Effects of Lifelong Moderate Caloric Restriction on Levels of Neuropeptide Y, Proopiomelanocortin, and Galanin mRNA

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CONTINUOUS moderate caloric restriction throughout life prolongs life span and delays the onset of pathological and deleterious conditions associated with aging (1,2). One of the physiological systems that declines relatively early during the aging process is the capacity of females to reproduce (reviewed in 3). We and others have shown that moderate caloric restriction delays loss in reproductive function with age in rodents (4-7). Our previous findings suggest that the mechanisms that allow caloric restriction to delay reproductive aging may involve alterations in hypothalamic function (4).

It appears that virtually all hypothalamic-hypophyseal-hypothalamic systems deteriorate with age including the reproductive axis (8), growth hormone axis (9,10), the stress axis (11), and the thyroid hormone axis (12,13). Estrous cyclicity and luteinizing hormone (LH) secretion (4), growth hormone secretory patterns (9), and the thyroid axis (14) are improved with moderate caloric restriction.

Many neuropeptides that regulate appetite/feeding behavior have also been implicated as neuromodulators of reproductive axis. We have chosen to focus our attention on the possibility that caloric restriction preserves female reproductive function by influencing neuropeptide Y (NPY), proopiomelanocortin (POMC), and galanin (GAL) gene expression because these neuropeptides influence reproductive function, respond to varying levels of dietary energy restriction, and regulate certain aspects of feeding behavior and intermediary metabolism (15). Thus, they may be the neurochemical mediators that communicate metabolic status to gonadotropin-releasing hormone (GnRH) neurons to alter reproductive function. We examined the effects of caloric restriction and age on specific populations of NPY, POMC, and GAL neurons. These neuropeptides serve multiple functions. However, only specific subpopulations of these neurons send efferents to GnRH-containing regions of the brain. Thus, they are the most likely to communicate metabolic information to GnRH neurons.

NPY is produced by neurons in the arcuate nucleus (AN) that project to the paraventricular nucleus (PVN) to regulate appetite (16). These neurons also project to the medial septal diagonal band of Broca (MS-DBB) and median eminence (17) where NPY may inhibit the episodic release of GnRH or facilitate preovulatory release of GnRH. NPY also acts as a neuroendocrine hormone: it is released into hypophysial portal blood and acts at the level of the pituitary to stimulate LH release and enhance gonadotrope responsiveness to GnRH (reviewed in 18). Severe food restriction or deprivation results in elevated levels of NPY in the AN of rats (19), and this has been associated with impaired reproductive function.

POMC-containing neurons, originating in the AN, project to the ventral preoptic area and MS-DBB at the level of the organum vasculosum of the lamina terminalis where they act either directly on GnRH neurons (20), or possibly via interneurons (21) to inhibit GnRH secretion. β-Endorphin, a cleavage product of the translation of the POMC...
gene, is synthesized in neurons in the AN. It stimulates appetite; severe food restriction or deprivation reduces POMC gene expression in the AN (19,22).

GAL-producing neurons in the AN terminate in the median eminence, and GAL is secreted into the portal vasculature where it is suspected to directly regulate gonadotropes (23). GAL is also colocalized with GnRH in the MS-DBB and median eminence where it is thought to play a role in stimulation of GnRH secretory activity (24). Undernutrition results in decreased GAL mRNA levels in the AN (19). GAL also plays a role in both reproduction and eating behavior (25). GAL levels in the PVN closely parallel the level of fat intake (26). Furthermore, many GAL-containing neurons of the PVN project to the median eminence (23) where they can influence GnRH function.

There is some evidence for an age-related decline in the activity of neuronal populations which synthesize NPY (27), POMC (28,29), and GAL (30). Attenuation of the age-related decline or alterations in normal levels of activity of hypothalamic neuropeptide cell populations may provide important clues regarding the neural mechanisms through which moderate caloric restriction delays age-related processes. Therefore, we conducted a study to determine the effects of lifelong moderate caloric restriction on levels of mRNA for NPY, POMC, and GAL in the AN and GAL in the MS-DBB and PVN, using in situ hybridization histochemistry in ovarioctomized young, middle aged, and old rats.

**Materials and Methods**

**Animals**

Female Sprague-Dawley rats (7 weeks old) were randomly allotted to receive Purina Laboratory Rat Chow 5001 (Ralston-Purina, St. Louis, MO) ad libitum (AL; n = 70) or to be calorically restricted (CR; n = 70) to 60% of the mean daily food intake of AL-fed rats, which was calculated once a month by weighing pellets before and after a 24-hour period for each AL rat. All rats were individually housed under a 14:10 light/dark cycle (lights-on at 0400 hour), and CR rats were fed daily within 1.5 hours of lights-off, which was approximately the time when AL rats tended to begin to eat. Effects of CR on body weight, reproductive cycle history, and characteristics of LH secretion in these rats have been described previously (4).

Rats were bilaterally ovarioctomized 2.5 weeks prior to tissue collection at 4, 12, or 18 months of age. Ovarioctomized rats were used to eliminate the confounding interacting effects of varying steroidal milieux in these different experimental groups. In addition, we have previously shown that CR spares age-related changes in the pattern of GnRH secretion, as monitored by LH secretion, in ovarioctomized rats (4). These studies were intended to test the hypothesis that such changes in secretion correlate with changes in the neurotransmitters that regulate GnRH secretion. These particular age groups were chosen because female rats are sexually mature and the vast majority cycle regularly by 4 months of age. Twelve-month-old rats are considered middle-aged from a reproductive standpoint. That is, the majority begin to exhibit the initial stages of reproductive aging between the ages of 10–12 months. At 18 months of age, the majority are acyclic and exhibit either constant estrous or persistent diestrous vaginal smears. At each age, rats were decapitated and brains quickly dissected and frozen on dry ice between 1100–1300 hour on the day of sacrifice. Brain sections (12 μm thick) were sliced using a cryostat and stored at -70°C until use. The anatomical regions of interest in this study included the anterior and medial portions of the AN (A4380–4620) and the MS-DBB (A7890–7470) (31). We chose these two brain regions because (i) the AN is the site of perikarya for NPY- and POMC-containing neurons which arborize the region of the hypothalamus where GnRH cell bodies are located, and these neuropeptides are thought to modulate pulsatile LH secretory patterns; and (ii) the MS-DBB is the site of both GAL and GnRH perikarya. This overlapping localization suggests that interactions between these two peptides could occur at this site. We also analyzed GAL mRNA levels in the PVN because this region sends efferents to the preoptic region and the median eminence where they may modulate GnRH neuronal activity and secretion.

**In Situ Hybridization Histochemistry**

Region-matched sections were selected and processed for in situ hybridization as described by Wise and coworkers (32) with some modifications. Briefly, all slides to be analyzed for the expression of a given gene in a given brain region were included in a single in situ hybridization assay. They were equilibrated to room temperature, fixed for 5 minutes in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS; pH 7.4), rinsed twice in PBS for 1 minute followed by a brief rinse in water, and another brief rinse in triethanolamine (TEA; pH 8.0). The sections were then acetylated in TEA containing 0.25% acetic anhydride for 10 minutes, followed by a 2-minute rinse in 2× sodium chloride sodium citrate buffer (SSC) with 2 mM dithiothreitol (DTT), then dehydrated in ethanol rinses (70 and 95%), and allowed to air dry.

A 511-base pair cDNA clone of the rat preproNPY gene (33) was provided by Dr. Steven Sabol (NIH) and was used as a template to transcribe NPY cRNA. A 923-base pair cDNA clone of the mouse POMC gene was provided by Dr. James Douglass (Vollum Institute) and described by Chown-Breed and coworkers (34). Dr. James F. Hyde (University of Kentucky) provided the rat GAL cDNA clone of the GAL gene. Linearized cDNA template—containing plasmids were transcribed in vitro in the presence of a 1:4 35S-UTP:α-thio-UTP ratio for NPY and GAL cRNA and a 1:14 ratio for POMC.

We used 200 ng/mL NPY cRNA or 300 ng/mL POMC or GAL cRNA, in a 50% formamide hybridization cocktail (Amresco, Solon, OH) to which 10 mg/mL tRNA and 40 mM DTT were added, because this concentration of cRNA probe was adequate to saturate endogenous mRNA for each neuropeptide in the tissue sections as determined in preliminary studies (data not shown). A 50-μL volume of probe in buffer was placed on each slide containing two brain sections. This was covered with a glass coverslip and incubated overnight at 60°C. After 18 hours, the sections were rinsed for 30 minutes at room temperature in 2× SSC (pH 7.2) containing 4 mM DTT, followed by a 30-minute rinse at
37°C with light agitation. Slides were then treated with RNase A (40 mg/L in 10 mM Tris, pH 7.4; 0.5 M NaCl; 100 mM EDTA) for 30 minutes at 37°C, followed by 30 minutes in buffer without RNase A added. Slides were then washed twice at room temperature in 0.2× SSC containing 2 mM DTT for 15 minutes, placed in 0.2× SSC with 2 mM DTT at 60°C for 1 hour with light agitation. Slides were rinsed briefly in 0.2× SSC with 2 mM DTT at room temperature, followed by water, and dehydrated in ethanol rinses. Within 1–3 days, slides were dipped in a liquid photographic emulsion (NTB2; Kodak, Rochester, NY) diluted 1:1 with distilled water, allowed to air dry, exposed for 10–14 days, and developed using conventional methods. Slides were lightly counterstained using 0.05% toluidine blue.

**Image Analysis**

Slides were selected for analyses based on anatomical region. If the region of interest (AN, MS-DBB, or PVN) was damaged or had poor morphology, the slide was excluded. Preanalysis selection of slides resulted in 1–4 slides analyzed per rat. The methods of quantitation of hybridization signal have been previously described (32). Briefly, we used a BioQuant IV MEG program (BQ; R and M Biometrics, Nashville, TN) to quantitate silver grains. Under light microscopy, the image was captured onto a color monitor and digitized. A threshold was set such that silver grains were computer-digitized, but the histological stain used to identify the cell nucleus did not interfere with quantification. The area of digitized grains over a cell was expressed as pixels. An area adjacent to the labeled cells was considered background and subtracted from the level of hybridization.

**Statistical Analyses**

Body weights were compared using analysis of variance (ANOVA) for a two-way factorial design. The average number of cells per section and average area of thresholded pixels per cell were subjected to ANOVA for a multifactorial design. Variability due to main effects of age (4, 12, vs 18), diet (CR vs AL), previous estrous cycle history (regular, irregular, or persistent estrus), and the Age × Diet interaction were accounted for in the model. Main effects of age, diet, and Age × Diet interaction were tested for significance using the mean squares for rat within each Age × Diet group as the error term. Because previous cycle history did not influence the majority of our results, data were presented as the mean ± standard error for each Age × Diet group. In the analysis for GAL in the PVN, a three-way ANOVA (Diet × Age × Cycle History) indicated an effect of cycle. Therefore, cycle history was included in the further analysis. p < .05 was considered statistically significant.

**RESULTS**

Moderate caloric restriction resulted in lower body weights at all ages (p < .01; Table 1).

Figure 1 is a composite of micrographs of representative cells labeled with riboprobes complementary to NPY (panel a), POMC (panel b) and GAL (panel c) in the AN. Low power photomicrographs show numerous cells that were analyzed. The insert in the upper left corner of each panel is

![Figure 1](image-url)
an enlargement of the area that is enclosed in the box of the main photomicrograph.

Average levels of NPY mRNA per cell were higher in CR versus AL rats overall \( (p < .004) \), but did not change with age \( (p = .91; \text{Figure 2}) \). Previous cycle history had no effect \( (p = .72) \), and there was no Diet \( \times \) Age interaction \( (p = .72) \). The average number of NPY cells detected by in situ hybridization in the AN did not differ with respect to age \( (p = .47) \), diet \( (p = .10) \), cycle, or Age \( \times \) Diet interaction \( (p = .77; \text{Figure 2}) \).

Average levels of POMC mRNA per cell were lower in CR versus AL rats overall \( (p < .02) \), but did not change with age \( (p = .21; \text{Figure 3}) \) or previous cycle history \( (p = .91) \). Although it appears that levels of POMC mRNA declined between 12 and 18 months, it did not result in a significant Age \( \times \) Diet interaction \( (p = .60) \). The average number of cells containing POMC mRNA did not change with age \( (p = .29) \), diet \( (p = .10) \), cycle \( (p = .60) \), or Age \( \times \) Diet interaction \( (p = .95; \text{Figure 3}) \).

Levels of GAL mRNA per cell and the number of cells detected in the AN (Figure 4, left panel) and MS-DBB (Figure 4, right panel) were not different due to age, diet, or previous cycle history. (Number of cells in the AN: age: \( p = .22 \); diet: \( p = .60 \); cycle: \( p = .53 \); Age \( \times \) Diet: \( p = .96 \). mRNA/cell in the AN: age: \( p = .87 \); diet: \( p = .46 \); cycle: \( p = .39 \); cycle: \( p = .61 \); Age \( \times \) Diet: \( p = .53 \). Number of cells in the MS-DBB: age: \( p = .52 \); diet: \( p = .54 \); cycle: \( p = .73 \); Age \( \times \) Diet: \( p = .97 \). mRNA/cell in the MS-DBB: age: \( p = .27 \); diet: \( p = .57 \); Age \( \times \) Diet, \( p = .44 \).) In the PVN, the level of GAL mRNA/cell was greater in regularly cycling animals as compared to irregular cyclers and rats in persistent estrus \( (p < .004) \) (Figure 5). There was no overall effect

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<th>Table 1. Mean Body Weights (Grams)</th>
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Values are mean ± SE (n). **Different from ad-libitum-fed rats of the same age \( (p < .01) \)

Figure 2. Mean levels of NPY mRNA/cell (top) and average number of cells per section (bottom) detected in the arcuate nucleus in ad-libitum-fed (AL) and calorically restricted (CR) rats. Means ± SE are presented. Numbers within each bar are the number of rats in each group. Levels of NPY mRNA/cell were higher in CR versus AL rats overall \( (p < .004) \).

Figure 3. Mean levels of POMC mRNA/cell (top) and average number of cells per section (bottom) detected in the arcuate nucleus in ad-libitum-fed (AL) and calorically restricted (CR) rats. Means ± SE are presented. Numbers within each bar are the number of rats in each group. Levels of POMC mRNA/cell were lower in CR versus AL rats overall \( (p < .02) \).
of age ($p = .11$), diet ($p = .56$), or Age $\times$ Diet interaction ($p = .92$). The number of cells expressing GAL mRNA in the PVN (data not shown) also decreased in middle-aged irregular cycling and middle-aged and old persistent estrus rats ($p < .02$). Again, there was no effect of age ($p = .16$), diet ($p = .56$), or Age $\times$ Diet interaction ($p = .34$).

**DISCUSSION**

**Neuropeptide Y**

There are several studies showing short-term food deprivation or severe undernutrition (19,35) that leads to decreased reproductive function are associated with elevated NPY biosynthesis and secretion in the AN. In the present study, we found enhanced NPY mRNA levels in response to a moderate form of caloric restriction known to increase longevity (2), enhance gonadotropin secretion, and delay age-related loss of reproductive cycles (4,6). Our previous observations that age-related decreases in mean LH concentrations and pulse amplitude in ovariectomized rats were attenuated when rats received restricted yet adequate nutrition (4), and the elevated levels of NPY mRNA that we report in the present study, add an interesting caveat to our understanding of NPY's involvement in modulating LH secretion. In this animal model, it is possible that increased NPY gene expression contributes to enhanced LH secretion in the absence of ovarian steroids.

Ours is the first study to investigate changes in NPY gene expression with age in female rats. We did not observe an age-related decline in NPY mRNA levels between 4 and 18 months of age. Several other studies have examined age-related changes in males with variable results (27,36–38). It is possible that a decline in NPY biosynthesis with aging is a trend that does not always achieve statistical significance and is not detected in female rats until ages well beyond the onset of reproductive decline.

**Proopiomelanocortin**

We observed a suppression in POMC mRNA levels as a result of caloric restriction at all ages studied. $\beta$-Endorphinergic neurons tonically inhibit LH secretion in the rat through direct effects on GnRH release (39). The primary
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Figure 5. Mean levels of GAL mRNA/cell in the paraventricular nucleus in ad-libitum-fed (AL) and calorically restricted (CR) rats. Means ± SE are presented. Numbers within each bar are the number of rats in each group. Levels of GAL mRNA/cell decreased in irregularly cycling or persistent estrus compared to regularly cycling rats (p < .004).

action of endogenous opioid peptides in the rat is to suppress LH pulse amplitude via reduction of the amount of GnRH release per pulse episode (40). Thus, a suppression in POMC mRNA levels in AN neurons is consistent with the enhanced pulse amplitude previously reported in our model (4).

Severe forms of food restriction or food deprivation, associated with inhibition of reproductive function, have been associated with decrease levels of POMC mRNA in the AN (19). In contrast, in the present model, a moderate form of caloric restriction resulted in suppressed POMC, increased NPY gene expression and enhanced LH secretion in ovariectomized females. Together, decreased opioid inhibitory tone and increased the stimulatory effects of NPY may enhance LH secretion and thereby prolong reproductive lifespan on CR rats.

We did not observe an age-related decline in levels of POMC mRNA per cell between 4 and 18 months of age. Other studies (28,29,41) have suggested POMC gene expression declines with age. However, careful comparisons between the animal models and methods of analysis in the present study and past studies of POMC mRNA reveal that it is difficult to compare the results of these studies with each other because previous studies have used different species, measured POMC mRNA levels at different times of day, or used different methods of quantifying gene expression. We conclude from comparing the results of all of these studies that age-related steroid-independent changes in POMC gene expression per cell are probably relatively small and, therefore, may be difficult to replicate consistently.

Galanin

GAL gene expression in the AN, MS-DBB, and PVN did not change with age and was not affected by moderate caloric restriction. Interestingly, GAL mRNA levels in the PVN correlated with reproductive aging regardless of the chronological age of the animals or whether they were AL or CR. Several investigations implicate GAL as an important neuromodulator of LH release, acting both centrally and at the level of the pituitary (24). Together, these data suggest that this population of GAL neurons in the PVN may be a “barometer” of the integrity of the reproductive axis; that is, GAL mRNA levels may decrease in association with the deterioration of reproductive function.

We found no changes between 4 and 18 months of age in
female rats in GAL mRNA levels in the AN or the MS-DBB. The number of immunopositive cells for GAL in the MS-DBB declines between 3 and 25–30 months of age in male rats (30); however, we did not see this trend by 18 months of age in ovariectomized females. Severe forms of undernutrition have been reported to decrease levels of GAL mRNA in the AN (19); however, acute food deprivation does not alter GAL mRNA or protein levels (19,42). The level of caloric restriction in the present study did not alter GAL gene expression.

In summary, we found that moderate caloric restriction throughout life enhanced NPY mRNA, suppressed POMC mRNA in the ARC, and did not alter levels of GAL mRNA in the AN, MS-DBB, or PVN. It is possible that these changes contribute to the enhanced gonadotropin secretion previously reported in these animals, and may indicate a compensatory hypothalamic response to caloric restriction aiding in the preservation of hypothalamic function with age.

ACKNOWLEDGMENTS

Supported by NIH AG02224 (PMW) and AG05257 (TMM).

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REFERENCES


Received December 18, 1997
Accepted July 13, 1998

Nominations for Editors

Journal of Gerontology: Biological Sciences
Journal of Gerontology: Medical Sciences
Journal of Gerontology: Psychological Sciences

The Gerontological Society of America Publications Committee is seeking nominations for three new editors. The positions are Editor of the Journal of Gerontology: Biological Sciences; Editor of the Journal of Gerontology: Medical Sciences; and Editor of the Journal of Gerontology: Psychological Sciences.

The positions will become effective January 1, 2000. Each Editor makes appointments to the Journal’s editorial board and develops policies in accord with the scope statement prepared by the Publications Committee and approved by Council. Each Editor works with reviewers and has the final responsibility for the acceptance of articles for his/her respective Journal. Editorships are voluntary positions. Candidates must be members of The Gerontological Society of America and dedicated to developing a premier scientific journal.

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