Blunted hypothalamic neuropeptide gene expression in response to fasting, but preservation of feeding responses to AGRP in aging male Brown Norway rats

Tami Wolden-Hanson¹, ², Brett T. Marck¹ and Alvin M. Matsumoto ¹, ²

¹Geriatric Research, Education and Clinical Center, Veterans Administration Puget Sound Health Care System, and ²Division of Gerontology and Geriatric Medicine, University of Washington School of Medicine, Seattle, Washington 98108-1597

Running title: Aging, neuropeptide regulation and melanocortin response

Key words: melanocortin, energy balance, food intake

Address all correspondence and reprint requests to:

Tami Wolden-Hanson, Ph.D.
VA Puget Sound Health Care System (S-182-GRECC)
1660 South Columbian Way
Seattle WA 98108-1597
phone: 206-768-5394, fax: 206-764-2569
email: twh@u.washington.edu
Abstract

Aging mammals lose the ability to maintain energy balance, exhibiting decreased appetite (anorexia) and impaired ability to maintain body weight. To determine the contribution of hypothalamic neuropeptides, two experiments were performed in male Brown Norway rats. To assess the hypothalamic neuropeptide response to food deprivation, young (Y; 4 mo old), middle-aged (M; 13 mo) and old (O; 25 mo) rats were either ad libitum fed or fasted for 72 h (n=10/group) and sacrificed. Hypothalamic levels of agouti-related peptide (AGRP), pro-opiomelanocortin (POMC) and cocaine-amphetamine-regulated transcript (CART) mRNA were assessed by in situ hybridization. With aging, arcuate AGRP gene expression decreased, and CART mRNA increased, but POMC mRNA did not change. Fasting-induced changes in gene expression of all neuropeptides studied were attenuated with aging. To test the food intake response to appetite-stimulating neuropeptides, Y, M, O and very old (VO; 33 mo) rats (n=4-8/group) received one intracerebroventricular injection of each of three treatments: 0.1 nmol AGRP, 2.34 nmol NPY and saline control. AGRP increased food intake of all groups by 10-20%, compared to saline, and this effect persisted up to 7d after injection. VO animals were more sensitive to the effects of AGRP than younger animals. In contrast, NPY increased food intake more in Y than in older animals and its effects did not last more than 24h. We conclude that the mechanisms by which arcuate nucleus neurons influence appetite are differentially affected by age, and speculate that the melanocortin system may be a useful target for treatment of the anorexia of aging.
Introduction
Aging is associated with impairment in the ability to maintain energy balance in response to homeostatic perturbations. The reasons for this loss of regulation of energy balance are multi-factorial. However, one of the key factors appears to be a loss of appetite, termed “the anorexia of aging” (8, 38, 43, 44). In humans, anorexia of aging is caused by numerous factors, including sensory impairment, social isolation, psychological and physiologic factors, in addition to the presence of disease (19, 38, 44). Studies have shown that healthy older people fail to respond to over- or under-feeding with the compensatory changes in eating that are observed in younger people (41-44). In addition, older people report less hunger after an overnight fast, and report greater degrees of satiation to meals than do younger subjects (8, 44, 56). This apparent insensitivity to metabolic cues can lead to inappropriate weight loss in response to acute or chronic illness or other stressors, resulting in greater morbidity and mortality in geriatric populations (19, 25, 38).

Our laboratory uses the Brown Norway rat to study aging-related decrements in reproductive function and regulation of body weight (22, 36). Male Brown Norway rats are an excellent model for human aging: they have long disease-free survival rates (52), and a maximum lifespan of around 36 months, with 50% surviving to 30 months (comparable to 80 years of age in humans), and 10% surviving to 32 months of age (52). Male Brown Norway rats exhibit age-related changes in circulating levels of trophic and metabolic hormones, and alteration of body composition, food intake and body weight that are consistent with those observed in aging men (36, 55).

We use the metabolic stressor of a 72h period of food deprivation to stimulate the neuroendocrine system in order to reveal aging-related differences in the functioning of the hypothalamic circuitry involved in energy homeostasis. This period of food deprivation is well tolerated by all animals, and no animal has ever lost more than 20% of body weight under these conditions. We (21, 54) and others (30) have consistently found that, compared to young animals, aging male rats fail to increase food intake after a 72h fast, and are slow to regain lost body weight upon re-feeding. We have observed that this inability to defend body weight is associated with an age-related deficiency of NPY mRNA and a blunted fasting-induced increase in NPY
gene expression (21, 30, 54). This decreased NPY gene expression may be due to blunting of counter-regulatory hormonal responses to fasting. Old Brown Norway rats are twice as fat as young (20% body fat compared to 10%), with correspondingly higher levels of the metabolic hormones leptin and insulin (55), and they remain hyperinsulinemic and hyperleptinemic at the end of a 72h fast (21, 54).

Leptin is an adipocyte-derived hormone that provides a signal of energy sufficiency (circulating levels are proportional to energy stored in adipose tissue) to hypothalamic nuclei involved in energy homeostasis (17). In young mice and rats, high levels of exogenous leptin decrease food intake, increase energy expenditure, and decrease gene expression of appetite-stimulating (orexigenic) neuropeptides (e.g., NPY and agouti-related peptide [AGRP]) and reciprocally increase gene expression of appetite-suppressing (anorexigenic) neuropeptides that decrease food intake and increase energy expenditure (e.g., alpha-melanocyte stimulating hormone [α-MSH] and cocaine-amphetamine regulated transcript [CART]) (1, 3, 26, 33). Aging and obesity appear to produce a leptin resistance, which blunts these responses to exogenous leptin and leads to hyperleptinemia as the system strives towards homeostasis (21, 23, 30, 45, 48, 50, 54). Fasting causes a rapid decrease in endogenous leptin levels; it is this acute decrease in leptin that signals to the hypothalamus that energy stores may be compromised, resulting in increases in NPY and AGRP gene expression, which the organism interprets as increased hunger and, when food is present, stimulates food intake (1, 17). It is likely that the relatively hyperleptinemic state of aging animals blunts the sensitivity of the hypothalamic energy regulatory system, thus decreasing appetite even during episodes of negative energy balance, such as would occur during an acute illness or caused by other stressors.

Although NPY has long been recognized to play a role in the control of energy balance, the presence of normal feeding and body weight in NPY knockout mice indicate that NPY is not required for maintenance of energy balance when food is freely available (2, 35, 40). Recent evidence has implicated the hypothalamic melanocortin system as a key regulator in the maintenance of energy balance (7, 9, 11, 12, 27). Work from a number of laboratories has identified a circuitry of neurons in the arcuate nucleus that integrates peripheral
metabolic signals and regulates appetite and energy expenditure through actions upon distal effector neurons (7, 9, 11, 12, 27). This circuit consists of the neurons co-expressing the anabolic neuropeptides AGRP and NPY, which synapse upon and inhibit the firing of neurons co-expressing the catabolic neuropeptides \( \alpha \)-MSH (derived from pro-opiomelanocortin, or POMC) and CART. The AGRP/NPY and POMC/CART neurons synapse upon each other; both project rostrally to second-order neurons in the paraventricular nucleus (PVN), and caudally to other second-order hypothalamic and extra-hypothalamic sites that are involved in the autonomic and behavioral processes that regulate energy balance (9, 11, 15, 17, 28).

Neuronal integration in this complex network of neurons is under intensive investigation, and it is clear that this network responds to many hormonal and metabolic signals, including leptin, insulin, ghrelin, corticosterone, and glucose.

NPY and AGRP are anabolic neuropeptides that stimulate feeding, and inhibit sympathetic activity, when exogenously applied to the hypothalamus of rats (4, 24, 37), although the feeding response to NPY is diminished in senescent rats that are undergoing terminal weight loss (4). Leptin, the catabolic hormone that decreases food intake and increases energy expenditure in young rats, also has blunted effectiveness in aging F344 and F344 x BN F1 hybrid rats (45-48, 53). The ability of AGRP to increase food intake of aging rats has not been investigated.

We hypothesized that the dysregulation of energy balance associated with aging results from reduced anabolic signaling through AGRP/NPY neurons, and increased catabolic signaling through POMC/CART neurons. Experiment 1 assessed the effect of age upon changes in AGRP, POMC and CART gene expression in response to a 72 h fast in young, middle aged, and old male Brown Norway rats (ages 4-25 months), and correlated neuropeptide expression with levels of their hormone mediators. We further hypothesized that the age-associated alterations in energy regulation result from an inherent inability of the aging system to appropriately respond to neuroendocrine signals. Experiment 2 compared the ability of
centrally administered AGRP and NPY to increase food intake in young, middle aged, old and very old male Brown Norway rats (ages 4-33 months).
Methods

Animals

Adult male inbred, specific-pathogen-free (SPF) Brown Norway BN/Bi (BN) rats were purchased under NIA contract from Harlan Sprague-Dawley (Indianapolis IN). Animals were isolated in a dedicated room containing no other animals, in a modified specific-pathogen free AAALAC-accredited facility at the VA Puget Sound Health Care System, in Seattle, WA. Animals were individually housed in polycarbonate rat cages containing corncob bedding, in a light- and temperature- controlled room on a 12h light-dark cycle (lights off from 1800-0600 h). Except where noted, animals were allowed ad libitum access to Purina rodent chow (5001, Ralston Purina, St. Louis, MO) and tap water. All animal experiments were conducted in accordance with the principles and procedures outlined in the NIH Guide for the Care and Use of Laboratory Animals, and were approved by the VA institutional animal care and use committee.

Experiment 1

Three age groups (n=20/age group) of male BN rats (Y, young: 3 mo; MA, middle-aged: 12 mo; O, old: 24 mo) were studied. After a 30d baseline period, half of the animals were subjected to a 72h fast, while the other half continued to be fed ad libitum. Body weights were monitored daily and no animal lost more than 20% of pre-fasting body weight. At the end of 72h, all rats were killed by decapitation between 0930-1200 h; brains were quickly removed, frozen on dry ice, and stored intact at –70°C for sectioning and in situ hybridization. Trunk blood was collected and serum separated for assay of leptin, insulin, corticosterone, testosterone, ghrelin, and thyroxine.

Tissue Processing

Serial 20-µm coronal sections (1:3 series) were prepared from each frozen brain using a Jung Frigocut cryostat. Brains were cut through the hypothalamus, from the point rostral to the flattening of the optic chiasm to the rostral pole of the mammillary bodies. Sections were thaw-mounted onto RNase-free microscope slides (Superfrost Plus, Fisher Scientific, Pittsburgh, PA) and stored at -70 °C until they were
prepared for hybridization as previously described (21, 50, 54). Adjacent sets of sections were used for the following in situ hybridizations.

**AGRP in situ hybridization**

DNA complimentary to nucleotides 1-396 of rat AGRP mRNA (U89484) was synthesized by PCR amplification (gift from TM Hahn and MW Schwartz, University of Washington (3, 26)) and used for riboprobe preparation. Purified linearized DNA (0.5µg/µl) was transcribed (Riboprobe® system T-7, Promega #P1440, Madison, WI) with [35S] dUTP (New England Nuclear). Labeled probe mixture (including yeast tRNA, TED and dithiothreitol) was diluted in hybridization buffer to a concentration of 0.6 pmol/ml and applied to each slide. Coverslipped slides were incubated overnight at 63°C. Coverslips were removed in 1x SSC, slides were loaded into slide racks and washed in 1x SSC for 30 min at room temperature, followed by 30 min wash in RNase buffer at 37°C, and a further 30 min wash in 1x SSC at room temperature. Three 20 min stringent washes (0.1x SSC at 68°C) were followed by a final 30 min wash in 0.1x SSC at room temperature. Sections were dehydrated through a series of ethanol solutions and air-dried. When dried, slides were apposed to Hyperfilm-ßMax film for 6 d and films were processed using Kodak Developer D19 and Rapid Fix. AGRP mRNA levels were quantitated throughout the entire arcuate nucleus of each brain using film autoradiography digitized with an image analysis system (MCID; Imaging Research, Inc., St Catharines Ontario, CANADA). One operator, who was blinded to the age and condition of the subjects, performed image analysis. For each brain section showing signal, hybridization area (mm²) was quantified by establishing a threshold value and determining the suprathreshold area (corrected for background labeling) with a predetermined template encompassing only the area of interest. The average optical density and hybridization area for each section were determined by the image analysis software, and the product of the optical density and hybridization area (expressed as hybridization units) was used as an index of the total amount of hybridization in the arcuate (ARC) nucleus (21, 50, 54).

**POMC in situ hybridization**
A 24-base oligodeoxynucleotide probe, complementary to the region of rat POMC coding for corticotrophin residues 8-15, was synthesized by the Molecular Biology Core facility at the Seattle VA. Probe was purified in our laboratory, 3'-end labeled with \[^{35}\text{S}]\text{dATP}\) (New England Nuclear, Boston, MA), further purified (NESSORB 20, New England Nuclear) and reconstituted as described previously (21, 23). Labeled probe mixture (including yeast tRNA, TED and dithiothreitol) was diluted in hybridization buffer. Aliquots were applied to each slide, coverslipped and incubated overnight at 27°C. Coverslips were removed in 1x SSC, slides were loaded into slide racks and washed four times for 15 min each at 55°C in 1x SSC, and again for 30 min at room temperature. Sections were then dehydrated and placed on Hyperfilm-\(\beta\)-Max film for 78 h and films were processed as above. POMC mRNA levels were quantitated throughout the entire arcuate nucleus of each brain using film autoradiography digitized using the MCID image analysis system, as described above.

\textbf{CART in situ hybridization}

DNA complimentary to nucleotides 20-409 of rat ppCART mRNA (accession number U10071) was synthesized by PCR amplification (gift from TM Hahn and MW Schwartz, University of Washington) and used for riboprobe preparation. Purified linearized DNA (0.5\(\mu\text{g/\mu l}\)) was transcribied (Riboprobe\textsuperscript{®} system T-7, Promega \#P1440, Madison, WI) with \[^{35}\text{S}]\text{dUTP}\) (New England Nuclear). Labeled probe mixture (0.4pmol/ml) was applied to each slide, and slides were incubated overnight at 65°C, as previously described (50). Dried slides were apposed to Hyperfilm-\(\beta\)-Max film for 26 h and films were processed as above. Total CART mRNA levels were quantitated throughout the paraventricular nucleus (PVN), arcuate nucleus (ARC) and dorsomedial hypothalamus (DMH) of each brain, using film autoradiography digitized with the MCID image analysis system, as described above. The patterns of CART gene expression in this study are consistent with previous studies demonstrating the distribution of CART mRNA in the hypothalamus (10, 13, 16, 34).

\textit{Hormone Assays}
Serum was stored at -30 °C until hormone assays were performed on duplicate samples. Serum leptin, insulin, corticosterone and ghrelin levels were determined by double antibody radioimmunoassay kits (rat leptin: RL-83K; rat insulin: RI-13K, Linco, St. Louis, MO; rat corticosterone: 07-120103, ICN Diagnostics, Costa Mesa, CA; rat ghrelin RK-031-31, Phoenix Pharmaceuticals, Belmont, CA). The detection limit of the rat leptin assay is 0.5 ng/ml; intra-assay variability is 7.2%. The detection limit of the rat insulin assay is 0.1 ng/ml, and the intra-assay variability is 3%. The detection limit of the rat corticosterone assay is 0.2 ng/ml and is 0.16 ng/ml for rat ghrelin assay. Total serum testosterone and thyroxine were measured by fluoroimmunoassay (Delfia® testosterone: A050-101; thyroxine: 1244-030, Wallac Oy, Turku, Finland). Detection limit of the testosterone assay is 0.1 ng/ml and intra-assay variability is 3.1%. The limit of detection of the thyroxine assay is 39 ng/ml.

Statistical Analyses

Leptin and other hormone levels were compared by two-way ANOVA, as were body weights, weight change during the fast, and CART, AGRP and POMC in situ hybridization data. Results are reported as mean ± SEM, and level of significance was set at p<0.05. Post-hoc testing by Fisher’s PLSD was performed for between-treatment differences separately for each age group. The statistical software package used was StatView Version 4.57 for Windows (Abacus Concepts, Inc. and SAS Institute, Inc., Berkeley, CA).

Experiment 2

Young (Y, 4mo; n=5), Middle-aged (M, 13mo; n=5), Old (O, 25mo; n=4) and very old (VO, 33 mo; n=8) weight-stable male BN rats were implanted with 3rd ventricular cannulas (Plastics One; 2.2mm caudal to Bregma and 7.5mm below dura) while under isoflurane anesthesia. Surgical recovery was supported with warmed subcutaneous fluids (lactated Ringer saline; 5ml/250g BW) and cages were placed on circulating-water heating pads until animals were completely awake and ambulatory. Antibiotic (gentamicin; 8mg/rat) and anti-inflammatory (ketoprofen; 5mg/kg) therapies were administered for 3d. Cannula patency was
tested using the dipsogenic hormone Angiotensin II, no less than one week after surgery. Animals were considered to have a functioning cannula if they drank at least 5ml of water within 15 minutes of injection of 50-100ng Angiotensin. Following cannula patency testing, each animal received one injection (2-5µl) of each treatment, administered in the early part of the light phase, 3-4h after lights-on. Animals were injected with 0.1 nmol AGRP (AGRP 86-132; Peptides International), 2.34 nmol NPY (10 µg, Sigma) and saline vehicle as control. The AGRP dosage that was chosen has been shown to stimulate food intake for up to 7 d in young rats (14, 24, 31, 32, 49, 57). The NPY dosage that was chosen has been shown to be the maximal dosage that stimulates food intake in old F344 rats but that fails to do so in animals undergoing senescent terminal weight loss (4). Treatment order was counterbalanced and treatments were separated by at least 7d. After injection, animals were returned to their home cages and given a weighed supply of regular rodent chow, which was weighed to the nearest 0.1g after 4h and 24h, and daily thereafter (fresh food was supplied daily and all animals were accustomed to the feeding protocol). Body weights were also measured daily.

Statistical Analyses

Food intake measurements are reported as mean ± SEM. Results were compared by two-way repeated measures ANOVA; the level of significance was set at p<0.05. Post-hoc testing by paired Student’s t-tests was performed for between-treatment differences separately for each age group. The statistical software package used was StatView Version 4.57 for Windows (Abacus Concepts, Inc. and SAS Institute, Inc., Berkeley, CA).
Results

Experiment 1

Body weights

Initial body weights of rats were significantly greater (p<0.0001) with increasing age (Table 1). Animals that were subjected to a 72-h fast lost around 50 g of body weight, regardless of their age, resulting in a greater percentage of body weight loss in Young animals, when compared to Middle-aged and Old rats (Table 1). Little change in weight was observed in the *ad libitum* fed animals during this 72-h period.

*Agouti-related peptide (AGRP) gene expression*

AGRP mRNA hybridization in the entire arcuate nucleus decreased with age (p<0.0001) and increased with fasting (p<0.0001; Figure 1); both signal density and signal area were increased. Total hybridization units in fasted rats were significantly increased by two-fold in all groups of rats, compared to *ad libitum* fed animals.

*Pro-opiomelanocortin (POMC) gene expression*

POMC mRNA hybridization in the arcuate nucleus was similar between all ages of *ad libitum* fed rats (Figure 1). 72h of fasting significantly (p<0.0001) decreased POMC gene expression (both decreased signal density and decreased signal area). Total hybridization units in fasted rats were 57%, 27% and 13% lower in Young, Middle-aged and Old fed rats, respectively; this decrease was significant for Young and Middle-aged groups only.

*Cocaine-amphetamine regulated transcript (CART) gene expression*

CART mRNA hybridization in the entire arcuate nucleus increased with age (p<0.0001) and decreased with fasting (p<0.0001; Figure 1). Total hybridization units in fasted rats were 48%, 33% and 19% lower in Young, Middle-aged and Old fed rats, respectively; this decrease was significant for each age group.

In the PVN, CART mRNA hybridization decreased with age (p<0.0001) and fasting (p=0.0003; Figure 2). Both signal density and signal area were decreased, although signal density was less sensitive to fasting than
to age, while signal area was less sensitive to age than to fasting. Total hybridization units in fasted rats were 6%, 25% and 15% lower in Young, Middle-aged and Old rats, respectively; this decrease was significant only for the Middle-aged group.

CART mRNA hybridization in the perifornical region of the dorsomedial hypothalamus (DMH), was not altered either by age or by fasting (data not shown).

*Serum hormone levels*

Levels of serum insulin were increased with age (p<0.0001; Table 1) in both fed and fasted groups. Insulin levels of Old animals in the fed group were three-fold higher than in Young fed rats, and two-fold higher than in Middle-aged fed rats. Fasting significantly (p<0.0001) decreased insulin by 70-80% in all age groups.

Serum leptin levels were also increased with age (p<0.0001; Table 1) in both fed and fasted groups, however, age-related differences were more pronounced than for insulin. Leptin levels of Old animals in the fed group were seven-fold higher than in Young, and three-fold higher than Middle-aged rats. Fasting significantly (p<0.0001) decreased leptin by 60-70% in all age groups.

Serum corticosterone levels were significantly lower with increasing age (p<0.0001; Table 1) in both fed and fasted groups. Fasting significantly (p<0.002) increased corticosterone by 166%, 183% and 126% in Young, Middle-aged and Old rats, respectively.

Levels of serum testosterone were similar between fed groups of Old and Middle-aged animals, and were decreased compared to Young rats (p<0.07; Table 1). Fasting significantly (p<0.0001) decreased testosterone by 94%, 65% and 72% in Young, Middle-aged and Old rats, respectively. Testosterone levels of the Young fasted animals were reduced to or below the lower limit of the assay.
Serum thyroxine levels were similar between groups of Old and Middle-aged animals, and were decreased compared to Young rats (p<0.05; Table 1). Fasting significantly (p<0.0001) decreased thyroxine by 20%, 10% and 8% in Young, Middle-aged and Old rats, respectively.

Serum ghrelin levels were similar between fed groups of rats. Fasting significantly (p<0.0001; Table 1) increased serum ghrelin by 70%, 41% and 25% Young, Middle-aged and Old rats, respectively.

*Correlations between mRNA levels and hormones*

Correlations between mRNA levels and serum hormones (Table 2) indicate that leptin and insulin were positively associated with gene expression of the anorexigenic neuropeptides POMC and CART in the arcuate nucleus, and negatively associated with gene expression of the orexigenic neuropeptide AGRP. In contrast, corticosterone and ghrelin were positively associated with AGRP mRNA and negatively correlated with POMC and CART mRNA levels in the arcuate nucleus. Testosterone and thyroxine were negatively correlated with arcuate AGRP, positively associated with POMC gene expression, and positively associated with CART mRNA levels in the PVN and the dorsomedial hypothalamus.

*Experiment 2*

There was no significant effect of age on food intake of the saline group at any time point, although food intake tended to be lower in Young, and greater in Old, compared to Middle-aged and Very Old animals (Figures 3 and 4). Both AGRP and NPY stimulated feeding at 4 and 24h after 3rd ventricular injections during the light phase, compared to saline (p<0.001; Figure 3). Cumulative daytime food intake 4h after 2.34 nmol injection of NPY was greater than food intake after 0.1nmol AGRP, but by 24h the effect of NPY was abolished in all but the Young animals (Figure 3). In contrast, AGRP continued to stimulate food intake for 24h (p<0.001; Figure 3) and a single 3rd ventricular injection significantly increased 3d and 7d cumulative food intake by 10-20% compared to saline injection (p<0.001; Figure 4). The ability of AGRP to increase food intake was more pronounced in Very Old animals than it was in Young (Figure 4).
Individual daily food intake was increased by 2-4g for as many as 7d after the single injection, compared to average food intake after saline (p<0.001). Body weights of Young animals were significantly lower than older animals, whose weights did not differ significantly. Body weights were not affected by any treatment for any age group. Final body weights per age group: 3-4mo (Y), 285 ± 11, range 245-316g; 12-13mo (M), 415 ± 3, range 404-424g; 24-25mo (O), 457 ± 16, range 407-500g; 32-33mo (VO), 450 ± 11, range 394-495g.
Discussion

These studies have revealed an age-associated decrease in basal arcuate nucleus AGRP gene expression that is of the same magnitude as we have previously shown for arcuate NPY gene expression (21, 23, 50, 54). We also found that POMC mRNA levels in the arcuate nucleus were not altered with age in the basal state and we have confirmed and extended our earlier (50) finding of an age-associated increase in CART mRNA in the arcuate nucleus, and an age-associated decrease in CART mRNA in the PVN. We confirmed the finding (4) that the food intake response evoked by exogenous NPY is blunted in aging animals, and we showed for the first time that the prolonged feeding response to exogenous AGRP was maintained in old Brown Norway rats. The results of this study confirmed our hypothesis that age-associated decrements in levels of orexigenic signaling through AGRP/NPY neurons in the arcuate nucleus are accompanied by increased levels of anorexigenic signaling through POMC/CART neurons. This pattern of neuropeptide gene expression may contribute to a loss of appetite and the anorexia of aging.

Old animals may remain weight-stable when food is freely available, but experiments using metabolic perturbations have demonstrated failures in the ability of aging animals to regulate energy balance (4, 21, 23, 30, 45, 48, 54). The negative energy balance induced by a 72h fast increased gene expression of the orexigenic neuropeptide AGRP (a similar magnitude as the fasting-induced NPY increase (21, 54)), and decreased expression of anorexigenic neuropeptides CART and POMC. Food deprivation for 72h revealed that old animals failed to reduce POMC mRNA, and that fasting-induced decreases in CART mRNA were blunted in the old animals, compared to young and middle-aged rats. Although AGRP mRNA levels doubled with fasting in all age groups, levels in fasted old rats were not different from those in ad libitum fed young animals, similar to the pattern seen for NPY mRNA in previous studies (21, 23, 54). These findings indicate that in addition to the attenuated orexigenic drive, aging animals also showed incomplete suppression of anorexigenic drive, possibly driven by blunted endocrine responses to fasting.
Because AGRP gene expression was reduced in old animals, we sought to determine whether the aging melanocortin system was able to respond to centrally administered AGRP, or whether aging would dampen responsivity to exogenous AGRP, as is the case for NPY (4). We found that, contrary to our hypothesis that the aging system has an inherent inability to appropriately respond to neuroendocrine signals, the aging melanocortin system is able to respond appropriately to exogenous AGRP, while at the same time showing blunted responses to NPY. We observed that a dose of AGRP, shown by others to be submaximal but long-lasting (14, 24, 49), stimulated food intake equally well in aged rats as in young rats. In contrast, exogenous NPY increased short-term food intake more than did AGRP, but was far less effective in older animals than in young, a finding that confirms studies in other strains of aging rats (4, 39). The loss of responsivity to exogenous NPY may be due, in part, to pharmacologic actions upon different NPY receptor subtypes, suppressing endogenous NPY release via Y2 autoreceptors on arcuate NPY/AGRP neurons, while simultaneously stimulating second-order neurons through actions on the Y1- and Y5-receptors (9, 11).

Aging mammals have increased total and peripheral fat mass, with a proportional increase in leptin and insulin. We previously showed that total adiposity (as determined by dual energy X-ray absorptiometry) increases from 9% at 3mo to 20% at 29mo of age in male Brown Norway rats (55). Although body fat is twice as high in aged compared to young male Brown Norway rats, circulating leptin levels are six to seven fold higher in the old animals, even at the end of the 72h fast. Thus, aging male Brown Norway rats appear to be in a chronically hyperleptinemic state. Our findings of decreased orexigenic and increased anorexigenic signaling with advancing age may indicate that aging Brown Norway (BN) rats maintain at least partial responsiveness to endogenous leptin, in contrast to aged F344 x BN F1 hybrid rats, which are resistant to the effects of exogenous leptin (45-48). This leptin resistance may be due to a leptin dosage effect, as the F344/BN males are fatter and have higher levels of leptin, compared to the BN males (47).

Energy balance results from a homeostatic feedback system that is regulated, in part, by endocrine signals acting upon neurons in the arcuate nucleus that integrate peripheral metabolic signals and regulate appetite and energy expenditure (1, 7, 12, 27). When leptin and insulin levels are high, such as in a fed or satiated
state, metabolic rate is increased and food intake is decreased. When food is scarce, leptin and insulin levels decrease, hunger signals increase, and metabolic rate is decreased in order to conserve energy stores. Compared to young animals, the aging male Brown Norway rat has high levels of insulin and leptin and lower levels of testosterone, thyroxine, ghrelin and corticosterone. When confronted with a lack of food, insulin, leptin, testosterone and thyroxine are reduced, and ghrelin and corticosterone are increased. These appropriate responses to food restriction reduce energy expenditure and increase hunger and food-seeking behavior, because the hypothalamic systems that regulate energy balance are responsive to changes in the levels of these hormones. The metabolic challenge of a 72h fast resulted in altered levels of these hormone mediators of energy balance, although fasting-induced changes were attenuated in aging rats. We showed previously (54) that treatment with the insulin sensitizer troglitazone ameliorated hyperinsulinemia and hyperleptinemia in old male Brown Norway rats, and improved food intake and body weight gain after a 72h fast, suggesting that age-related increases in these hormonal signals of energy sufficiency play a role in the attenuated food intake that we observe in old animals after fasting. Troglitazone-induced improvements in food intake and body weight recovery (54) occurred without changes in arcuate NPY gene expression suggesting that other neuropeptide systems may have been involved.

Exogenous leptin and insulin activate POMC/CART neurons and suppress activity of NPY/AGRP neurons (3, 15), whereas exogenous ghrelin activates NPY/AGRP neurons and suppresses activity of POMC/CART neurons (9, 29). The degree to which this circuitry is influenced by other endocrine mediators is just beginning to be understood. We found that high levels of the catabolic neuroendocrine mediators leptin and insulin were correlated with suppressed levels of AGRP mRNA and with increased levels of CART and POMC mRNA, both in the basal and fasted state. Similarly, reduced anabolic signaling (via ghrelin and corticosterone) was correlated with suppressed orexigenic and increased anorexigenic neuropeptide gene expression. Serum testosterone and thyroxine levels were negatively correlated with arcuate AGRP, positively correlated with arcuate POMC, and positively associated with CART gene expression in the PVN and DMH, suggesting that the neuromodulatory actions of these hormones may be more complex, perhaps because of their roles in growth, reproduction and autonomic function. CART is co-localized with a number
of neuropeptides in the PVN (6, 18, 51), including TRH, vasopressin and oxytocin, all of which are decreased with aging, or with low levels of testosterone resulting from castration or aging (5, 20). ICV injections of CART have been shown to prevent the fasting-induced decrease in TRH mRNA (18), and it may be that the decreased CART gene expression that we observed in the PVN of older, fasted animals reflected a counter-regulatory response to decrease energy expenditure via thyroxine’s effects upon the autonomic nervous system.

In summary, using in situ hybridization, we found decreased gene expression of orexigenic (AGRP) and increased gene expression of anorexigenic (POMC and CART) neuropeptides in the arcuate nucleus with advancing age. AGRP gene expression doubled with fasting in all age groups, but levels in old fasted rats were attenuated and similar to those of young fed animals, and fasting-related reductions in POMC and CART were blunted with aging. Using intracerebroventricular injections of orexigenic neuropeptides, we found that, in contrast to a blunted and acute feeding response to NPY administration, aged animals were as sensitive to the effects of AGRP as young rats, and showed stimulation of food intake for up to one week after one treatment. We conclude that the mechanisms by which the co-localized AGRP and NPY systems in arcuate neurons influence appetite are differentially affected by age. Our finding of long-lasting effects of AGRP to stimulate food intake of aged rats lead us to speculate that the melanocortin system may be a useful target for treatment of the anorexia of aging.
Acknowledgements

This work was supported by Veterans Affairs Medical Research Funds and NIH grant P50-HD-12629. Work in this report has been presented and published in part at the 82nd Annual meeting of the Endocrine Society, Denver, CO (2000) and at the Fifth International Melanocortin Meeting, Sunriver, OR (2002).
<table>
<thead>
<tr>
<th></th>
<th>Young (4mo)</th>
<th>Middle-Aged (13mo)</th>
<th>Old (25mo)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fed</td>
<td>72-Fasted</td>
<td>Fed</td>
</tr>
<tr>
<td>n</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Pre-fast weight (g)</td>
<td>289 ± 7</td>
<td>288 ± 11</td>
<td>410 ± 10 a</td>
</tr>
<tr>
<td>Weight change-72h (g)</td>
<td>5 ± 1</td>
<td>-56 ± 2 *</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>% Weight change-72h</td>
<td>2 ± 0.4</td>
<td>-20 ± 0.3 *</td>
<td>1 ± 0.3 b</td>
</tr>
<tr>
<td>Insulin (pg/ml)</td>
<td>1.03 ± 0.16</td>
<td>0.24 ± 0.03 *</td>
<td>1.77 ± 0.28</td>
</tr>
<tr>
<td>Leptin (ng/ml)</td>
<td>2.1 ± 0.2</td>
<td>0.7 ± 0.1 *</td>
<td>4.0 ± 0.6</td>
</tr>
<tr>
<td>Corticosterone (ng/ml)</td>
<td>209 ± 35</td>
<td>348 ± 47 ‡</td>
<td>116 ± 23 c</td>
</tr>
<tr>
<td>Testosterone (ng/ml)</td>
<td>2.5 ± 0.6</td>
<td>0.14 ± 0 †</td>
<td>1.0 ± 0.2 c</td>
</tr>
<tr>
<td>Thyroxine (ng/ml)</td>
<td>129 ± 6</td>
<td>103 ± 5 †</td>
<td>112 ± 3 b</td>
</tr>
<tr>
<td>Ghrelin (ng/ml)</td>
<td>0.42 ± 0.05</td>
<td>0.70 ± 0.10 ‡</td>
<td>0.56 ± 0.06 c</td>
</tr>
</tbody>
</table>

Data are mean ± SEM. Post-hoc testing by Fisher’s PLSD a=p<0.001, b=p<0.01, c=p<0.05 compared to young rats in same feeding condition, * p<0.001, †=p<0.01, ‡=p<0.05 compared to fed rats of same age.
Table 2: Significant R values for correlations between mRNA and serum hormone levels

<table>
<thead>
<tr>
<th></th>
<th>Insulin</th>
<th>Leptin</th>
<th>Corticosterone</th>
<th>Testosterone</th>
<th>Thyroxine</th>
<th>Ghrelin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arcuate AGRP</td>
<td>-0.62*</td>
<td>-0.58*</td>
<td>0.62*</td>
<td>-0.35†</td>
<td>-0.27‡</td>
<td>0.44†</td>
</tr>
<tr>
<td>Arcuate POMC</td>
<td>0.41*</td>
<td>0.36†</td>
<td>-0.53*</td>
<td>0.40*</td>
<td>0.30‡</td>
<td>-0.47†</td>
</tr>
<tr>
<td>Arcuate CART</td>
<td>0.59*</td>
<td>0.66*</td>
<td>-0.62*</td>
<td>ns</td>
<td>ns</td>
<td>-0.47†</td>
</tr>
<tr>
<td>PVN CART</td>
<td>ns</td>
<td>-0.27‡</td>
<td>ns</td>
<td>0.31‡</td>
<td>0.35†</td>
<td>ns</td>
</tr>
<tr>
<td>DMH CART</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>0.26‡</td>
<td>0.33‡</td>
<td>ns</td>
</tr>
</tbody>
</table>

Correlation (r) values for data from fed and 72h fasted young (4mo), middle-aged (13mo) and old (25mo) male BN rats (n=10/group) * p<0.001; † p<0.01; ‡ p<0.05
A: Arc AgRP mRNA (hybridization units)

B: Arc POMC mRNA (hybridization units)

C: Arc CART mRNA (hybridization units)

4mo (Y) 13mo (M) 25mo (O)
PVN CART mRNA (hybridization units)

4mo (Y) 13mo (M) 25mo (O)
**4h Cumulative Food Intake**

- 3-4mo (Y)
- 13mo (M)
- 25mo (O)
- 33mo (VO)

**24h Cumulative Food Intake**

- 3-4mo (Y)
- 13mo (M)
- 25mo (O)
- 33mo (VO)
Figure Legends

Figure 1. Total hybridization units (signal area x signal optical density) throughout arcuate nucleus of (A) AGRP mRNA (mean ± SEM). (B) POMC mRNA (mean ± SEM). (C) CART mRNA (mean ± SEM), in *ad libitum* fed, compared to 72h fasted, young (4mo), middle-aged (13mo) and old (25mo) male Brown Norway rats (n=10/group). Post-hoc testing by Fisher’s PLSD; a=p<0.001, b=p<0.01, c=p<0.05 compared to young rats in same feeding condition, * =p<0.001, †=p<0.01, ‡=p<0.05 compared to fed rats of same age.

Figure 2. Total hybridization units (signal area x signal density) CART mRNA (mean ± SEM) throughout paraventricular nucleus (PVN) in *ad libitum* fed, compared to 72h fasted, young (4mo), middle-aged (13mo) and old (25mo) male Brown Norway rats (n=10/group). Post-hoc testing by Fisher’s PLSD; a=p<0.001, b=p<0.01, c=p<0.05 compared to young rats in same feeding condition, * =p<0.001, †=p<0.01, ‡=p<0.05 compared to fed rats of same age.

Figure 3. 4h (**upper**; g, mean ± SEM) and 24h food intake (**lower**) after one 3rd ventricular injection, 3-4h after lights-on, of 0.1nmol AGRP, 2.34nmol NPY or saline in young (Y, 3-4mo; n=5), middle-aged (M, 13mo; n=5), old (O, 25mo; n=4) and very old (VO, 33 mo; n=8) male Brown Norway rats. Data were analyzed by repeated-measures ANOVA with post-hoc testing by Student’s T test for effect of treatment within age groups; *=p<0.05 compared to saline.

Figure 4. Cumulative food intake (**left**; g, mean ± SEM) and as % of food eaten after saline (**right**; %, mean ± SEM) at 3d (**upper**) and 7d (**lower**) after one 3rd ventricular injection of 0.1nmol AGRP or saline in young (Y, 3-4mo; n=5), middle-aged (M, 13mo; n=5), old (O, 25mo; n=4) and very old (VO, 33mo; n=8) male Brown Norway rats. Data were analyzed by repeated-measures ANOVA with post-hoc testing by Student’s T test for effect of treatment within age groups after 3 and 7d of injection; *=p<0.05 compared to saline.
Literature Cited


50. Sohn, E. H., T. Wolden-Hanson, and A. M. Matsumoto. Testosterone (T)-induced changes in arcuate nucleus cocaine-amphetamine-regulated transcript and NPY mRNA are attenuated in old compared to young male brown Norway rats: contribution of T to age-related changes in cocaine-amphetamine-regulated transcript and NPY gene expression. *Endocrinology* 143: 954-63, 2002.


