Published in final edited form as:

Nutrition. 2007 September ; 23(9): 665–671.

Chronic Consumption of Low-Fat Diet Leads to Increased Hypothalamic Agouti-Related Protein and Reduced Leptin

Jaroslav Staszkievicz, Ph.D.^{1,2,*}, Ronald Horswell, Ph.D.¹, and George Argyropoulos, Ph.D.¹

¹Pennington Biomedical Research Center, LSU System, 6400 Perkins Road, Baton Rouge, LA, 70808, USA

²Department of Animal Physiology, University of Warmia and Mazury in Olsztyn, ul. Oczapowskiego 1A, 10-719 Olsztyn, Poland

Abstract

Objective—The study was performed to examine the hypothesis that dietary fat under *ad libitum* feeding conditions influences the expression levels (mRNA) of the mouse AgRP, leptin, leptin receptor (OBRb), and NPY at early stages of development.

Research Methods & Procedures—C57Bl/6J male mice were placed on either High Fat Diet (HFD) or Low Fat Diet (LFD) shortly after weaning. Groups of mice were euthanized at various ages and real-time one-step RT-PCR was used to analyze the gene expression in the hypothalamus (AgRP, NPY, OBRb), the adrenal gland (AgRP), the testis (AgRP), and epididymal fat (leptin)

Results and Conclusions—Leptin expression increased linearly with age but only under the HFD despite body-weight gain under both diets. This pattern of expression coincided with reduced expression of hypothalamic AgRP under HFD, while OBRb and NPY did not fluctuate in response to diet. By contrast, consumption of LFD (i.e. high carbohydrate) increased hypothalamic AgRP and suppressed adipose leptin which is consistent with the notion that leptin could regulate AgRP centrally. In contrast, AgRP expression in the adrenal gland initially decreased and then increased with age, under both diets, suggesting that dietary fat could have a tissue-dependent effect on AgRP that may be unfettered by leptin under the HFD.

Keywords

AgRP; leptin; hypothalamus; fat; food; chronic; appetite

Introduction

The orexigenic peptides Agouti-Related Protein (AgRP) and Neuropeptide Y (NPY) are co-expressed in neurons of the ARC [1,2]. NPY and AgRP neurons also contain leptin-receptor

* Correspondence to: Jaroslav Staszkievicz, Ph.D., Department of Animal Physiology, ul. Oczapowskiego 1A, 10-719 Olsztyn, Poland, Tel: (+48 89) 523 42 26, Fax: (+48 89) 523 39 37, E-mail: jarex@uwm.edu.pl.

role of authors:

J. Staszkievicz participated in the conceptualization and design of the study and performed all the experimental procedures

R. Horswell performed all statistical analyses

G. Argyropoulos co-designed the project and supervised all experimental approaches.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

mRNA, and evidence points to their co-regulation by leptin [3,4]. AgRP knockout mice do not differ in food intake behavior from wild type mice [5] but exhibit an age-related lean phenotype [6]. Several recent studies have demonstrated that AgRP/NPY neurons in the hypothalamus are essential for controlling energy homeostasis [7,8] but the exact functions of AgRP remain under scrutiny [9]. AgRP can regulate feeding behavior when overexpressed [10,11] or perhaps in peripheral organs. Indeed, AgRP is also expressed widely in peripheral organs such as the adrenal gland, testis and epididymal fat and is regulated by fasting in a similar fashion that it is in the brain [12]. Moreover, peripherally-administered AgRP is selectively taken up by various tissues but fails to effectively cross the blood brain barrier [13].

Leptin is essentially produced by adipocytes [14] and the hypothalamus is a major site of its action [15]. Both, AgRP and NPY are downregulated by leptin [16-18]. The action of leptin has been proposed to be mediated by its receptor in the brain (OBRb) which binds directly to the signal transducer and activator of transcription 3 (STAT3) [19]. A recent study showed that the circadian expression of leptin and its hypothalamic receptor could play a prevalent role in the regulation of nocturnal eating in mice [20].

Consumption of high-fat diets for a prolonged period results in positive energy balance and the development of obesity in certain strains of mice. Detailed information on the response of leptin and leptin-regulated neuropeptides to high fat feeding is still limited. Studies undertaken previously focused only on hypothalamic mRNA expression and that mostly after short-term feeding conditions [21,22]. The ontogeny of the AgRP/NPY system has been studied in rodents at young ages and shows that maturation and formation of mechanisms controlling appetite occurs mainly during the first three postnatal weeks [23,24].

To gain further insight into the physiological response of normal mice to prolonged usage of LFD and HFD from young age, we performed a longitudinal study to examine the influence of these diets on AgRP mRNA levels in the hypothalamus and peripheral tissues (adrenal gland and the testis), NPY mRNA in the hypothalamus, leptin receptor mRNA in the hypothalamus, and leptin mRNA in epididymal fat. Expression was thus measured in 2-, 3.4-, 6-, 12- and 16-week-old mice fed a LFD or HFD.

Methods

Animals

C57Bl/6J male mice were used in the study. Mice were bred at the Pennington Biomedical Research Center (PBRC). Experiments involving animals followed the guidelines approved by the PBRC Institutional Animal Care and Use Committee. All procedures were designed to minimize suffering of experimental animals. Pregnant mice were housed in a temperature and humidity controlled room ($22 \pm 2^\circ\text{C}$, 30-70%, respectively) with a 12:12-h light/dark cycle (lights on at 06.00 AM) and were given ad libitum access to prescribed diets and tap water throughout the study. Mice were checked daily for the presence of pups; the day of delivery was considered day 0. Mice were sacrificed by cervical dislocation.

Diets and experimental design

Male mice were grouped into several categories. Gene expression was measured initially in 5 mice at 2 weeks of age. Immediately after weaning (3 weeks of age) all the mice were placed on regular chow (Labdiet 5001; Purina Mills, St. Louis, MO) and at 3.4 weeks of age a group of 5 mice was sacrificed to take the first post-weaning measurement of gene expression. The remaining mice were assigned randomly to low-fat diet (LFD) or high-fat diet (HFD) (D12450B: 10 kcal% fat at 3.8 kcal/gm, and D12492: 60 kcal% fat at 5.2 kcal/gm, Research Diets, Inc., New Brunswick, NJ). At 6 weeks of age, 5 mice from each dietary regiment were

sacrificed. In other words, a total of 10 mice (5 from LFD and 5 from HFD) were sacrificed. This process was repeated at 12 and then at 16 weeks of age, each time using different groups of 5 mice per diet. Mice were sacrificed by cervical dislocation. The total carcass was weighed, and adipose tissue (epididymal), testis, adrenal and hypothalamus were removed quickly and snap-frozen by immersion into liquid nitrogen. Tissues were stored frozen at -80°C until being processed for RNA isolation.

RNA isolation and real-time RT-PCR (qPCR)

Total RNA was isolated using the trizol method (Invitrogen, Carlsbad, CA) and purified with RNeasy columns according to the manufacturer's instruction (QIAGEN, Valencia, CA). The TaqMan one-step RT-PCR core reagents kit (Applied Biosystems, CA) was used with cyclophilin B as endogenous control. RT-PCR was performed in MicroAmp Optic 384-well Reaction Plates (Applied Biosystems) on an ABI PRISM 7700 Sequence Detection system (Applied Biosystems), with the condition of 30 min at 48°C , 10 min at 95°C , then 40 cycles of 15 s at 95°C and 1 min at 60°C . qPCR was performed in duplicate for each sample, and each run included a standard curve, non-template control and negative RT control. The levels of AgRP, NPY, leptin and leptin receptor gene expression were quantified relative to the level of cyclophilin B using a standard curve method. The following primers were used to amplify mouse mRNAs: mAgRP – forward: 5'-GCT CCA CTG AAG GGC ATC A-3', reverse: 5'-GTG GAT CTA GCA CCT CCG C-3' and probe: 5'-6-FAM TTC CCA GGT CTA AGT C MGBNFQ-3'; mNPY – forward: 5'-CTC CGC TCT GCG ACA CTA CA-3', reverse: 5'-AAT CAG TGT CTC AGG GCT GGA-3', and probe: 5'-6-FAM d(CAA TCT CAT CAC CAG ACA GAG ATA TGG CAA GAT) BHQ-1-3'; leptin – forward: 5'-ATT TCA CAC ACG CAG TCG GTA T-3', reverse: 5'-AAG CCC AGG AAT GAA GTC CA-3', and probe: 5'-6-FAM GCC AGT GAC CCT CTG CTT GGC G) BHQ-1-3'; leptin receptor – forward: 5'-TGT TCC TGG GCA CAA GGA CT-3', reverse: 5'-TGA TTC TGC GTG CTT GGT AAA-3', and probe: 5'-6-FAM d(AAT TTC CAA AAG CCT GAA ACA TTT GAG CAT CTT A) BHQ-1-3'; and cyclophilin B – forward: 5'-TAG AGG GCA TGG ATG TGG TAC-3', reverse: 5'-GCC GGA GTC GAC AAT GAT G-3', and probe: 5'-6-FAM d(AGC CGG GAC AAG CCA CTG AAG GAT) BHQ-1-3'. One hundred nanograms of total RNA was used for qPCR. mRNA expression is presented throughout as arbitrary units, which is the standard form of presenting mRNA expression measured by qPCR.

Statistical analyses

For each outcome, an overall two-way ANOVA test was conducted for any effect of time or diet. In addition, a test was conducted for a difference (aggregated over 6, 12, and 16 weeks) between the LFD and HFD diets. Also, tests were conducted for changes over age within each diet (LFD and HFD) separately. Each of the above-described tests was conducted using $\alpha = 0.05$. Differences between treatment groups and differences over time were assessed using a two-way ANOVA model, with the two fixed factors in the model being (1) age (four levels = 3.4, 6, 12, and 16 weeks) and diet (three levels = chow, LFD, and HFD.) These two-way models are not completely crossed; in particular, the “chow” level of the diet factor appears with only one level of the age factor (i.e., 3.4 weeks), while the LFD and HFD levels of the diet factor each appear with only three levels of the age factor (6, 12, and 16 weeks.) For some outcome variables, ANOVA models were estimated after log transformations, so as to improve normality and reduce heteroskedasticity.

Additional statistical testing examined difference between LFD and HFD groups at each time point (i.e., at 6, 12, and 16 weeks) and differences between time points within the LFD and HFD groups. These additional pairwise comparisons were done using Tukey's honestly significant difference (HSD) approach to control for multiple a posteriori tests. For these tests, test size was controlled at $\alpha = 0.05$ for each of three subsets of comparisons: (1) the LFD versus

HFD comparisons at 6, 12, and 16 weeks, (2) the comparisons among age levels within the LFD group, and (3) the comparisons among age levels within the HFD group.

Correlations analyses were also performed to assess the associations between AgRP mRNA and Weight, between AgRP mRNA and Age, and between AgRP mRNA and epididymal fat leptin mRNA. These correlations were adjusted for group differences by pooling the correlations of the two diet groups (i.e. LFD & HFD).

Results

The purpose of these experiments was to test the hypothesis that dietary fat influences the central and peripheral expression (mRNA) levels of mouse AgRP, leptin, leptin receptor (OBRb), and NPY at early stages of development. Gene expression was measured initially in 5 mice at 2 weeks of age. Immediately after weaning (3 weeks of age) mice were placed on regular chow and at 3.4 weeks of age a group of 5 mice was sacrificed to take the first post-weaning measurement of gene expression. The remaining mice were placed on HFD or LFD. Subsequently, separate groups of 5 mice per diet were sacrificed at 6, 12, and 16-weeks of age and gene expression was measured in the hypothalamus (AgRP, NPY, OBRb), the adrenal gland (AgRP), the testis (AgRP), and epididymal fat (leptin). Body weight was also monitored during these time points. We wish to point out that the effect of the chow diet that was used between 3 and 3.4 weeks of age (i.e. a total of 3 days) on the remaining mice was not evaluated in this study.

The left-hand-side column in Table 1 contains results from the overall two-way ANOVA tests. These results show significant effects for all outcomes except OBRb. The next two columns show the results from testing for age effects within the LFD and HFD individually. With the exception of OBRb, outcomes show significant differences across age groups within each diet. Column D shows results for the comparison of LF to HF diets (aggregate comparisons combining weeks 6, 12, and 16.) Only hypothalamic AgRP, testis AgRP, and epididymal fat leptin showed significant overall diet effects, although the diet effect for adrenal AgRP ($p = 0.057$) approached statistical significance.

Figure 1 shows the effects of age and diet on body weight. Body weight increased under both diets and as the mice got older, but HFD had a more severe effect (Figure 1). In the hypothalamus, AgRP expression decreased between ages 3.4 and 6 weeks and then significantly increased only under the LFD at 16-weeks of age ($p < 0.05$) (Figure 2A). In contrast, the adrenal gland and the testis (Figure 2B&C) AgRP mRNA was affected significantly by the HFD at 12-weeks of age and to a lesser extent by the LFD ($p < 0.05$).

Leptin mRNA in epididymal fat was significantly affected mostly by the HFD (Figure 3A), starting at 6-weeks of age ($p < 0.01$). Under LFD, leptin mRNA increased marginally at 6 weeks and leveled off at 12 weeks of age without increasing further by 16 weeks of age.

Hypothalamic leptin receptor (Figure 3B) was not affected dramatically by either diet between 2 and 12 weeks of age but it increased significantly between 12 and 16 weeks of age under both diets. Hypothalamic NPY mRNA (Figure 3C) was not affected by diet. Under LFD NPY expression increased at the age of 6 weeks and was significantly different if compared to 3.4- and 16-week-old mice.

Correlation analyses were also performed to assess the association between Weight and AgRP and between Age and AgRP. These correlations were calculated by pooling the correlations of the two diet groups (i.e. LFD & HFD). Essentially, adrenal AgRP correlated strongly with both Weight ($r = 0.75$, $p < 0.001$) and Age ($r = 0.79$, $p < 0.001$). Hypothalamic AgRP correlated somewhat less strongly with Weight ($r = 0.37$, $p = 0.047$) and Age ($r = 0.43$, $p = 0.017$).

Correlations were weaker and not statistically significant between testis AgRP and both Age and Weight. The correlation between adrenal AgRP and leptin was also statistically significant, ($r = 0.59$, $p = 0.001$), while leptin mRNA was not statistically correlated with either hypothalamic AgRP ($r = 0.15$, $p = 0.437$) or testis AgRP ($r = 0.20$, $p = 0.286$). Hypothalamic NPY was negatively correlated with Weight ($r = -0.48$, $p = 0.008$) and Age ($r = -0.56$, $p = 0.001$), but did not correlate significantly with leptin ($r = -0.34$, $p = 0.068$). Hypothalamic OBRb showed no clear correlation with Weight ($r = 0.05$, $p = 0.085$), Age ($r = 0.09$, $p = 0.630$), or leptin ($r = -0.01$, $p = 0.940$).

Discussion

The present study investigated the effects of chronic consumption of LFD and HFD on the expression levels of AgRP, NPY, leptin, and its receptor in the hypothalamus and peripheral tissues. We wish to point out that the same 5 mice were used to measure gene expression at 2 weeks of age (i.e. during weaning) and another set of 5 mice at 3.4 weeks of age (representing the first post-weaning measurement). Standard chow diet was used between 3 and 3.4 weeks of age (i.e. 3 days) to acclimate the mice to ad-libitum feeding but its effects on gene expression were not evaluated in this study. We considered that the effect of the chow diet was similar on all the mice during those three days and that is why the first post-weaning measurement was taken at 3.4 weeks of age which represents the on-set of the dietary fat content intervention.

Body weight increased under both diets, as expected, since the mice were in the growing phase during this period. In previous studies, one-week of highly saturated fat diet resulted in 20% reduction of AgRP [25], whereas, two weeks on a high energy (HE) diet did not affect mRNA levels of hypothalamic NPY, AgRP, POMC, and CART of mature mice [26]. The latter finding is similar to our findings for hypothalamic AgRP and NPY under HFD during the first two weeks of feeding. Others have shown that diet-induced obese mice had significantly lower arcuate nucleus AgRP mRNA in mice [27] and in rats [28]. This is similar to our findings whereby HFD reduced hypothalamic AgRP between 3.4 and 6 weeks of age and remained at low levels between 6 and 16 weeks of age. LFD (i.e. high CHO diet) on the other hand, initially decreased expression of hypothalamic AgRP at 6 weeks of age and then increased its expression levels becoming significant at 16 weeks of age. Others have reported that injection of 5% glucose caused an initial reduction of AgRP mRNA at 30 and 60 min post-injection which was followed by a 50% increase at 90 minutes [29]. This pattern of effect by glucose is similar to the *ad libitum* feeding of high CHO (LFD) diet that we report here between the ages of 3.4 and 16 weeks.

A recent study showed that AgRP, NPY, and the leptin receptor are expressed in the mouse brain from fetal age 12 and feeding pregnant dams a protein-restricted diet resulted in alterations of expression of AgRP, NPY, and the leptin receptor in 12-day-old fetuses [30]. It would appear that AgRP expression is tightly regulated from embryonic ages either as a direct effect of dietary fat and/or effects by leptin. Both LFD and HFD in the adrenal gland led to increased AgRP expression between 6 and 16 weeks of age. In the testis the pattern was similar but weakened especially at 16 weeks of age which did not differ significantly from the effects of both diets at 6 weeks of age. This result suggests that adrenal AgRP may be regulated by different mechanisms than hypothalamic AgRP while testis AgRP may not change dramatically as a result of age or dietary fat content. Given that leptin increased at 16 weeks of age under HFD and remained low under LFD, whereas, hypothalamic AgRP displayed an exactly opposite pattern of expression, and assuming that leptin directly regulates hypothalamic AgRP, it is possible that such an effect may be taking place irrespective of the content of fat in the diet or body weight. In the adrenal gland, however, leptin may be influencing AgRP expression only under LFD. This raises the possibility that under HFD it is adrenal (and not hypothalamic) AgRP that could escape leptin regulation. This hypothesis would require extensive

experimentation to be confirmed. What appear to be certain is that hypothalamic and adrenal AgRP expression may be under the influence of differential regulatory mechanisms.

The potential role of AgRP in the periphery (if any) is still under investigation but it was recently shown that peripherally administered AgRP is taken up by various organs (mostly the liver, adrenal, and adipose) and this process is further modulated by fasting [13]. Both AgRP and Agouti have direct effects on adipocytes, influencing the expression of fatty acid synthase and leptin [31-33], and blocking α -MSH dependant effects on leptin gene expression [34]. AgRP also binds to MC3R in the adrenal gland [35,36], and adrenal-derived AgRP has paracrine functions, blocking the induction of corticosterone secretion by α -MSH [37,38]. In a recent study, overexpression of AgRP into leg muscle lead to a significant increase of food intake and body weight that lasted for three weeks [39], which presents a clear case of the effects of AgRP on energy balance via a peripheral action. Importantly, it was recently shown that AgRP acts as an inverse agonist in 293 HEK liver cells by inducing arrestin-mediated endocytosis of MC3R and MC4R [40].

The leptin receptor in the hypothalamus was not affected by age or diet up to 12 weeks of age but then increased significantly between at 16 weeks of age only under HFD. This increase of OBRb under HFD also coincided with the increase of leptin at the same age which is suggestive of a tightly linked regulation of OBRb and leptin expression levels under LFD. Similar to OBRb, NPY mRNA in neuronal cells was not affected dramatically by age or diet. NPY neuronal expression under LFD was in fact different from that of AgRP, suggesting that the two genes may be differentially regulated. The concept of the so-called “co-expressing AgRP/ NPY neurons” might need to be used with caution as the two genes do not seem to be co-regulated by dietary fat and NPY expression does not seem to follow a pattern indicating a regulation by leptin (in contrast to that of AgRP). This notion is further supported by data showing that after a stressful event, AgRP mRNA levels go down while those of NPY go up [41].

Food consumption was also measured (pellet weighing) in two separate groups of mice, involving six mice under HFD and six mice under LFD. Mice under LFD ate higher quantities of food but the caloric intake did not differ between groups (data not shown). This is similar to previous reports for C57BL/6J mice that, although eating more of a chow diet than mice on condensed milk, caloric intake was identical between the two diets [42]. Body weight increased linearly with age, under both diets, but more so under the high fat diet, as expected [43]. A recent study in humans reported that consumption of LFD did not significantly reduce the risk for cardiovascular disease, stroke, and coronary heart disease [44]. In the present study, chronic consumption of LFD resulted in reduction of leptin and an increase of AgRP, which could potentially lead to continuously elevated appetite, thus, offering no significant advantage against the development of cardiovascular disease.

In correlation analyses we found that adrenal AgRP correlated strongly with both Weight and Age while hypothalamic AgRP correlated somewhat less strongly with these two parameters. It should be noted that Weight and Age are also highly correlated during mouse developmental stages ($r = 0.92$, $p < 0.001$), which could explain the similar correlation coefficients between AgRP and Weight and AgRP and Age. Testis AgRP and hypothalamic OBRb were not significantly correlated with Weight, Age, or leptin, while hypothalamic NPY was negatively correlated with Weight, Age, but not leptin. Adrenal AgRP, on the other hand, was significantly correlated with leptin. Overall, these correlations suggest that adrenal and hypothalamic AgRP may be tightly regulated by developmental changes in body weight and fat content in the diet, while, leptin itself could further modulate AgRP under both dietary conditions.

In conclusion, dietary fat regulates leptin and AgRP expression in a more robust fashion than it regulates hypothalamic OBRb and NPY. Moreover, there were significant differences between the expression patterns of central and peripheral (adrenal & testis) AgRP by dietary fat suggesting that central AgRP may be under different regulatory mechanisms or that the same mechanisms may be having differential effects depending on the cellular environment (i.e. co-expressing signaling molecules and transcription factors). These findings point to the existence of intricate effects by dietary fat on the regulation of central and peripheral AgRP which may be further complicated by leptin's effects.

Acknowledgements

This study was supported by NIH grant number DK62156 to GA. Dr. Jaroslaw Staszkiwicz was supported by the Polish-U.S. Fulbright Commission, Warsaw, Poland (Grant #: PPLS/04/14). We thank Ms. Tammy Fairburn for excellent technical assistance.

References

1. Broberger C, Johansen J, Johansson C, Schalling M, Hokfelt T. The neuropeptide Y/agouti gene-related protein (AGRP) brain circuitry in normal, anorectic, and monosodium glutamate-treated mice. *Proc Natl Acad Sci U S A* 1998;95:15043–15048. [PubMed: 9844012]
2. Hahn TM, Breininger JF, Baskin DG, Schwartz MW. Coexpression of Agrp and NPY in fasting-activated hypothalamic neurons. *Nat Neurosci* 1998;1:271–272. [PubMed: 10195157]
3. Baskin DG, Hahn TM, Schwartz MW. Leptin sensitive neurons in the hypothalamus. *Horm Metab Res* 1999;31:345–350. [PubMed: 10422733]
4. Tritos NA, Vicent D, Gillette J, Ludwig DS, Flier ES, Maratos-Flier E. Functional interactions between melanin-concentrating hormone, neuropeptide Y, and anorectic neuropeptides in the rat hypothalamus. *Diabetes* 1998;47:1687–1692. [PubMed: 9792536]
5. Qian S, Chen H, Weingarth D, Trumbauer ME, Novi DE, Guan X, et al. Neither agouti-related protein nor neuropeptide Y is critically required for the regulation of energy homeostasis in mice. *Mol Cell Biol* 2002;22:5027–5035. [PubMed: 12077332]
6. Wortley KE, Anderson KD, Yasenchak J, Murphy A, Valenzuela D, Diano S, et al. Agouti-related protein-deficient mice display an age-related lean phenotype. *Cell Metab* 2005;2:421–427. [PubMed: 16330327]
7. Bewick GA, Gardiner JV, Dhillon WS, Kent AS, White NE, Webster Z, et al. Post-embryonic ablation of AgRP neurons in mice leads to a lean, hypophagic phenotype. *FASEB J* 2005;19:1680–1682. [PubMed: 16099943]
8. Luquet S, Perez FA, Hnasko TS, Palmiter RD. NPY/AgRP neurons are essential for feeding in adult mice but can be ablated in neonates. *Science* 2005;310:683–685. [PubMed: 16254186]
9. Flier JS. AgRP in energy balance: Will the real AgRP please stand up? *Cell Metab* 2006;3:83–85. [PubMed: 16459309]
10. Graham M, Shutter JR, Sarmiento U, Sarosi I, Stark KL. Overexpression of AgRP leads to obesity in transgenic mice. *Nat Genet* 1997;17:273–274. [PubMed: 9354787]
11. Ollmann MM, Wilson BD, Yang YK, Kerns JA, Chen Y, Gantz I, et al. Antagonism of central melanocortin receptors in vitro and in vivo by agouti-related protein. *Science* 1997;278:135–138. [PubMed: 9311920]
12. Charbonneau C, Bai F, Richards BS, Argyropoulos G. Central and peripheral interactions between the agouti-related protein and leptin. *Biochem Biophys Res Commun* 2004;319:518–524. [PubMed: 15178437]
13. Pan W, Kastin AJ, Yu Y, Cain CM, Fairburn T, Stutz AM, et al. Selective tissue uptake of agouti-related protein(82-131) and its modulation by fasting. *Endocrinology* 2005;146:5533–5539. [PubMed: 16141394]
14. Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM. Positional cloning of the mouse obese gene and its human homologue. *Nature* 1994;372:425–432. [PubMed: 7984236]
15. Elmquist JK, Maratos-Flier E, Saper CB, Flier JS. Unraveling the central nervous system pathways underlying responses to leptin. *Nat Neurosci* 1998;1:445–450. [PubMed: 10196541]

16. Cone RD, Cowley MA, Butler AA, Fan W, Marks DL, Low MJ. The arcuate nucleus as a conduit for diverse signals relevant to energy homeostasis. *Int J Obes Relat Metab Disord* 2001;25(Suppl 5):S63–67. [PubMed: 11840218]
17. Schwartz MW, Baskin DG, Bukowski TR, Kuijper JL, Foster D, Lasser G, et al. Specificity of leptin action on elevated blood glucose levels and hypothalamic neuropeptide Y gene expression in ob/ob mice. *Diabetes* 1996;45:531–535. [PubMed: 8603777]
18. Wilson BD, Bagnol D, Kaelin CB, Ollmann MM, Gantz I, Watson SJ, et al. Physiological and anatomical circuitry between Agouti-related protein and leptin signaling. *Endocrinology* 1999;140:2387–2397. [PubMed: 10218993]
19. Myers MG Jr. Leptin receptor signaling and the regulation of mammalian physiology. *Recent Prog Horm Res* 2004;59:287–304. [PubMed: 14749507]
20. Stutz AM, Staszkiwicz J, Ptitsyn A, Argyropoulos G. Circadian expression of genes regulating food intake. *Obesity (Silver Spring)* 2007;15:607–615. [PubMed: 17372310]
21. Beck B. Neuropeptides and obesity. *Nutrition* 2000;16:916–923. [PubMed: 11054597]
22. Wilding JP, Gilbey SG, Mannan M, Aslam N, Ghatei MA, Bloom SR. Increased neuropeptide Y content in individual hypothalamic nuclei, but not neuropeptide Y mRNA, in diet-induced obesity in rats. *J Endocrinol* 1992;132:299–304. [PubMed: 1541928]
23. Grove KL, Allen S, Grayson BE, Smith MS. Postnatal development of the hypothalamic neuropeptide Y system. *Neuroscience* 2003;116:393–406. [PubMed: 12559095]
24. Nilsson I, Johansen JE, Schalling M, Hokfelt T, Fetisov SO. Maturation of the hypothalamic arcuate agouti-related protein system during postnatal development in the mouse. *Brain Res Dev Brain Res* 2005;155:147–154.
25. Wang H, Storlien LH, Huang XF. Effects of dietary fat types on body fatness, leptin, and ARC leptin receptor, NPY, and AgRP mRNA expression. *Am J Physiol Endocrinol Metab* 2002;282:E1352–1359. [PubMed: 12006366]
26. Heijboer AC, Voshol PJ, Donga E, van Eden CG, Havekes LM, Romijn JA, et al. High fat diet induced hepatic insulin resistance is not related to changes in hypothalamic mRNA expression of NPY, AgRP, POMC and CART in mice. *Peptides* 2005;26:2554–2558. [PubMed: 15979206]
27. Huang XF, Han M, South T, Storlien L. Altered levels of POMC, AgRP and MC4-R mRNA expression in the hypothalamus and other parts of the limbic system of mice prone or resistant to chronic high-energy diet-induced obesity. *Brain Res* 2003;992:9–19. [PubMed: 14604768]
28. Leibowitz SF, Sepiashvili K, Akabayashi A, Karatayev O, Davydova Z, Alexander JT, et al. Function of neuropeptide Y and agouti-related protein at weaning: relation to corticosterone, dietary carbohydrate and body weight. *Brain Res* 2005;1036:180–191. [PubMed: 15725416]
29. Chang GQ, Karatayev O, Davydova Z, Wortley K, Leibowitz SF. Glucose injection reduces neuropeptide Y and agouti-related protein expression in the arcuate nucleus: a possible physiological role in eating behavior. *Brain Res Mol Brain Res* 2005;135:69–80. [PubMed: 15857670]
30. Terroni PL, Anthony FW, Hanson MA, Cagampang FR. Expression of agouti-related peptide, neuropeptide Y, pro-opiomelanocortin and the leptin receptor isoforms in fetal mouse brain from pregnant dams on a protein-restricted diet. *Brain Res Mol Brain Res* 2005;140:111–115. [PubMed: 16099070]
31. Claycombe KJ, Wang Y, Jones BH, Kim S, Wilkison WO, Zemel MB, et al. Transcriptional regulation of the adipocyte fatty acid synthase gene by agouti: interaction with insulin. *Physiol Genomics* 2000;3:157–162. [PubMed: 11015611]
32. Claycombe KJ, Xue BZ, Mynatt RL, Zemel MB, Moustaid-Moussa N. Regulation of leptin by agouti. *Physiol Genomics* 2000;2:101–105. [PubMed: 11015588]
33. Ebihara K, Ogawa Y, Katsuura G, Numata Y, Masuzaki H, Satoh N, et al. Involvement of agouti-related protein, an endogenous antagonist of hypothalamic melanocortin receptor, in leptin action. *Diabetes* 1999;48:2028–2033. [PubMed: 10512369]
34. Hoggard N, Hunter L, Duncan JS, Rayner DV. Regulation of adipose tissue leptin secretion by alpha-melanocyte-stimulating hormone and agouti-related protein: further evidence of an interaction between leptin and the melanocortin signalling system. *J Mol Endocrinol* 2004;32:145–153. [PubMed: 14765998]

35. Takeuchi S, Takahashi S. A possible involvement of melanocortin 3 receptor in the regulation of adrenal gland function in the chicken. *Biochim Biophys Acta* 1999;1448:512–518. [PubMed: 9990303]
36. Takeuchi S, Teshigawara K, Takahashi S. Widespread expression of Agouti-related protein (AGRP) in the chicken: a possible involvement of AGRP in regulating peripheral melanocortin systems in the chicken. *Biochim Biophys Acta* 2000;1496:261–269. [PubMed: 10771094]
37. Dhillo WS, Small CJ, Gardiner JV, Bewick GA, Whitworth EJ, Jethwa PH, et al. Agouti-related protein has an inhibitory paracrine role in the rat adrenal gland. *Biochem Biophys Res Commun* 2003;301:102–107. [PubMed: 12535647]
38. Doghman M, Delagrangé P, Blondet A, Berthelon MC, Durand P, Naville D, et al. Agouti-Related Protein Antagonizes Glucocorticoid Production Induced through Mc4-R Activation in Bovine Adrenal Cells: A Possible Autocrine Control. *Endocrinology*. 2003
39. Xiang L, Murai A, Muramatsu T. The effects of agouti-related protein gene transfer in vivo by electroporation in mice. *Neurosci Lett* 2004;370:108–113. [PubMed: 15488304]
40. Breit A, Wolff K, Kalwa H, Jarry H, Buch T, Gudermann T. The natural inverse agonist agouti-related protein induces arrestin-mediated endocytosis of melanocortin-3 and -4 receptors. *J Biol Chem*. 2006
41. Kas MJ, Buijnzeel AW, Haanstra JR, Wiegant VM, Adan RA. Differential regulation of agouti-related protein and neuropeptide Y in hypothalamic neurons following a stressful event. *J Mol Endocrinol* 2005;35:159–164. [PubMed: 16087729]
42. West DB, Boozer CN, Moody DL, Atkinson RL. Dietary obesity in nine inbred mouse strains. *Am J Physiol* 1992;262:R1025–1032. [PubMed: 1621856]
43. Bachmanov AA, Reed DR, Tordoff MG, Price RA, Beauchamp GK. Nutrient preference and diet-induced adiposity in C57BL/6ByJ and 129P3/J mice. *Physiol Behav* 2001;72:603–613. [PubMed: 11282146]
44. Howard BV, Van Horn L, Hsia J, Manson JE, Stefanick ML, Wassertheil-Smoller S, et al. Low-fat dietary pattern and risk of cardiovascular disease: the Women's Health Initiative Randomized Controlled Dietary Modification Trial. *JAMA* 2006;295:655–666. [PubMed: 16467234]

Fig. 1. Effects of LFD or HFD on body weight at different ages. Asterisks indicate significant differences between LFD and HFD (* – $P < 0.05$).

Fig. 2. Effects of LFD or HFD on AgRP expression (mRNA) in the hypothalamus (A), the adrenal gland (B), and the testis (C) at different ages. Asterisk indicate significant differences between LFD and HFD (* – $P < 0.05$). Time points with different lower case letters are significantly different ($P < 0.05$).

Fig. 3. Effects of LFD or HFD on leptin (A), leptin receptor (B), and NPY expression (C) mRNA at different ages. Asterisks indicate significant differences between LFD or HFD (* – $P < 0.05$). Time points with different lower case letters are significantly different ($P < 0.05$).

TABLE 1

Results from two-way ANOVA models for each outcome. The overall test (left column) shows results of testing for any difference in the outcome (Age-related or diet-related difference.) The next two columns show results of tests for differences across Age groups within LFD and HFD groups. The right-hand-side column shows results of testing for a difference between LFD and HFD diet groups aggregated over age. The numbers in parenthesis for each F-value are the numerator and denominator degrees of freedom associated with that particular test.

Outcome	Overall test	Age (LFD)	Age (HFD)	LFD vs. HFD
AgRP-adrenal	F(6,28) = 73.98 p < 0.001	F(3,28) = 17.06 p < 0.001	F(3,28) = 106.79 p < 0.001	F(1,28) = 3.94 p = 0.057
AgRP-hypothalamus	F(6,28) = 6.59 p < 0.001	F(3,28) = 5.86 p = 0.003	F(3,28) = 9.41 p = 0.002	F(1,28) = 4.39 p = 0.045
AgRP-testis	F(6,28) = 31.80 p < 0.001	F(3,28) = 39.33 p < 0.001	F(3,28) = 62.67 p < 0.001	F(1,28) = 18.86 p < 0.001
Leptin	F(6,28) = 34.40 p < 0.001	F(3,28) = 43.90 p < 0.001	F(3,28) = 50.19 p < 0.001	F(1,28) = 54.89 p < 0.001
NPY	F(6,28) = 7.04 p < 0.001	F(3,28) = 10.87 p < 0.001	F(3,28) = 4.35 p = 0.012	F(1,28) = 0.48 p = 0.0493
OBRb	F(6,28) = 3.43 p = 0.001	F(3,28) = 1.58 p = 0.217	F(3,28) = 4.87 p = 0.008	F(1,28) = 0.01 p = 0.928