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.

Student #1

SPECIFIC AIMS

Energy balance changes in females during pregnancy and lactation (1-3) are crucial to meet exceedingly high metabolic demands required for fetus development and development of fat stores that are utilized during lactation. Two important energy balance changes are hyperphagia and increased fat storage (conservative metabolism). To undergo these necessary energy balance changes, mechanisms regulating anorectic (appetite suppressing) signaling change. For example, anorectic oxytocin neurons undergo many changes in their function such as signaling and release mechanisms.(4-6) Oxytocin is a hormone that has widely been described as having a strong influence in suppressing food intake.(7, 8) There is evidence that prolactin acts directly on magnocellular oxytocin neurons to decrease oxytocin release in the supraoptic nucleus (SON) of the hypothalamus (5) and thereby increase food intake. Moreover, impaired oxytocin signaling has been implicated in increased fat storage and obesity.(9) In addition, prolactin levels are elevated during pregnancy and lactation (10) and administration of prolactin induced hyperphagia in virgin female rats.(11-13) Therefore, it is likely that elevated levels of prolactin reduce oxytocin release in the SON ultimately leading to hyperphagia and a conservative metabolism during pregnancy and lactation.

Recently, our group has obtained a valuable rodent model from the rain forest of Brazil. The male species offers a unique opportunity to study energy balance changes because preliminary data from our group showed that males undergo similar changes in feeding and energy balance compared to those of their mate during pregnancy and lactation such as salient hyperphagia and conservative metabolism. The mechanisms underlying changes in energy balance in the male sex are poorly understood. Therefore, *the goal of this research is to determine the underlying mechanisms responsible for hyperphagia and a conservative metabolism in the male Brazil rodent.* Importantly, this rodent species is highly monogamous and biparental. There is evidence that monogamous, biparental male rodent species show elevated prolactin levels, much like those of the pregnant/lactating females (14, 15) and thus it is likely that prolactin underlies the mechanisms of hyperphagia and a conservative metabolism in the male Brazil rodent. Therefore, *the central hypothesis is elevated prolactin levels in the male Brazil rodent during the female's pregnancy and lactation induces hyperphagia and a conservative metabolism by directly impairing oxytocin release from the SON.* The central hypothesis will be tested with two specific aims.

Specific Aim 1: Are elevated levels of prolactin responsible for inducing hyperphagia and a conservative metabolism in the male Brazil rodent?

Prolactin levels will be determined in the male Brazil rodent over the course of the female's reproductive cycle from copulation until pup weaning. Prolactin levels will be compared to changes in food intake and body weight. In addition, changes in food intake and body weight will be determined after administration of a prolactin receptor antagonist. Testosterone may also mediate these energy changes since testosterone has been negatively correlated with parental care and increased fat storage.(16, 17) However, monogamous, biparental rodents exhibit increased testosterone during the reproductive cycle.(18) Thus, this aim will investigate if prolactin or testosterone mediates hyperphagia and a conservative metabolism. I expect prolactin levels in the male Brazil rodent to be elevated during the course of the reproductive cycle and for these levels and not testosterone levels to correlate with increased food intake and body weight.

Specific Aim 2: Do elevated prolactin levels inhibit oxytocin release from magnocellular oxytocin neurons in the SON to contribute to hyperphagia and a conservative metabolism?

Prolactin receptor expression on oxytocin neurons in the SON will be determined in the male Brazil rodent upon observed hyperphagia and weight gain. Basal oxytocin release and release after prolactin receptor antagonist administration within the SON will be determined. This aim will determine if prolactin directly inhibits oxytocin release. Prolactin may also mediate other anorectic signaling pathways and thus prolactin receptor expression on these neurons will be determined. I expect that the prolactin receptor is expressed on oxytocin neurons and oxytocin release in the SON will be significantly reduced during hyperphagia and weight gain.

The proposed experiments will greatly add to the current knowledge of energy balance that has focused primarily in female rats by elucidating the mechanisms that underlie how hormonal changes influence energy balance in the male sex and have the capability to further understand the mechanisms of hyperphagia

and conservative metabolism. Furthermore, these experiments will enable us to discern the direct effects of prolactin that likely mediate changes in energy balance.

BACKGROUND AND SIGNIFICANCE

Prolactin-dependent regulation of oxytocin neuronal activity mediates hyperphagia and a conservative metabolism in pregnant females

Much research has been done to understand energy balance in females during pregnancy and lactation.(1-3) During pregnancy, females undergo energy balance changes in which they must meet exceedingly high metabolic demands. These changes are crucial both for fetus development and development of fat stores that are utilized during lactation. In order to meet these high metabolic demands, females become hyperphagic and continue to increase body fat levels during pregnancy. A crucial physiological adaptation that is necessary to maintain increased food intake and increased fat deposition is a change in anorectic (appetite suppressing) signaling. As such, during pregnancy and lactation, oxytocin neurons undergo many changes in their function such as their response to stress, their electrical properties, signaling and release mechanisms.(4-6) Oxytocin is a hormone that has widely been described as having a strong influence in inhibiting food intake.(7, 8) For example, oxytocin directly administered to the cerebral lateral ventricles by intracerebroventricular (icv) injections diminished food intake in rats.(19) In addition, by blocking appetite-suppressing effects of oxytocin by using an oxytocin receptor antagonist administered by icv, food intake was significantly increased in rats receiving an appetite suppressor.(20) Results from this study suggest that oxytocin has the capability to mediate hyperphagia when its actions are impaired. Likewise, oxytocin has been implicated in mechanisms leading to a conservative metabolism. For example, Camerino observed increased fat storage and obesity in oxytocin knock-out (KO) mice.(9) As stated previously, during pregnancy and lactation there are changes in oxytocin neuronal activity and there is evidence that prolactin may mediate changes in oxytocin release in the supraoptic nucleus (SON) of the hypothalamus (5) and thereby influencing food intake. For example, administration of prolactin to lactating rats and induced-hyperprolactinemia inhibited stress-dependent oxytocin release.(21, 22) Furthermore, the prolactin receptor is expressed on magnocellular oxytocin neurons in the SON and prolactin directly acting on magnocellular oxytocin neurons has the capability to decrease their firing rate.(5) The results of these studies, suggest that prolactin can directly affect oxytocin

neuronal release and thereby influence hyperphagia and conservative metabolism. Moreover, prolactin levels are elevated during pregnancy and lactation (10) and administration of prolactin induced hyperphagia in virgin female rats in a dose dependent manner.(11-13) Therefore, it is likely that elevated levels of prolactin during pregnancy and lactation reduce oxytocin release in the SON by acting directly on oxytocin neurons thereby contributing to the development and maintenance of hyperphagia and a conservative metabolism (Fig. 1).

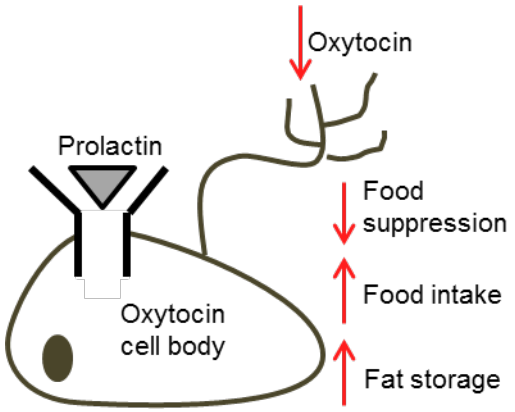

Fig. 1 Prolactin actions on magnocellular oxytocin neuron in the SON

Magnocellular oxytocin neurons project to the posterior pituitary where they release oxytocin into the circulation. In addition, oxytocin may be released centrally by dendrites and cell bodies of these neurons allowing oxytocin to act locally at its receptors in the SON. Importantly, dendritic oxytocin release is significant and likely to diffuse throughout the hypothalamus (8), where it can act at its receptors in brain regions implicated in food intake and body weight regulation such as the ventromedial hypothalamus (VMH) and paraventricular hypothalamus (PVN).(23)

Oxytocin is also an important signaling molecule in sexual reproduction in which parturition and lactation depend on increased oxytocin secretion from the posterior pituitary.(24) Intriguingly, the release of oxytocin is reduced during mid-late pregnancy.(24) Regardless, the amount of oxytocin released from the pituitary gland that enters the brain is minimal (25) and thus oxytocin effects on food intake in the hypothalamus rely on centrally released oxytocin from dendrites of magnocellular oxytocin neurons in the SON.

<u>Monogamous, biparental males exhibit elevated levels of prolactin during female's pregnancy and lactation</u>

Monogamy is characterized by the pair-bond formed between one male and one female and together provide biparental care. Biparental behavior is associated with several hormones that may help to initiate or maintain biparental care. Specifically, circulating prolactin levels are involved in rodent parental behavior; however, the involvement of prolactin is species dependent. Regardless, monogamous males show elevated

levels of prolactin during the pregnancy and lactation of their female mate.(14, 15, 26-28) For example, Brown et al. determined plasma prolactin levels in the male monogamous, biparental Mongolian gerbil over the course of the female's reproductive cycle beginning from mating and ending at pup weaning and found plasma prolactin levels to be elevated throughout this time period compared to unmated non-paired males.(14) Furthermore, males of the monogamous, biparental California mouse exhibited similar elevated circulating prolactin levels compared to lactating females.(15) In addition, prolactin receptors, both the long (PRL-RL) and short (PRL-RS) forms, are expressed throughout the hypothalamus in the male rat.(29) Therefore, it seems imperative that elevated prolactin levels have the capability to modulate energy changes in males involving similar mechanisms to those observed in pregnant and lactating females.

Significance

Recently, our group has obtained a small rodent species from the rain forest of Brazil that is strongly monogamous and biparental. Moreover, both male and pregnant female share the role in building nests and in hoarding food. Preliminary data from our group showed that males undergo similar changes in feeding and energy balance compared to those of their mate during pregnancy and lactation.(My lab, unpublished data) These changes in energy balance are salient hyperphagia and portioning of fuels in favor of storage (conservative metabolism). To date, mechanisms explaining the energy balance changes observed in the male sex are rare. Therefore, *the goal of this research is to determine the underlying mechanisms responsible for hyperphagia and a conservative metabolism in the male sex of the rodent species obtained from the rain forest of Brazil.*

Innovation

Given this unique species, our group has the ability to elucidate mechanisms underlying how hormonal changes influence energy balance in the male sex. Moreover, the proposed experiments will add depth to the current research in energy balance that has focused primarily in female rats and importantly, provide evidence of direct prolactin effects on changes in energy balance.

DESIGN AND METHODS

Specific Aim 1: Are elevated levels of prolactin responsible for inducing hyperphagia and a conservative metabolism in the male Brazil rodent?

Rationale: Preliminary data show that when habituated with the pregnant and lactating female, the male Brazil rodent concurrently exhibits changes in energy balance; namely, hyperphagia and a conservative metabolism. Based on evidence (described above) from pregnant and lactating female rats, it is likely that prolactin may underlie hyperphagia and a conservative metabolism. In monogamous, biparental males, circulating prolactin levels are elevated along the course of pregnancy and lactation. Furthermore, Le et al. demonstrated severe increased lipid storage and obesity in male transgenic mice only expressing PRL-RL.(30) Therefore, I will investigate if prolactin levels in the male Brazil rodent over the entire reproductive cycle of the female starting with mating until pup weaning are elevated and if these levels correlate with increased food intake and body weight.

Alternative hypothesis

It may be that prolactin levels are not elevated during pregnancy and lactation in this species. Another possible mediator of hyperphagia and conserved metabolism is testosterone. Specifically, low testosterone levels were associated with increased biparental behaviors in various species and moreover, it has been implied that testosterone levels impede parental behavior.(16, 31, 32) In addition, low levels of testosterone have been shown to correlate with increased food intake and obesity in males.(17) However, it may not be the case that low testosterone levels in the male Brazil rodent mediate hyperphagia and a conservative metabolism since biparental care in the monogamous male California mouse was associated with higher testosterone levels compared to controls.(18) Regardless, testosterone levels will be measured at the same time points that prolactin levels are measured in order to conclude that testosterone does not mediate hyperphagia and a conservative metabolism.

Animals

All animal use protocols were approved by the Institutional Animal Care and Use Committee at Michigan State University. Animals will be housed in male-female pairs in standard cages maintained in a temperature (22 ± 1˚C) and light controlled (12L:12D) room provided with food and water *ad libitum*. Males

will be reproductively experienced. Animals (n = 7) will be split into different groups. Prolactin and testosterone levels will be determined in the following 7 groups. These levels will be determined over the entire reproductive cycle starting with mating until pup weaning. The 7 groups will be the following: group 1) unmated and housed independently (control), group 2) paired with the female for 24 h (copulation takes place), group 3) paired with the female for 10 d (early-pregnancy), group 4) paired with the female for 20 day (late-pregnancy), group 5) paired with female until 3 days after pups born, group 6) paired with female until 10 days after pups born, group 7) paired with female until 20 days after pups born.(14) The gestation period for the Brazil rodent is around 25 days and pups are weaned after 21 days. Prolactin antagonism effects will be determined in 3 groups; one group will receive prolactin antagonist, the second group will receive vehicle and the third will receive no treatment (control). In these 3 groups, the male will be paired with the female for 20 day as at this time hyperphagia is fully observed and body weight is significantly increased. Microdialysis oxytocin levels, prolactin receptor mRNA and oxytocin mRNA will be determined in 2 groups where in 1 group the male is paired with the female for 20 day and in other group the male is unpaired (control). Lastly, prolactin receptor mRNA and POMC mRNA will be determined in 2 groups where in 1 group the male is paired with the female for 20 day and in other group the male is unpaired (control).

Prolactin and testosterone levels

Plasma prolactin levels will be determined at the time of sacrifice for each of the 7 groups using a Mouse/Rat Prolactin ELISA according to manufacturer's specifications (GenWay Biotech, Inc. San Diego, CA USA; sensitivity 0.2 ng mL^{-1}). At this same time, plasma testosterone levels will be determined using a Rodent Testosterone ELISA (ICN Pharmaceuticals, Orangeburg, New York USA; sensitivity 0.1 ng mL^{-1}). All samples will be run in triplicate.

Total food intake and body weight

To determine if elevated prolactin levels correspond with hyperphagia and a conservative metabolism, total food intake and body weight will be assessed in each of the 7 groups. Total food intake will be assessed for 1 h during normal feeding times 4 separate times the week of sacrifice and averaged for each animal. Body weights will be taken before sacrifice.

Student #1

Prolactin antagonism

To determine if prolactin mediates hyperphagia exhibited by the male Brazil rodent, a prolactin antagonist will be administered. The pure antagonist Δ1-9-G129R-hPRL has been successfully used to block prolactin effects both *in vivo* and *in vitro* by acting as a full competitive antagonist.(33, 34) Δ1-9-G129R-hPRL (1.78 μg) and vehicle will be administered by icv injections 30 min prior to normal eating times. The concentration of antagonist to be administered, previously gave the largest number of responders.(34) Total food intake will be determined after 30 min in control, vehicle-treated and Δ1-9-G129R-hPRL treated animals.

Expected results

Throughout the course of pregnancy and lactation in the female Brazil rodent, I expect plasma prolactin levels to be elevated in the male compared to controls that would likely correspond with hyperphagia and a conservative metabolism presented as an increased food intake and increased body weight. I do not expect testosterone levels to correspond with increased food intake and body weight since I predict that these levels will be elevated. Furthermore, I expect that after administration of the prolactin antagonist, total food intake during feeding times will be significantly reduced. The results from this aim will provide evidence that an elevated level of prolactin underlies hyperphagia and a conservative metabolism in the male Brazil rodent during the time of the female's pregnancy and lactation.

Potential pitfalls

Prolactin receptor antagonists have been shown to induce partial agonism.(35) However, the pure antagonist Δ1-9-G129R-hPRL has not shown to have this property in rats at low concentrations; however, it is not known at what concentration of this antagonist will provide full antagonistic properties in the Brazil rodent. In addition, the prolactin receptor antagonist may not have a profound effect on food intake due to a present sex difference in prolactin receptor expression. Pi and Voogt demonstrated a sex difference in prolactin receptor expression in the hypothalamus, noting that the expression was less pronounced in male rats compared to cycling female rats.(29) Regardless, Scotland et al. demonstrated that the antagonist Δ1-9-G129R-hPRL exhibits antagonistic effects at low concentrations and at these concentrations the antagonist inhibited prolactin effects efficiently in the adult male rat.(34) In order to determine the concentration at which

the antagonist affects the greatest number of responders, I will construct a dose response curve using 0.1-10 μg.

Statistics

All data will be analyzed using a one-way ANOVA with Bonferroni post-hoc test. A correlation analysis will be used to determine if the change in prolactin levels is associated with a consistent change in food intake and bodyweight separately and to determine the strength of the association between prolactin levels and food intake and prolactin levels and bodyweight. Outliers for this analysis will be determined by the Grubb's test. Results will be presented as the mean ± the S.E.M. Results will be deemed significant if the P value is < 0.05.

Specific Aim 2: Do elevated prolactin levels inhibit oxytocin release from magnocellular oxytocin neurons in the SON to contribute to hyperphagia and a conservative metabolism?

Rationale: There is evidence showing that prolactin inhibits oxytocin release in lactating rats and furthermore that prolactin administration reduces the firing rate of oxytocin neurons in the SON.(5, 21) In addition, prolactin receptors are localized on magnocellular oxytocin neurons in the SON.(5) Furthermore, low levels of oxytocin and inhibition of oxytocin signaling lead to hyperphagia and a conservative metabolism.(9, 20) Therefore, it is likely that elevated prolactin levels in the male Brazil rodent have potential to decrease oxytocin release from the SON that would contribute to hyperphagia and a conservative metabolism. To determine if elevated prolactin levels acting at prolactin receptors on magnocellular oxytocin neurons in the SON decrease oxytocin release driving hyperphagia and a conservative metabolism, I will determine if prolactin receptors are present on magnocellular oxytocin neurons and measure basal oxytocin levels and oxytocin levels after prolactin antagonist administration in the SON in the male Brazil rodent paired with a female for 20 days and control animals.

Alternative hypothesis

There is evidence that prolactin increases the suppressor of cytokine signaling molecule (SOCS3) mRNA in another brain region implicated in food intake and body weight regulation, the arcuate nucleus (ARC).(36) The SOCS3 is important in the downstream Janus kinase (JAK)/signal transducer and activator of

transcription (STAT) signaling pathway of the prolactin receptor. SOCS3 acts to inhibit phosphorylation of STAT3 (pSTAT3) that is required for leptin-dependent pro-opiomelanocortin (POMC) neuronal activity.(37) Leptin receptors use the JAK/STAT signaling pathway as well and are expressed on POMC neurons. Leptin activation of POMC neurons by pSTAT3 drives anorectic (appetite suppressing) responses. Impaired POMC neuronal activity would ultimately reduce appetite suppression. This suggests that prolactin may work to inhibit POMC neuronal activity and ultimately lead to hyperphagia and a conservative metabolism. However, Kokay and Grattan demonstrated that the numbers of POMC neurons expressing the prolactin receptor were very few and thus any prolactin-dependent effect on POMC neuronal activity mediating hyperphagia and conservative metabolism would be insignificant.(38) Therefore, it is unlikely that prolactin exhibits a direct, considerable effect on decreasing POMC neuronal activity. In agreement, Roy et al. demonstrated that prolactin-dependent decreases in pSTAT3 did not affect leptin-dependent increases in pSTAT3 (39) suggesting that even though these receptors use the same signaling pathway, their pathways do not converge and hence prolactin likely does not impair POMC neuronal activity directly. Regardless, prolactin receptor expression on POMC neurons will be determined in order to conclude that prolactin does not directly affect POMC activity that would lead to hyperphagia and a conservative metabolism.

Double-labeled in situ hybridization for simultaneous detection of the prolactin receptor, oxytocin and POMC

To determine if the prolactin receptor is expressed on oxytocin neurons and/or POMC neurons, I will use double-labeled *in situ* hybridization. This technique offers advantages as it allows for the simultaneous localization of two different mRNAs at the cellular level. In addition, this technique is superior to Northern blotting in that it offers a more precise quantification that requires smaller amounts of RNA. The protocol that will be used has been described elsewhere.(5, 38) Briefly, tissue will be fixed and coronal sections through the SON and ARC will be cut using a cryostat. Tissue will be hybridized with a ^{35}S-labeled prolactin receptor probe and a digoxigenin(DIG)-11-UTP-labeled RNA probe directed against oxytocin mRNA or ^{35}S-labeled prolactin receptor probe and a digoxigenin-11-UTP-labeled RNA probe directed against POMC. The ^{35}S radioactive probe offers sensitivity, but is biohazardous. DIG-labeled oxytocin and POMC will be detected by a blue-violet precipitate and the prolactin receptor will be detected by silver grains present within neurons. Cells positive for

DIG-labeled oxytocin or POMC and prolactin receptor will be identified in individual cells with a visible nucleus by imaging software that will determine if the prolactin receptor mRNA signal is greater than three times that of the background signal.

Basal oxytocin levels in the SON by microdialysis

To determine basal oxytocin levels in the SON during observed hyperphagia and increased body weight, I will use microdialysis to sample oxytocin dendritic release in the SON. It is implied that centrally dendritic oxytocin release is important in regulating food intake since oxytocin released into the posterior pituitary enters the circulation and cannot cross the blood brain barrier. Oxytocin could be measured in the plasma however, this concentration relates to oxytocin release into the posterior pituitary and not in the SON. In addition, the half-life for oxytocin in the blood is 2-3 min (8) and thus an accurate measure of oxytocin release is hindered. On the other hand, microdialysis is an *in vivo* sampling technique used to sample low molecular weight analytes from the extracellular space in specific brain regions providing valuable information on the general chemical environment of a specific brain region. Neurotransmitters and neuropeptides diffuse down their concentration gradient toward the probe, which is de-void of these compounds. Oxytocin is a large molecule (1007 Da) and it may be difficult to sample; however, the molecular weight cut off can be as great as 18 kDa for some microdialysis probes. Thus, this technique will provide an adequate measure of SON oxytocin concentration. Briefly, male Brazil rodents after being paired with the female for 20 days will be anesthetized and placed onto a stereotaxic frame. A burr hole will be drilled into the exposed skull. A guide cannula for a CMA/7 microdialysis probe (CMA/Microdialysis, Chelmsford, MA; molecular cut off is 6000 Da) will be implanted into the SON and anchored with dental cement. Dialysis samples will be analyzed using a commercial oxytocin immunoassay according to the manufacture's specifications (Enzo Life Sciences, Inc. Farmingdale, NY USA). Measurements will be made in triplicate.

Oxytocin levels in the SON after prolactin antagonist administration

To determine if prolactin acts directly on its receptor expressed by magnocellular oxytocin neurons, oxytocin levels will be determined in the SON 30 min after icv injection of the prolactin antagonist Δ1-9-G129R-

hPRL described in specific aim 1. Oxytocin release will be sampled by microdialysis and analyzed using an oxytocin immunoassay as described in the previous section.

Expected results

Based on evidence demonstrating that elevated prolactin levels can inhibit oxytocin release and furthermore that direct application of prolactin can inhibit oxytocin neuronal firing in the SON, I expect that oxytocin levels in the SON collected by microdiaylsis will be significantly reduced in the male paired with the female for 20 days compared to control. This result in addition to elevated prolactin levels found in specific aim 1 will suggest that elevated prolactin levels act to reduce centrally released oxytocin from the SON. Furthermore, I expect that prolactin receptors will be expressed on oxytocin neurons in the SON, which will coincide with the idea that prolactin has direct effects on central oxytocin release in the SON. I expect that administration of the prolactin antagonist will increase oxytocin levels in the SON to control levels. This experiment will provide direct evidence that prolactin acting on magnocellular oxytocin neurons has the capability to modulate centrally released oxytocin. I do not expect prolactin receptors to be expressed on a great number of POMC neurons in agreement with previous evidence.(38) Therefore, it will be highly unlikely that prolactin directly impairs POMC neuronal activity. The expected results will show that prolactin, by acting directly at its receptors expressed on oxytocin neurons in the SON, will act to inhibit oxytocin release thereby contributing to hyperphagia and a conservative metabolism that is exhibited in the male Brazil rodent during times of the female's pregnancy and lactation.

Potential pitfalls

In addition to regulating energy balance, circulating oxytocin is important in initiating and maintaining pair-bonds and biparental care in rodents. For example, circulating oxytocin levels are elevated just after copulation and remain elevated during early-pregnancy in monogamous, pair-bonded male rodents compared to controls.(40) Indeed, oxytocin's actions to mediate these behaviors are in brain regions such as the bed nucleus of the stria terminalis and the nucleus accumbens (41) and dendritic oxytocin release from the SON may not be able to reach these areas by diffusion only.

Student #1

There are potential problems using microdialysis. Firstly, microdialysis is an invasive technique and likely directly damages the tissue during probe placement. In order to minimize tissue damage, a probe with very small dimensions will be used. Secondly, microdialysis collection of neuropeptides likely will require a longer sampling time since the concentration of neuropeptides tends to be low and in addition neuropeptides have a low diffusion coefficient. Thus, in order to maximize the neuropeptide concentration I will have to use longer sampling times and couple this with a highly sensitive immunoassay. The assay that will be used has a sensitivity of < 12 pg mL^{-1} and it is predicted that the concentration of oxytocin in the SON is ~1000 pg mL^{-1}.(8) Thirdly, the concentration of analyte collected reflects the flow rate of the perfusate and thus to obtain a high analyte recover, the flow rate must be low leading to longer sampling times.

A potential problem using *in situ* hydridization is the specificity of the probe used. To overcome this possible pitfall, a tissue section will be hybridized with sense RNA probes as a control. These probes have the same transcripts of the oxytocin or POMC cellular mRNA sequences. In addition, to determine the preservation or mRNA and failure of the probe to detect RNA, I will use housekeeping β-actin as a control probe.

Statistics

All results will be analyzed using an unpaired Student's t-test. Results will be presented as the mean ± the S.E.M. Results will be deemed significant if the P value is < 0.05.

Literature Cited:

1. Ladyman SR, Augustine RA, Grattan DR. Hormone interactions regulating energy balance during pregnancy. Journal of neuroendocrinology. 2010;22(7):805-17. Epub 2010/05/12.
2. Augustine RA, Grattan DR. Induction of central leptin resistance in hyperphagic pseudopregnant rats by chronic prolactin infusion. Endocrinology. 2008;149(3):1049-55. Epub 2007/12/08.
3. Roberts SB, Coward WA. Lactation increases the efficiency of energy utilization in rats. The Journal of nutrition. 1984;114(12):2193-200. Epub 1984/12/01.
4. Lipschitz DL, Crowley WR, Armstrong WE, Bealer SL. Neurochemical bases of plasticity in the magnocellular oxytocin system during gestation. Experimental Neurology. 2005;196(2):210-23.
5. Kokay IC, Bull PM, Davis RL, Ludwig M, Grattan DR. Expression of the long form of the prolactin receptor in magnocellular oxytocin neurons is associated with specific prolactin regulation of oxytocin neurons. American journal of physiology Regulatory, integrative and comparative physiology. 2006;290(5):R1216-25. Epub 2006/01/18.
6. Patel H, Chowdrey HS, Lightman SL. Lactation abolishes corticotropin-releasing factor-induced oxytocin secretion in the conscious rat. Endocrinology. 1991;128(2):725-7. Epub 1991/02/01.

7. Parker JA, Bloom SR. Hypothalamic neuropeptides and the regulation of appetite. Neuropharmacology. (0).
8. Leng G, Ludwig M. Neurotransmitters and peptides: whispered secrets and public announcements. The Journal of physiology. 2008;586(Pt 23):5625-32. Epub 2008/10/11.
9. Camerino C. Low sympathetic tone and obese phenotype in oxytocin-deficient mice. Obesity (Silver Spring, Md). 2009;17(5):980-4. Epub 2009/02/28.
10. Garcia MC, Lopez M, Gualillo O, Seoane LM, Dieguez C, Senaris RM. Hypothalamic levels of NPY, MCH, and prepro-orexin mRNA during pregnancy and lactation in the rat: role of prolactin. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2003;17(11):1392-400. Epub 2003/08/02.
11. Naef L, Woodside B. Prolactin/Leptin interactions in the control of food intake in rats. Endocrinology. 2007;148(12):5977-83. Epub 2007/09/18.
12. Gerardo-Gettens T, Moore BJ, Stern JS, Horwitz BA. Prolactin stimulates food intake in a dose-dependent manner. The American journal of physiology. 1989;256(1 Pt 2):R276-80. Epub 1989/01/01.
13. Sauve´ D, Woodside B. The effect of central administration of prolactin on food intake in virgin female rats is dose-dependent, occurs in the absence of ovarian hormones and the latency to onset varies with feeding regimen. Brain research. 1996;729(1):75-81.
14. Brown RE, Murdoch T, Murphy PR, Moger WH. Hormonal Responses of Male Gerbils to Stimuli from Their Mate and Pups. Hormones and Behavior. 1995;29(4):474-91.
15. Gubernick DJ, Nelson RJ. Prolactin and paternal behavior in the biparental California mouse, Peromyscus californicus. Horm Behav. 1989;23(2):203-10. Epub 1989/06/01.
16. Gray PB, Kahlenberg SM, Barrett ES, Lipson SF, Ellison PT. Marriage and fatherhood are associated with lower testosterone in males. Evolution and Human Behavior. 2002;23(3):193-201.
17. Christoffersen BO, Gade LP, Golozoubova V, Svendsen O, Raun K. Influence of castration-induced testosterone and estradiol deficiency on obesity and glucose metabolism in male Gottingen minipigs. Steroids. 2010;75(10):676-84. Epub 2010/04/28.
18. Trainor BC, Marler CA. Testosterone, paternal behavior, and aggression in the monogamous California mouse (Peromyscus californicus). Horm Behav. 2001;40(1):32-42. Epub 2001/07/27.
19. Arletti R, Benelli A, Bertolini A. Influence of oxytocin on feeding behavior in the rat. Peptides. 1989;10(1):89-93.
20. Olson BR, Drutarosky MD, Stricker EM, Verbalis JG. Brain oxytocin receptor antagonism blunts the effects of anorexigenic treatments in rats: evidence for central oxytocin inhibition of food intake. Endocrinology. 1991;129(2):785-91. Epub 1991/08/01.
21. Torner L, Toschi N, Nava G, Clapp C, Neumann ID. Increased hypothalamic expression of prolactin in lactation: involvement in behavioural and neuroendocrine stress responses. The European journal of neuroscience. 2002;15(8):1381-9. Epub 2002/05/08.
22. Carter DA, Lightman SL. Oxytocin responses to stress in lactating and hyperprolactinaemic rats. Neuroendocrinology. 1987;46(6):532-7. Epub 1987/12/01.
23. Douglas AJ, Johnstone LE, Leng G. Neuroendocrine mechanisms of change in food intake during pregnancy: a potential role for brain oxytocin. Physiology & behavior. 2007;91(4):352-65. Epub 2007/05/22.
24. Russell JA, Leng G, Douglas AJ. The magnocellular oxytocin system, the fount of maternity: adaptations in pregnancy. Frontiers in neuroendocrinology. 2003;24(1):27-61. Epub 2003/03/01.
25. Ermisch A, Brust P, Kretzschmar R, Ruhle HJ. Peptides and blood-brain barrier transport. Physiological reviews. 1993;73(3):489-527. Epub 1993/07/01.
26. Ziegler TE, Snowdon CT. Preparental hormone levels and parenting experience in male cotton-top tamarins, Saguinus oedipus. Horm Behav. 2000;38(3):159-67. Epub 2000/10/20.
27. Ziegler TE, Wegner FH, Carlson AA, Lazaro-Perea C, Snowdon CT. Prolactin levels during the periparturitional period in the biparental cotton-top tamarin (Saguinus oedipus): interactions with gender, androgen levels, and parenting. Horm Behav. 2000;38(2):111-22. Epub 2000/08/31.
28. Ziegler TE, Wegner FH, Snowdon CT. Hormonal Responses to Parental and Nonparental Conditions in Male Cotton-Top Tamarins,Saguinus oedipus,a New World Primate. Hormones and Behavior. 1996;30(3):287-97.

29. Pi X, Voogt JL. Sex difference and estrous cycle: expression of prolactin receptor mRNA in rat brain. Molecular Brain Research. 2002;103(1–2):130-9.
30. Le JA, Wilson HM, Shehu A, Devi YS, Aguilar T, Gibori G. Prolactin activation of the long form of its cognate receptor causes increased visceral fat and obesity in males as shown in transgenic mice expressing only this receptor subtype. Hormone and metabolic research = Hormon- und Stoffwechselforschung = Hormones et metabolisme. 2011;43(13):931-7. Epub 2011/10/13.
31. Burnham TC, Chapman JF, Gray PB, McIntyre MH, Lipson SF, Ellison PT. Men in committed, romantic relationships have lower testosterone. Horm Behav. 2003;44(2):119-22. Epub 2003/09/18.
32. Clark MM, Galef BG, Jr. A testosterone-mediated trade-off between parental and sexual effort in male mongolian gerbils (Meriones unguiculatus). Journal of comparative psychology (Washington, DC : 1983). 1999;113(4):388-95. Epub 1999/12/23.
33. Bernichtein S, Kayser C, Dillner K, Moulin S, Kopchick JJ, Martial JA, et al. Development of pure prolactin receptor antagonists. The Journal of biological chemistry. 2003;278(38):35988-99. Epub 2003/06/26.
34. Scotland PE, Patil M, Belugin S, Henry MA, Goffin V, Hargreaves KM, et al. Endogenous prolactin generated during peripheral inflammation contributes to thermal hyperalgesia. The European journal of neuroscience. 2011;34(5):745-54. Epub 2011/07/23.
35. Goffin V, Bernichtein S, Touraine P, Kelly PA. Development and potential clinical uses of human prolactin receptor antagonists. Endocrine reviews. 2005;26(3):400-22. Epub 2005/04/09.
36. Anderson GM, Beijer P, Bang AS, Fenwick MA, Bunn SJ, Grattan DR. Suppression of prolactin-induced signal transducer and activator of transcription 5b signaling and induction of suppressors of cytokine signaling messenger ribonucleic acid in the hypothalamic arcuate nucleus of the rat during late pregnancy and lactation. Endocrinology. 2006;147(10):4996-5005. Epub 2006/07/22.
37. Munzberg H, Huo L, Nillni EA, Hollenberg AN, Bjorbaek C. Role of signal transducer and activator of transcription 3 in regulation of hypothalamic proopiomelanocortin gene expression by leptin. Endocrinology. 2003;144(5):2121-31. Epub 2003/04/17.
38. Kokay IC, Grattan DR. Expression of mRNA for prolactin receptor (long form) in dopamine and pro-opiomelanocortin neurones in the arcuate nucleus of non-pregnant and lactating rats. Journal of neuroendocrinology. 2005;17(12):827-35. Epub 2005/11/11.
39. Roy AF, Benomar Y, Bailleux V, Vacher CM, Aubourg A, Gertler A, et al. Lack of cross-desensitization between leptin and prolactin signaling pathways despite the induction of suppressor of cytokine signaling 3 and PTP-1B. The Journal of endocrinology. 2007;195(2):341-50. Epub 2007/10/24.
40. Gubernick DJ, Winslow JT, Jensen P, Jeanotte L, Bowen J. Oxytocin Changes in Males over the Reproductive Cycle in the Monogamous, Biparental California Mouse, Peromyscus californicus. Hormones and Behavior. 1995;29(1):59-73.
41. Young KA, Gobrogge KL, Liu Y, Wang Z. The neurobiology of pair bonding: Insights from a socially monogamous rodent. Frontiers in neuroendocrinology. 2011;32(1):53-69.

Student #1

Grade: Pass

The applicant presents a very perceptive review of the available data from other bi-parental mammalian species and uses it to generate the hypothesis that prolactin, acting on the supraoptic nucleus (SON), affects oxytocin (OT) production and that OT is then responsible for the changes in the males' energy balance. This is a logical extrapolation since males of several bi-parental species show changes in prolactin secretion as their partners go through pregnancy. The applicant is also aware of potential contributions of changes in steroid hormones, and considers this as an alternative or complementary hypothesis. The work is presented in a logical fashion and the rationale reflects a good understanding of the pertinent literature. One concern is the fact that the justification for Aim 2, which is the technically demanding component of this proposal, depends substantially upon the outcome of the work from Aim 1. However, considering that nothing is known about the endocrinology of this interesting species, even a simple description of the mechanisms controlling OT production/release by the SON would be valuable information for the development of this novel animal model. One apparent weakness of the application, which is independent of the approach, is the lack of a serious effort to identify what is innovative and significant about the development of this animal model. The applicant could strengthen this proposal by explaining how this particular model may be of value in understanding human obesity, particularly those aspects of this prevalent problem that may stem from the social interactions associated with parental care.

Minor things:

- The in-text citations are, to my knowledge, incorrect: the numbers should come before the period.
- Specific aim 1: since investigating the role of testosterone is a major part of that aim, I would consider mentioning this already in the 'headline' of the aim.
- Specific aim 2: I suggest wording this this more in the sense or 'To determine whether...', instead of asking a question 'Do elevated prolactin levels...'
- p 5, bottom: it is 'ad libidum', not 'ad libitum'.