular/plural incompatibilities). Also, given the syntactical structure in Latin, I doubt that the animal would be called 'Brazil rattus', but rather 'rattus Brasilensis'
- References: inconsistent capitalization