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Sex differences in the effect of prepubertal GALP infusion on growth, metabolism and LH secretion

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Abstract

Sex differences in the effect of prepubertal GALP infusion on growth, metabolism and LH secretion, *Physiol. Behav.* XX(XX): XXX. The hypothalamic neuropeptide, galanin-like peptide (GALP), is known to have an effect on energy expenditure and reproduction in adult male rats, but little work has been done on prepubertal rats. We hypothesized that hypothalamic GALP is involved in physiological changes associated with the onset of puberty. To test this hypothesis, we first determined the postnatal ontogeny of GALP gene expression via *in situ* hybridization of developing male and female rat pups through adulthood. GALP gene expression was not observed in either male or female rat pups until after postnatal day (PND) 10 and did not reach adult-like levels until after weaning (PND25). To determine if exogenous GALP could induce the onset of puberty, PND25 male and female rats were implanted with lateral ventricular cannulas connected to an osmotic minipump that delivered either GALP or vehicle. GALP infusion significantly ($p < 0.05$) increased body weight, food intake, and metabolic rate in male but not female rats compared to control infusion. After two weeks, GALP infusion had no significant effect on the onset of puberty, percent body fat, nor plasma levels of insulin, FSH or gonadal steroids in either sex; however, GALP did significantly ($p < 0.05$) increase plasma levels of LH and leptin in male but not female rats and increased plasma growth hormone (GH) in both sexes. Our observations further demonstrate a sex difference in GALP responsiveness in prepubertal rats. These data suggest that GALP may be involved with the prepubertal increase in circulating leptin, LH, and GH resulting in an increase in metabolic rate and lean growth associated with puberty.

Keywords

ontogeny; oxygen consumption; energy homeostasis; puberty

INTRODUCTION

The onset of puberty involves changes in feeding behavior, metabolic rate, and reproductive physiology. The onset of puberty is normally triggered by a hypothalamic increase in gonadotrophic releasing hormone (GnRH) release. The increase in GnRH secretion signals the activation of the hypothalamic-pituitary-gonadal axis (HPG) and sexual maturation. The onset of puberty is also associated with changes in food intake, metabolism and energy expenditure.

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Metabolic hormones, such as insulin and leptin, are thought to play a role in gating the timing of puberty and in the associated changes in energy expenditure [1–3], however the neural mechanisms that integrate metabolic signals are unknown. Galanin-like peptide (GALP) is a likely candidate for a neural modulator between energy expenditure and the onset of puberty (for reviews see [4,5]).

GALP has been identified as a neural mediator between metabolism and reproduction [4–9]. In the brain, GALP is only found in the arcuate nucleus [10]. In adult male rats, GALP stimulates both hormonal and behavioral reproduction [11], as well as increases energy expenditure [12]; however, there is little evidence for the actions of GALP in adult female rats. Negative metabolic states, such as fasting, diabetes, and glucoprivation significantly reduce GALP gene expression and central GALP replacement can restore normal reproduction [6,9]. These observations suggest that GALP is a neural mediator between metabolism/energy expenditure and reproduction. In rodents at weaning age, changes in leptin secretion and signaling have been implicated with eliciting changes in metabolism and energy expenditure associated with the onset of puberty [13]. Furthermore, studies have shown that leptin not only stimulates GALP gene expression, but leptin also has a gating effect on the onset of puberty. Observations of leptin's effects on puberty and on GALP gene expression suggest that GALP may be an important neuromodulator not only in the metabolic control of adult reproduction, but also in leptin's effects on the timing of the onset of puberty [14–16].

This manuscript describes investigations into the effects of GALP in prepubertal male and female rats. We predicted that GALP gene expression in the Arc would increase prior to the onset of puberty and that central GALP infusion into prepubertal rats would initiate the metabolic and hormonal changes associated with the onset of puberty. Our observations suggest that like leptin, GALP may not directly stimulate the onset of puberty. However, GALP may be involved in the hormonal and energy homeostatic changes associated with the onset of puberty.

Materials and Methods

Animals

Prepubescent male and female Long Evans rats (45–60 g) were purchased from Harlan (Indianapolis, IN) on postnatal day (PND) 21 that were weaned at PND 20. They were given individual cages and housed in the vivarium at Hope College (Holland, MI). Animals were individually housed in microenvironment chambers to prevent hormonal influence. They were given a standard rodent diet and water *ad libitum*. They were kept on a 12:12 light-dark cycle with lights on at 0700 hr. All procedures were approved by the University of Washington Animal Care and Use Committee (Experiment #1) and the Hope College Animal Care and Use Committee (Experiments #2), in accordance with the National Institutes of Health Guide for Care and Use of Laboratory animals.

Lateral Ventricle Cannulation and Osmotic Minipump Implantation

All surgeries were performed on postnatal day 25 (weaning). Prior to surgery, osmotic mini pumps (223 μ l) were weighed, and filled with appropriate solutions and reweighed to the manufacturer's recommendations (Alzet Inc.). Rats were injected (0.1 ml/100 g body weight) with a ketamine cocktail (ketamine, xylazine, and acepromazine in a 5.0:2.5:1.0 ratio respectively) for anesthesia and ketofen (5mg/kg, intramuscularly) for analgesia. Rats were then placed in a stereotaxic frame and secured. A vertical incision along the top of the skull was made and a stainless steel 26-gauge cannula (Brain Infusion Kit, Alzet) was inserted into the lateral ventricle (AP = –1.2 mm, Lat = –1.0 mm, DV = –3.5 mm relative to bregma). The minipumps were attached to 1.5 cm of polyethylene tubing (0.69 mm internal diameter, 1.14

external diameter) filled with either experimental or control solution and the other end of the tubing was attached to the cannula. Following the installation of the cannula, the minipump was implanted subcutaneously and all incisions were sealed with TissueTek (Websters Veterinary Supply, Milwaukee, WI). The osmotic minipumps were pre-charged in physiologic saline and the PE tubing filled with the same solution as within the minipump—thus ensuring the immediate infusion of GALP or vehicle as described previously by our laboratory [9].

Metabolic Rate

Approximately 1 week after surgery (prior to puberty in either sex), rats were placed in a 13.5 cm wide × 30.5 cm long × 21.5 cm deep plexiglass chamber. Oxygen consumption was utilized as an indirect measure of metabolic rate. Air from the room was drawn through the chambers at 1.3 L/min and the oxygen content of the room air was determined by using Amatec S-3A/II. Oxygen consumption was monitored for 1.5 hours with no personnel present in the room. The first 30 min of each test was not included for analyses, to allow for habituation to the test chamber. After the oxygen consumption test was complete, rats were returned to their cages in the vivarium. Tests were performed between 1000 and 1200 hrs during the beginning of the animals day cycle at a time when the rats would least likely be active or eating. No food was given during the 90 minute test.

In Situ Hybridization for GALP mRNA

Plasmids containing a partial cDNA for rat GALP were cloned as previously described [17]. Linearized GALP cDNA was transcribed for antisense riboprobes with SP6 RNA polymerase (New England Biologicals, Beverly, MA) in the presence of ³³P-UTP (Perkin-Elmer, Indianapolis, IN). Sense transcriptions were carried out with T7 RNA polymerase (Roche Biochemicals, Indianapolis, IN). Appropriate riboprobe length was determined with formaldehyde gel electrophoresis. Riboprobes were quantified following purification in a scintillation counter.

One in three sets of hypothalamic sections (20 μm,) was removed from the freezer, pretreated, and allowed to air-dry prior to the hybridization procedure. The final volume of probe mix plus hybridization buffer was equal to 100 μl/slide. The volume of probe was calculated (0.2 pM/slide) and allowed to thaw on ice. The probe was combined with 1/20 volume yeast tRNA (Roche Biochemicals, Indianapolis, IN) in TE (0.1M tris/0.01M EDTA, pH = 8.0) to produce the probe mix. Probe mix was heat denatured by placing into boiling water for 3 min, then returned to ice for 5 min. The denatured probe mix was added to pre-warmed hybridization buffer at a ratio of 1:4. After adding the hybridization mix to the slides, the sections were covered with glass coverslips. Slides were placed in humidity chambers, which were then put into ovens at 55 C for 16 h.

Following hybridization, humidity chambers were removed from the ovens and allowed to return to room temperature. Coverslips were removed and the slides were returned to slide racks, then washed twice in 4X SSC for 15 min each at room temperature. Slides were then placed into RNase (Roche Biochemicals, Indianapolis, IN), 37 mg/ml RNase in 0.15 M sodium chloride, 10 mM Tris, 1 mM EDTA, pH 8.0) for 30 min at 37 C, then in RNase buffer without RNase at 37 C for another 30 min. After a 3 min wash in 2X SSC at room temperature, slides were washed twice with agitation in 0.1X SSC at 62 C for 30 min each. After a 3 min room temperature wash in 0.1X SSC, sections were dehydrated in graded ethanols (3 min each) and allowed to air-dry. The slides were dipped in Kodak NTB emulsion (VWR, Inc., Seattle, WA), air dried, and stored at 4 C for approximately one week. They were then removed under safe light conditions, developed, dehydrated in graded ethanols, cleared in Citrasol (VWR, Inc., Seattle, WA), and coverslips were applied with DPX (Sigma Biologicals, St. Louis, MO).

GALP mRNA Quantification and Analysis

All GALP mRNA-containing Arc sections were analyzed unilaterally. Slides from all of the animals are assigned a random three-letter code, alphabetized, and read by an operator unaware of the animals' experimental group (age and sex) with an automated image processing system under darkfield illumination. The image processing system consisted of a Leica DMC480 digital camera mounted on a Leica DM5000b microscope. The camera was attached to a Scion VG-5 Frame Grabber (Scion Corp., Frederic, MD) mounted on a Macintosh G5 computer (Apple Computer, Cupertino, CA) running UW Grains Software [18]. Cells are counted as GALP mRNA-positive if a tight cluster of silver grains exceeded that of background. Analyses were made of the number of clusters at each age per sex, as well as the number of silver grains per cluster. Any baseline for comparisons selected would be completely arbitrary for an ontogenetic study, thus data are presented qualitatively.

Radioimmunoassays

Plasma LH, GH, estrogen, testosterone and FSH serum concentrations were measured by Ms. Brigitte Mann at Northwestern University (Evanston, Ill., USA). The LH assay was run with reagents from the NIH and the standard LH antiserum was Rlh-rp3, the antiserum used was anti-Rlh-s11, the assay sensitivity was 0.1 ng/ml, and the intra assay coefficient of variation was 2.2%. The FSH was also measured with reagents from the NIH. The intra-assay CV was 6.85% and sensitivity was 0.05 ng/tube. GH was performed with a double antibody RIA kit (Linco Research, St. Charles, MO) with an intra-assay variance of 4.34%. Insulin and Leptin assays were sent to Linco Diagnostic Services, Inc., with a sensitivity of 0.2 ng/ml.

Experiment #1: Ontogeny of GALP Gene Expression

On the day of birth (PND1) litters were culled to an $n = 8$ (4 male and 4 female when possible). Male and female rat pups ($n = 5$ per sex, age) were collected on PND1, 5, 10, 12, 15, 25, day of puberty (day of preputial separation in males (~PND42) or vaginal opening in females (~PND38)), and adult (PND75). Other male and female rats at PND 1, 5, and 10 ($n = 4$ per sex, per age, per treatment) were injected with either insulin (1.0 U porcine insulin in 0.05 ml saline, subcutaneous), leptin (5.0 mg/kg in saline, subcutaneous), or vehicle. Injection sites were sealed with TissueTek to prevent leakage. Doses were chosen as those that stimulated GALP gene expression in adult male rats [6,9]. Rats were killed by decapitation at approximately 0900 hr during the rats light cycle (food and water were available *ad lib* until decapitation), brains collected and stored at -80°C until processed for *in situ* hybridization for GALP mRNA. *Ad hoc* analyses of male and female rat brains were also done as described above at PND28, 32, 38 and 44 (early post-pubertal).

Experiment #2: Effects of GALP Infusion in Prepubertal Rats

Control infusions consisted of a phosphate buffer solution and experimental infusions contained galanin-like peptide (GALP). The minipump provided a constant flow of solution to the lateral ventricle 0.5 $\mu\text{l/hr}$, 15nmol/day of GALP or control for 14 days. This dose has been reported previously to restore normal reproductive behavior and physiology and food intake in diabetic rats [9]. Cannulas and minipumps were implanted on PND25 since this was the age that showed the first adult-like levels of GALP mRNA. Following surgery, all rats were weighed and allotted approximately 300g of food daily. Each day, the remaining food within the cage was collected and weighed to calculate the amount of food intake. Another 300 g aliquot of food was then provided. Pubertal signs were monitored in all rats each day (preputial separation in males, vaginal opening in females). Metabolic rates were measured one week following minipump implantation (prior to puberty in both sexes as determined by preputial separation or vaginal opening). After the 14 day period had passed (PND 39) all rats were euthanized with an overdose (400 mg/kg) of Fatal Sure Pentobarbital (concentration of 400 mg/

ml). All rats were decapitated at approximately 0900 hr during the rats light cycle (food and water were available *ad lib* until decapitation), and their trunk blood was collected, centrifuged, and plasma was removed for analysis of GH, LH, FSH, insulin, and leptin by radioimmunoassay. Body fat was collected from intrascapular, perigonadal, and abdominal fat pads and weighed collectively. Total body fat and gonadal weights were compared relative to total body weight. Minipump function was determined by the manufacturers suggestions and as reported previously by this laboratory [9]. The final N for each group was: male control, n = 7; male GALP, n = 9; female control, n = 7; female GALP, n = 10.

Statistical Analyses

Oxygen consumption was measured as ml oxygen consumed/min-kg^{0.75}, and was analyzed by determining the average oxygen consumption on 15 minute cycles that was used for graphing and statistical analyses. Food intake and body weight were measured daily at approximately 0900 hrs throughout the experiment. All data were analyzed by a two-way, or one way repeated measures ANOVA, or a two-way ANOVA as appropriate. A Fisher's PLSD posthoc test was performed where appropriate. A $p < 0.05$ was considered significant and all data were presented as means plus/minus standard error about the mean. Data from daily measures are presented beginning 48 hrs after surgery (PND27), thus allowing for surgical recovery. Due to differences in the day of the onset of puberty between male and females, data were not statistically analyzed between the sexes except for Experiment #1, and the body weight and metabolic measures prior to the onset of puberty in Experiment #2.

Results

Experiment #1: Ontogeny of GALP Gene Expression

No sex differences were observed in the first day that GALP mRNA was seen, the number of cells containing GALP mRNA or the number of silver grains per cell. Thus for presentation (Figs. 1, 2), the sexes were combined. GALP mRNA was not observed in the arcuate nucleus until at least PND10. A significant increase ($p < 0.05$) in GALP gene expression was observed beginning with PND12 and continuing until Puberty. GALP mRNA levels reached their highest levels statistically at weaning (PND25, $p < 0.001$). There were no significant differences in GALP mRNA levels after weaning (PND25). The *ad hoc* analyzed ages also showed no significant differences from GALP gene expression from those at weaning to adult ages (data not shown). Neither insulin or leptin treatment was not able to induce any increase in GALP gene expression denoted by silver grain clusters at PND1, 5 or 10. The data are not illustrated since no observable clusters were analyzed (thus identical to same ages presented in Figs. 1 & 2).

Experiment #2: Effects of GALP Infusion in Prepubertal Rats on Food Intake, Body Weight, Oxygen Consumption and the Onset of Puberty

All female rats, regardless of treatment, showed signs of vaginal opening prior to the end of the experiment (by ~PND38–39). None of the male rats showed preputial separation at the end of the experiment. GALP infusion significantly increased both food intake ($p < 0.01$, Fig. 3) and body weight ($p < 0.05$, Fig. 4) in male, but not female, rats compared to vehicle infusion.

One week following surgery (PND32, prior to the onset of puberty in both sexes) metabolic rate was measured. GALP-treated male rats showed a significant increase ($p < 0.001$) in oxygen consumption compared to control male rats (Fig. 5) suggesting a higher metabolic rate. Female rats showed no significant differences between treatments in oxygen consumption (Fig. 5). At the end of the experiment, no significant differences were observed in relative testes or ovarian weights or in percent body fat in either male or female rats treated with either GALP or vehicle (Table 1).

Hormone Analyses

Female rats showed no significant differences in plasma levels of insulin, leptin, LH, or FSH between GALP and vehicle treatment (Table #2). Female rats treated with GALP did show a significant ($p < 0.05$) increase in plasma GH levels compared to control female rats. Male rats also showed no significant differences in plasma levels of insulin or FSH between treatment groups. There were no significant differences in gonadal steroid levels among rats treated with either control or GALP solution in either sex (Table #2). However, male rats infused with GALP did show significant differences in plasma levels of LH ($p < 0.01$), GH ($p < 0.05$) and leptin ($p < 0.05$), but not insulin or FSH, compared to vehicle-infused males (Table #2).

Discussion

The purpose of these studies was to determine GALP's role in the onset of puberty in male and female rats. A significant increase in GALP mRNA was observed in both sexes at weaning (PND25), but did not achieve adult-like levels until the onset of puberty. Infusion of GALP did not advance the onset of puberty in either sex, despite the significant increase in plasma LH observed in males. Similar to a recent report, a sex difference in the LH response to GALP treatment was observed. Young—post-weaning—female rats were much less responsive to central GALP infusion than were males. In addition, female rat pups appeared to be relatively non-responsive to GALP in regards to any variable measured in this study other than GH secretion. GALP treatment had no effect on male rats in regards to plasma levels of FSH or insulin, however, GALP infusion in male rats did significantly increase food intake, body weight, oxygen consumption—suggesting metabolic rate—and plasma levels of GH, LH, and leptin. These novel findings suggest that GALP is involved in the alterations in energy expenditure associated with puberty, but exogenous GALP may not stimulate the onset of puberty.

The ontogeny of neuropeptide gene expression in the Arcuate nucleus (Arc) is critical for the neuroendocrine control of feeding, metabolism, and reproduction [19–29]. In adult rats, GALP is expressed in the Arc of the hypothalamus and GALP gene expression is regulated by leptin, insulin, and metabolic fuels [6,30,31]. Our study has shown that in neonatal rats, GALP gene expression does not appear until late postnatally (~PND12). Although the Arc is generally considered a sexually dimorphic structure, there is no sex difference in GALP gene expression—not surprising given that GALP gene expression is not regulated by gonadal steroids [32]. In both sexes however, GALP mRNA did not appear until after PND10, a relatively late time point compared to other neuropeptides in the Arc [13,33,34]. There are differences in leptin and insulin effects between neonatal and adult rats [35,36], and sex steroids may play a role in the programming of insulin sensitivity as adults [37]. Metabolic hormones stimulate GALP gene expression in adult rats, therefore the relative insensitivity to leptin in neonates [13] may explain the lack of GALP gene expression prior to PND10. Leptin secretion is also known to increase around the time of weaning [38], which may explain the increase in GALP gene expression we observed at PND25. A lack of leptin and insulin activity prior to weaning is supported by our study in that we showed that these two metabolic hormones could not induce GALP gene expression until after PND10. Alternatively, the time course of GALP appearance in the Arc is similar to that reported by others for hindbrain catecholaminergic innervation of the ventromedial hypothalamus [39,40]. A recent report demonstrated that GALP gene expression is also regulated by catecholaminergic afferents from the hindbrain [30]. It is possible that GALP gene expression is initially triggered by the appearance of hindbrain catecholaminergic neurons in the hypothalamus. However, it is not known if GALP neurons co-express catecholamine receptors.

GALP is a large peptide (60 amino acids) and thirteen of GALP's amino acid residues (9–21) are identical to the N-terminal portion (1–13) of galanin. GALP interacts with the galanin

receptor subtypes Gal-R1 and Gal-R2 *in vitro*, with a higher affinity for Gal-R2 ($IC_{50}=0.24$ nM) than for Gal-R1 ($IC_{50}=4.3$ nM) [41]. However, a study by Krasnow *et al* [42] demonstrated that exogenous GALP's effects were not dependant upon the *in vivo* presence of either Gal-R1, Gal-R2 or the combination of the two receptors. There is also evidence to suggest the existence of a specific GALP receptor; however, this putative receptor has yet to be identified. There is currently no evidence regarding GALP's interactions with the third galanin receptor, Gal-R3. However, the distribution of the Gal-R3 receptor [43] in the rat brain does overlap with some of the putative functions for GALP in regulating reproduction, feeding, and other homeostatic processes associated with the onset of puberty.

The timing of the onset of puberty is somewhat variable and is tightly coupled to the individual's nutritional and metabolic state [3,44–46]. It has long been known that a relationship exists between body weight and the timing of sexual development (for reviews see [47,48]). Nevertheless, the mechanisms for the regulation of the timing of puberty are still unknown. Leptin is a protein secreted as a hormone from adipocytes that plays an important role in the regulation of body weight and metabolism. It may seem paradoxical that increased leptin secretion at puberty is coincident with an increase in food intake at that time (for example as was observed in the male rats in *Experiment #2*). However, as puberty approaches, increased leptin levels signal the brain that sufficient energy stores are available to meet the caloric demands for reproduction to occur. This results in an increase in feeding and energy expenditure (metabolic rate) and allows for the initial GnRH surge denoting the onset of puberty [2,3,46,49–51]. Mutant *ob/ob* mice are unable to produce leptin and show extreme levels of obesity and abnormal sexual maturation. Treatment with leptin reverses their obesity, and allows for normal sexual function to ensue [52–54]. Central GALP treatment in *ob/ob* mice has also been shown to increase body temperature and autonomic activity, both of which indicate an increase in metabolic rate [55]. GALP infusion has also restores sexual function in diabetic male rats, and increases metabolic rate and sexual behavior in normal adult male rats [9]. These data suggest that GALP is an important mediator between adipocyte signaling to the brain and the brain's regulation of reproductive status. Similar to previous studies with leptin, we have demonstrated that GALP administration was of itself not able to advance the onset of puberty. However, GALP treatment did elicit many of the physiologic changes associated with the onset of puberty, such as an increase in feeding, metabolic rate, and LH & GH secretion. The lack of advancement of puberty could be due to GALP's inability to increase FSH secretion. The fact that GALP did not elicit many of these changes in the female rat pups is quite fascinating and emphasizes the potentially different neuroendocrine mechanisms between male and female mammals that signal the onset of puberty—possibly indicative of the sex differences associated with eating or metabolic disorders associated with adolescence.

Growth hormone secretion increases around the time of the onset of puberty [14,22], and this leads to increased food intake and lean body mass (particularly in males); and, the increased GH secretion may also be related to increased levels of circulating leptin associated with onset of puberty [2,14]. GALP is known to have bimodal effects on food intake in the rat. Initially, ICV GALP stimulates food intake [56–59] but reduces food intake and body weight over 24 hours [5,6,9,60]. The mechanism of GALP's bimodal action on food intake is not clear, although actions within the preoptic area may stimulate food intake [56] while the hindbrain may mediate GALP's anorexic actions [60]. The increased food intake in response to GALP infusion in prepubertal male rats may be due to increased central NPY secretion that is also associated with increased plasma levels of GH [61–64]. It is interesting that both male and female pups infused with GALP showed significantly increased levels of leptin and GH, but males only showed increases in body weight and food intake. This may reflect differential sensitivity between the sexes to GALP administration as reported by others [65]. Alternatively, female rats may have required more time to increase subcutaneous adiposity relative to controls than allowed for by our experimental design. It would be interesting to investigate the long-

term effects of prepubertal GALP administration on adiposity levels and feeding as adults. The sex difference in the effect of GALP on prepubertal growth and energy homeostasis is clearly interesting and worthy of further study.

Insulin, like leptin, is a hormone involved in the regulation of bodyweight, glucose homeostasis, and is also a necessary signal to support normal reproductive function and energy homeostasis [66–75]. It has been shown that animals with genetically abnormal insulin levels are reproductively dysfunctional (for review see [76]). Insulin, as well as leptin, also regulates the expression of GALP in the hypothalamus and evidence supports that insulin also regulates GALP gene expression and that central administration of GALP corrects reproductive dysfunction associated with diabetes in the male rat [6,9]. Our study also demonstrates that GALP infusion significantly increased plasma leptin levels in prepubertal male rats. Previous reports have demonstrated that ICV GALP injections in *ob/ob* mice increase autonomic activity and UCP-1 expression in brown adipose tissue—suggesting an increase in metabolic activity [55]. Though plasma leptin levels were not analyzed in that experiment, the increased autonomic activity in the adipose tissue could have led to an increase in leptin secretion. This opinion is bolstered by a recent study from our laboratory that demonstrated that acute GALP injections increases plasma leptin levels in adult male rats [77].

In summary, this study supports a study from another lab that demonstrated a sex difference in the neonatal responsiveness to GALP. In male rats, GALP not only stimulates LH secretion, but also metabolic rate—as measured by oxygen consumption—and GH and leptin secretion. Furthermore, male but not female rats show an increase in food intake and lean body mass. These exciting findings may suggest possible neuroendocrine mechanisms responsible for the sex differences in the onset of metabolic diseases, such as anorexia nervosa, in developing animals.

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Fig. 1.

Postnatal ontogeny of GALP gene expression. The presence of hypothalamic GALP mRNA was determined via *in situ* hybridization. GALP gene expression was not observed until after PND10. Analysis by two way ANOVA (sex $\times$ age) shows a significant ($p < 0.001$) increase in GALP gene expression over time. No sex difference was observed in GALP gene expression at any postnatal age. * = $p < 0.05$, *** = $p < 0.001$.

Fig. 2.

Photomicrographs of representative GALP ISH. The right column (bar = 40 μ m) is a higher power image of the panels in the left column (bar = 100 μ m). All photomicrographs are from similar coronal sections of the Arc at the level of the median eminence and ventromedial hypothalamic nucleus. 3v = third ventricle, arrows indicate silver grain clusters denoting GALP-expressing cells.

Fig. 3. Central GALP infusion significantly increased food intake in male (A) but not female (B) prepubescent rats compared to control infusion. Asterisk indicates statistical significance, $p < 0.01$.

Fig. 4. Central GALP infusion significantly increased body weight in male (A) but not female prepubescent rats compared to control infusions. By the end of the infusion period, GALP-treated male rats weighed significantly ($p < 0.05$) more than all other groups. Error bars are presented but are too small to resolve. Asterisk indicates $p < 0.05$.

Fig. 5.

Rats were habituated to the metabolic chamber for 30 minutes, then oxygen consumption measured for one hour. Central GALP infusion significantly increase male (A) but not female (B) oxygen consumption suggesting an increase in metabolic rate. Asterisks indicate statistical significance, $p < 0.001$.

Table 1

Anatomical Data Following GALP treatment, Experiment #2

Sex	Treatment	Final BW (grams)	% Body Fat	% Gonadal Wt
Male	Control	135.0 $\pm$ 2.78	0.8 $\pm$ 0.01	0.9 $\pm$ 0.06
	GALP	148.8 $\pm$ 5.44 *	0.8 $\pm$ 0.02	1.0 $\pm$ 0.04
Female	Control	123.5 $\pm$ 4.31	0.7 $\pm$ 0.05	0.06 $\pm$ 0.006
	GALP	122.4 $\pm$ 2.63	0.8 $\pm$ 0.04	0.07 $\pm$ 0.005

* Statistically different compared to control of same sex, $p < 0.05$.

Table 2
Hormonal Data from GALP Infusion in Prepubertal Rat Pups.

Treatment	Sex	Insulin (ng/ml Plasma)	Leptin (ng/ml Plasma)	LH (ng/ml Plasma)	FSH (ng/ml Plasma)	GS ^I (ng/ml Plasma/	GH (ng/ml Plasma)
Control	Male	85.7 ± 27.48	82.5 ± 28.67	0.6 ± 0.04	10.6 ± 1.69	0.2 ± 0.06	11.1 ± 4.44
GALP		110.3 ± 23.43	140.6 ± 14.15 [*]	1.3 ± 0.19 [*]	12.3 ± 0.95	0.3 ± 0.07	39.2 ± 8.57 [*]
Control	Female	105.4 ± 26.18	101.2 ± 17.18	0.7 ± 0.08	4.4 ± 0.18	9.4 ± 1.88	9.1 ± 3.85
GALP		167.4 ± 40.20	192.8 ± 40.25 [*]	0.7 ± 0.12	4.1 ± 0.18	9.5 ± 1.38	27.2 ± 5.04 [*]

Data are expressed as mean ± standard error of the mean.

^{*} Statistically significant compared to control injection within the same sex. p < 0.05

^I GS = gonadal steroids, male = testosterone, female = estrogen