Published in final edited form as:

Aging Cell. 2011 June ; 10(3): 483–492. doi:10.1111/j.1474-9726.2011.00693.x.

The arcuate nucleus and NPY contribute to the antitumorigenic effect of calorie restriction

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Summary

Calorie restriction (CR) is known to have profound effects on tumor incidence. A typical consequence of CR is hunger, and we hypothesized that the neuroendocrine response to CR might in part mediate CR's antitumor effects. We tested CR under appetite suppression using two models: neuropeptide Y (NPY) knockout mice and monosodium glutamate (MSG)-injected mice. While CR was protective in control mice challenged with a two-stage skin carcinogenesis model, papilloma development was neither delayed nor reduced by CR in the MSG-treated and NPY knockout mice. Adiponectin levels were also not increased by CR in the appetite-suppressed mice. We propose that some of CR's beneficial effects cannot be separated from those imposed on appetite, and that NPY neurons in the arcuate nucleus of the hypothalamus (ARC) are involved in the translation of reduced intake to downstream physiological and functional benefits.

Keywords

calorie restriction; hypothalamus; MSG; neuroendocrine; NPY; tumorigenesis

Introduction

Since the 1930s calorie restriction (CR) has been well established to delay the onset of age-related disorders and extend the lifespan of multiple species (McCay & Crowell, 1934; McCay *et al.*, 1935; Weindruch & Walford, 1988). Hunger is a fundamental response to CR that triggers a multitude of alterations in the neuroendocrine milieu, and CR modulation of the neuropeptide profile may mediate some of the beneficial changes associated with

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Author contributions

Animal experiments were designed by RKM, KJP, RMA, and RdeC and carried out by RKM, CMY, BJ, KJP and RdeC. The manuscript was prepared by RKM, CYM, RMA and RdeC. ML and CD performed the *in situ* hybridization analysis and assisted with manuscript preparation. MRI analysis of body fat distribution was performed by BJ.

restrictive diets (Minor *et al.*, 2009). Signals like ghrelin from the gut and leptin from adipose tissue converge on the arcuate nucleus of the hypothalamus (ARC) where they are translated into a suite of neuropeptides, both orexigenic (e.g. neuropeptide Y (NPY) and agouti-related protein (AgRP)) and anorexigenic (e.g. cocaine- and amphetamine-regulated transcript (CART) and pro-opiomelanocortin (POMC)). These neuropeptides exert potent downstream effects on feeding behavior and other physiological processes including metabolism (Kalra, 2008) and circadian rhythms (Kallungal & Mintz, 2007). NPY in particular stands out as a prime candidate for sensing and responding to signals of energy homeostasis as NPY expression levels respond to both short-term and long-term fasting conditions (Bi, 2007).

Among the mechanisms through which CR has been postulated to extend lifespan, modulation of endocrine systems and glucose homeostasis are recurring, interrelated themes. It has been known for any years that CR results in reduced blood glucose levels and altered hormone levels, and that these alterations are associated with longevity (see reviews by Bartke, 2005 and Tatar *et al.*, 2003). Reducing adipose stores is another way CR may act to alter circulating hormone levels as adipose tissue is increasingly recognized for its impact on health through chronic disease by acting as an endocrine tissue and secreting numerous factors with diverse physiological effects (Ahima & Flier, 2000). Among these, leptin and adiponectin are expressed by adipocytes differentially depending on adiposity and aging. Leptin is known to increase with adiposity (Frederich *et al.*, 1995) and age (Ma *et al.*, 2002); adiponectin conversely decreases with adiposity (Combs *et al.*, 2002; Stefan *et al.*, 2002) and age (Zhu *et al.*, 2007). Maintenance of youthful adipokine levels by CR (Zhu *et al.*, 2007) may forestall development of insulin resistance (Berg *et al.*, 2001) and its consequences with aging. Because NPY expression in the ARC is responsive to circulating glucose levels (Mizuno *et al.*, 1999), insulin (Schwartz *et al.*, 1992), leptin (Stephens *et al.*, 1995) and adiponectin (Guilod-Maximin *et al.*, 2009), the NPY response may be critical for changes in glucose homeostasis under CR.

In addition to improvements to glucose homeostasis and endocrine profiles, CR also has the ability to stall tumor formation and growth. In fact, while the lifespan-extending properties of CR were not well appreciated until the 1930s (McCay & Crowell, 1934; McCay *et al.*, 1935), observations that CR is protective against transplanted and induced tumors had been made two decades earlier (Rous, 1914). Since then the evidence for CR's antitumorigenic effects has been expanded to include, among others, induced skin papillomas (see reviews by Birt *et al.*, 2004; Kritchevsky, 2002). While the mechanisms behind the protective effects of CR against tumorigenesis are unknown, CR's effects on tumorigenesis through alterations to metabolism, glucose homeostasis and endocrine profiles are active areas of study (Finkel *et al.*, 2007; Hursting, 2003).

If the ARC neuropeptide response to CR is compulsory for some of the physiological benefits associated with the diet, then impairing the ability of an animal to sense or respond to a food shortage (i.e. impairing hunger sensing) through alterations to ARC neuropeptides should negate these beneficial effects. We tested this hypothesis by assessing the effects of CR in two models of altered ARC function (Minor *et al.*, 2008) by using monosodium glutamate (MSG) to induce global ARC lesioning and neuropeptide suppression (Olney, 1969; Broberger *et al.*, 1998; Broberger, 1999) and mutation of *Npy* to induce a specific knockout condition (Erickson *et al.*, 1996). For the MSG-induced ARC lesioning, C57BL/6J (B6) mice were injected subcutaneously with either 4 mg/g MSG or physiological saline (SAL) on postnatal day 5. For the NPY conditions, male 129S-*Npy*^{tm1Rpa}/J mice were used as the knockouts and 129S1/SvImJ males (129S) were used as controls.

Results

CR-driven increases in NPY expression are not seen in ARC-lesioned or NPY-knockout mice

Weight gain in the B6 CR mice was attenuated in both SAL and MSG mice (Fig. 1A) and remained below the weight of the AL groups throughout the duration of the study. Relative body weight was also reduced by CR in the 129S groups (Fig. 1B). After 11 wk on the diets the mice were divided into two subsets ($n = 6$ each), one of which started the carcinogenesis regimen, the other was reserved for body composition analysis (see arrows in Fig. 1 A and B). Nuclear magnetic resonance (NMR) analysis of body composition revealed a significant reduction in both lean and fat mass by CR in the B6 mice (Fig. S1 A and B). To further assess the regional changes to fat mass in the B6 mice we used magnetic resonance imaging (MRI) (Fig. S1C) to quantify the subcutaneous and visceral fat masses (Fig. S1D), which were increased in MSG AL mice compared with SAL ALs. Both fat depots were reduced by CR regardless of treatment. Among the 129S groups, lean and fat mass were reduced by CR regardless of genotype (Fig. S1 E and F).

Declines in body weight in both strains reflected the reduced food intake of CR mice compared with AL controls (Fig. 1 C and D). Considering rate of food consumption as a gauge for appetite, MSG CR mice were much less likely to consume their entire day's allotment of food within 2 h compared with SAL CR mice (Fig. S2A), and $Npy^{-/-}$ CR mice were also slightly less quick to consume their food than $Npy^{+/+}$ CR mice within 3 h (Fig. S2B).

To assess changes to neuropeptide expression levels in the brains of the altered models we performed *in situ* hybridization. Representative sections from B6 sections probed for NPY are presented in Figure 2A. Relative levels of NPY calculated as a percentage of SAL AL expression (Fig. 2C) show NPY expression was significantly affected by both diet ($P < 0.001$) and ARC condition ($P < 0.001$). Further post hoc analysis revealed a significant increase in NPY expression in the SAL CR group compared with SAL AL ($P < 0.001$). NPY expression was decreased in both MSG AL ($P = 0.002$) and CR ($P < 0.001$) mice compared to SAL controls, and there was no increase subsequent to CR in the MSG-treated mice. We also assessed levels of orexigenic AgRP and anorexigenic CART and POMC in the ARC (Fig. S3A). Expression of each of these neuropeptide transcripts was significantly repressed by MSG treatment in AL-fed mice, and the increase in AgRP and decreases in CART and POMC expected following CR were ablated by MSG treatment. We also assessed neuropeptide expression in two hypothalamic regions surrounding the ARC, namely the lateral hypothalamic area (LHA) and the ventromedial hypothalamic nucleus (VMH) (Fig. S3C). Fatty acid synthase (FAS) mRNA levels in the VMH were not changed by MSG treatment, nor were levels of melanin-concentrating hormone (MCH) or orexin (OX) changed in the LHA. To better understand the peripheral effects of MSG treatment and CR on NPY we assayed serum NPY levels (Fig. 2E). Circulating levels of NPY were not affected by MSG treatment, however there was a significant negative effect of CR on NPY concentrations ($P < 0.001$).

In situ hybridization for NPY in representative sections from the 129S mice are shown in Figure 2B. Relative expression compared with $Npy^{+/+}$ AL showed NPY expression was increased roughly 3-fold by CR in the wild type mice ($P < 0.001$; unpaired t test), while NPY was undetectable in either of the knockout groups (Fig. 2D). In this strain expression of CART was not affected by NPY knockout (Fig. S3B) while expression of orexigenic AgRP was decreased and anorexigenic POMC was increased in $Npy^{-/-}$ AL. Importantly, the response to CR was not affected by genotype for any of the neuropeptides. Serum NPY

levels were significantly reduced by CR in wild type mice while knockout mice did not produce appreciable expression values (Fig. 2F).

Taken together these data show that while ARC-ablated and NPY knockout mice still lose body weight and fat mass in response to reduced food intake, they do not show a typical appetite response in that ARC NPY levels are not increased and their rate of food consumption is not equal to normal CR mice.

Tumorigenesis is not reduced by CR after ARC disruption or NPY knockout

Following treatment with the two-stage tumor induction protocol, papilloma development was repressed by CR in SAL mice with regards to both initial time of onset (12 wk versus 13 wk, respectively) and in tumor incidence after 18 wk of treatment (33% versus 50%, respectively) (Fig. 3A). Overall tumor rate as represented by the area under the curve (AUC; Fig. 3A inset) was reduced by CR in SAL mice by approximately half. Tumor suppression by CR was not achieved in the MSG-treated groups; CR did not delay tumor onset but rather MSG CR mice presented with tumors 3 wk before their AL counterparts (12 wk versus 15 wk, respectively). Moreover, tumor incidence was the greatest for all groups in MSG CR mice at 18 wk (83% in MSG CR versus 67% in MSG AL). This is also reflected in the AUC for tumor incidence where MSG CR mice had more than double overall tumor incidence.

Papilloma development over time in the 129S mice is plotted in Figure 3B. While time of initial tumor onset was equivalent within the wild types (11 wk), CR was protective against tumor growth over time with only 58% *Npy*^{+/+} CR mice presenting with tumors at 18 wks versus 100% of *Npy*^{+/+} ALs. Reduced tumorigenesis in the wild type CRs is further demonstrated by the AUC bar graph in the inset, showing overall tumor incidence in the wild type CRs was less than half of the wild type CRs. By contrast, *Npy*^{-/-} CR mice displayed advanced tumor onset compared with *Npy*^{-/-} ALs (9 versus 11 wks, respectively), although both AL and CR groups reached 100% at 17 wks. Overall tumor incidence in the knockout mice was not affected by CR as the AUC of tumor incidence within the knockout mice reveals that overall tumor incidence was equivalent.

The number of tumors present on each mouse was counted at 18 wk of treatment. Pairwise comparison tests revealed no significant difference in final tumor count among B6 mice (Fig. 3C). However, in 129S mice, tumor count was significantly affected by diet ($P = 0.002$) and pairwise comparison tests showed *Npy*^{+/+} CR mice had significantly fewer ($P = 0.002$) tumors than *Npy*^{+/+} AL mice (Fig. 3D) after 18 wk of the carcinogen regimen. CR had no effect on tumor count in the *Npy*^{-/-} mice.

These observations show tumor onset in an environmentally-induced skin cancer model is not slowed by CR when the ARC is damaged by MSG injection or NPY is knocked out.

Circulating glucose and adipokines are differentially affected by ARC ablation and single NPY knockout

Fasting blood glucose in the B6 mice (Fig. 4A) showed significantly lower ($P < 0.001$) blood glucose in the SAL CR group compared with SAL AL, however this diet effect was absent in the MSG-treated groups. Insulin levels were not significantly altered by either diet or MSG treatment in B6 mice (Fig. S4A). In the 129S mice, CR significantly lowered glucose in both wild type and knockout mice (Fig. 4B), however insulin levels were significantly reduced by CR only in wild type mice (Fig. S4B).

Regarding leptin, circulating levels of the adipose hormone were significantly lowered by diet ($P < 0.001$) in B6 mice (Fig. 4C) and MSG AL mice showed significantly higher serum leptin than the SAL AL group ($P = 0.006$). Leptin levels in 129S mice were also

significantly decreased by CR diet ($P < 0.001$) (Fig. 4D), with no effect attributable to genotype. Circulating levels of another adipokine, adiponectin, were significantly affected by diet ($P < 0.001$) and ARC condition ($P = 0.006$) in B6 mice (Fig. 4E). There was also a significant interaction between ARC condition and diet ($P = 0.037$), with pairwise comparisons revealing a significant increase by the CR in SAL mice ($P < 0.001$) and a significant increase in the MSG ARC condition within the AL groups ($P = 0.001$). Adiponectin concentrations in the 129S mice (Fig. 4F) were significantly affected by diet ($P = 0.001$) with no significant effect by genotype or significant interaction between genotype and diet. Within genotype, *Npy*^{+/+} CR mice had significantly higher adiponectin levels compared to *Npy*^{+/+} AL mice ($P < 0.001$), but there was no similar diet effect in the *Npy*^{-/-} groups.

Discussion

This study was performed to determine the role of hunger signaling in the effects of CR on tumorigenesis in mice. Because the ARC is the central point of convergence for internal and external cues related to feeding and NPY plays a critical role in this task (Kalra, 2008) we employed these two models of hunger suppression to assess the role of the ARC and NPY in mediating downstream beneficial effects of CR. Indications that these models did indeed experience altered hunger sensing include depressed NPY expression levels and slower rates of food consumption in response to CR. The current findings further suggest that the ARC is integral in mediating at least some of the beneficial effects associated with CR on skin tumorigenesis and that NPY is a key component in the response to CR.

In the tumorigenesis experiment, the mice responding most rapidly to treatment were the *Npy*^{-/-} CR mice. The role of NPY in tumorigenesis is not yet well understood, but NPY localizes within tumor cells (Cohen *et al.*, 1990; deS Senanayake *et al.*, 1995; Körner *et al.*, 2004) and has been shown to be both repressive (Kitlinska *et al.*, 2005; Ruscica *et al.*, 2006) and stimulatory (Kitlinska *et al.*, 2005; Rämö *et al.*, 1990) to growth of different *in vitro* cancer cell lines. The current data appear to support the repressive role for NPY in tumorigenesis. Moreover, tumor progression in the B6 mice corroborates the involvement of the ARC and NPY in tumorigenesis as CR was similarly unprotective in the MSG condition. Given that the MSG-treated mice are not only deficient in NPY but also in other neuropeptides involved in appetite regulation, further studies are needed to determine the extent of involvement of other neuropeptides and neuroendocrine factors besides NPY in tumor resistance under CR. It should be noted that while our data indicate disruption of the ARC and NPY can have a significant effect on CR's ability to mitigate DMBA/TPA-induced skin papilloma growth, the extent of neuroendocrine involvement in other models of induced tumors and cancer also warrants further study.

Given our hypothesis that neuroendocrine changes subsequent to CR mediate beneficial effects of CR like reduced tumorigenesis, another critical finding of this study is the inconsistency in the CR response in the models of hunger suppression with regards to changes to circulating adipokines. While CR led to reduced serum leptin in all models, as would be expected (Zhu *et al.*, 2007), CR did not lead to increases in serum adiponectin levels in the ARC-impaired (MSG or *Npy*^{-/-}) mice. While it has been shown that adiponectin knockout mice have reduced NPY levels and appetites (Kubota *et al.*, 2007), the current finding where NPY knockout ablates the effect of CR on adiponectin levels is novel and suggests these proteins may be mutually regulatory. In the B6 mice, the increase in adiponectin in MSG AL mice relative to SAL AL mice further supports the hypothesis that ARC signaling impacts adipose function because models of non-hypothalamic-dependent obesity are characterized by reduced adiponectin output (Kadowaki & Yamauchi, 2005). It has been hypothesized that leptin controls adiponectin production through its action on

hypothalamic activity (Huypens, 2007), which is consistent with the results of this study. In normal models, high leptin produced during obesity would repress adiponectin expression, whereas weight loss would reduce leptin production and thereby promote adiponectin production. In the case of ARC-lesioned MSG mice, leptin is produced in abundance but the signal is not transduced by the ARC and therefore adiponectin production goes unrepressed. While CR reduces adiposity and leptin output in these mice, it has no effect on adiponectin levels because leptin is not a functional regulator without active ARC signaling. Given that adiponectin has been proposed to play a role in tumorigenesis—indeed, adiponectin concentration has been inversely associated with a variety of human cancers (Barb *et al.*, 2007)—the failure of CR to elicit increased adiponectin output in the ARC-compromised models strengthens this assertion.

It is important to note that the abovementioned changes occurred despite equivalent reductions to body weight in both B6 and 129S CR groups compared with their AL counterparts. Furthermore, both strains saw significant reductions in fat mass following CR regardless of treatment or genotype. MSG AL mice have proportionately more body fat than their SAL AL counterparts, a well-known feature of these mice (Pizzi & Barnhart, 1976) so it is unsurprising, then, that the MSG CR mice also have more fat than SAL CR mice. Regarding functional implications of weight loss in the mice, the MSG-treated and NPY knockout CR mice did not show reductions to tumor incidence regardless of CR-driven reductions to adiposity. These results suggest that loss of fat mass *per se* is not sufficient to suppress tumorigenesis, but that rather the profiles of circulating adipokines, particularly adiponectin, may be more influential on tumorigenesis.

Taken together the current results show at least some of the protective effects associated with CR are contingent upon the ARC response to CR, and in particular where NPY is concerned. It is of further interest to elucidate the mechanisms behind the association between NPY levels, adiponectin and tumor onset under conditions of food restriction. The extent of involvement of non-orexigenic NPY actions and contributions from related appetite-regulatory neuropeptides like AgRP, CART and POMC in tumor protection by CR also warrant additional studies. Regardless, the activation of ARC NPY signaling is a promising target for CR mimetics in the search for preventive strategies against environmentally-induced tumorigenesis.

Experimental procedures

Animals

Animal care and experimental protocols followed procedures outlined and approved by the Animal Care and Use Committee at the National Institute on Aging, NIH. Male and female *Npy*^{-/-} mice (129S-*Npy*^{tm1Rpa}/J) were obtained from the Jackson Laboratory (Bar Harbor, ME) and bred in a vivarium maintained by the Intramural Research Program at the National Institute on Aging (Baltimore, MD). After two generations of breeding, offspring were genotyped and all animals were confirmed to be homozygous knockout for NPY. Male wild type (*Npy*^{+/+}) controls (129S1/SvImJ) were purchased from the Jackson Laboratory directly. The C57BL/6J (B6) mice were bred at the Jackson Laboratory and injected subcutaneously with either 4 mg/g MSG or physiological SAL on postnatal day 5. The 129S mice were 3–5 mo of age, and the B6 mice were 2 mo of age when they were divided randomly into diet groups. Male mice were used exclusively and they were housed individually in ventilated caging on a 12 h light/dark cycle at 22°C and 35% humidity with *ad libitum* access to water. The diet, AIN93-G, was obtained from Bio-Serv (Frenchtown, NJ) and was used for both AL and CR diets. AL mice were fed unlimited quantities of the diet, and the CR animals were fed daily weighed amounts that were reduced to 70% of AL intake gradually with full CR achieved after wk 6. Body weights and food intake were recorded twice monthly and the

mice were maintained on the study diets for a minimum of 6 wk before experiments were performed.

Food consumption

AL mice were given unrestricted access to weighed quantities of chow, the remainder of which was weighed every other week. CR animals were fed daily weighed amounts equivalent to 70% of AL intake. During weeks 5–6 of the feeding study the CR mice were monitored for the time to consume all of their meal. Food was given to CR mice at the regular feeding time, 9am, and mice were checked at the end of 2 or 3 hours and data were collected on which mice had finished their daily meal and which mice had food remaining.

In situ hybridization

Coronal hypothalamic sections (16 μ m) were cut on a cryostat and immediately stored at -80°C until hybridization. A specific antisense oligonucleotide for NPY detection was used (Gen Bank Accession Number AF273768, sequence 5'-GGGCGTTTTCTGTGCTTTCCTTCATTAAGAGGTCTG-3'). The probe was 3'-end labeled with 35S- α ATP using terminal deoxynucleotidyl transferase (Amersham Biosciences, Little Chalfont, UK). *In situ* hybridizations were performed as previously published (López *et al.*, 2006; Chakravarthy *et al.*, 2007; López *et al.*, 2008; Lage *et al.*, 2010; López *et al.*, 2010).

Similar anatomical regions were analyzed using the rat brain atlas of Paxinos & Watson (1986). The slides from all experimental groups were exposed to the same autoradiographic film. All sections were scanned and the hybridization signal was quantified by densitometry using a digital imaging system (ImageJ 1.33, NIH) (López *et al.*, 2006; Chakravarthy *et al.*, 2007; López *et al.*, 2008; Lage *et al.*, 2010; López *et al.*, 2010). The optical density of the hybridization signal was determined and subsequently corrected by the optical density of its adjacent background value. A rectangle, with the same dimensions in each case, was drawn enclosing the hybridization signal over each nucleus and over adjacent brain areas of each section (background). For the *in situ* analysis we used between 6–8 animals per experimental group and 12 sections for each animal. The mean of these 12 values was used as the densitometry value for each animal.

Serum markers

During the eleventh week of the study mice were fasted overnight (16 h) and blood was collected by retro-orbital puncture. Glucose was measured in whole blood using an Ascensia Elite glucose meter (Bayer, Mishawaka, IN). After centrifugation sera were stored at -80°C until assay. Leptin measurements were taken with the Luminex-based bead array method using the LINCOplex simultaneous multianalyte detection system (Linco Research, Inc., St. Charles, MO) following the manufacturer's instructions. Adiponectin levels were assayed using the mouse ELISA kit from Alpco Diagnostics (Salem, NH) following the manufacturer's protocol except sera were diluted 1:40,000 (instead of 1:20,000) prior to assay.

Tumorigenesis

Skin tumor formation was promoted using an established model that employs 7,12-dimethylbenz[a]anthracene (DMBA) to initiate tumor formation followed by repeated treatments with 12-O-tetradecanoylphorbol-13-acetate (TPA) to promote tumor development (Abel *et al.*, 2009). Immediately before DMBA tumor initiation a 2 cm² treatment area was shaved into the back just above the base of the tail. All mice were treated with a single dose of 25 μ g DMBA dissolved in 100 μ L of acetone. Tumor promotion with

TPA (4 µg dissolved in 100 µL acetone) began 2 wk after DMBA initiation and continued twice weekly until at least 1 papilloma with a radius > 1 mm was recorded for tumor incidence data. Papilloma-positive mice were euthanized 15 wk after DMBA for 129S mice and 18 wk after DMBA for B6 mice. Final papilloma count was measured upon euthanization.

Statistical analysis

Data are expressed as means ± standard deviation from the mean (SD). Two-factor analyses of variance (ANOVA) were used to assess the effects of ARC condition (*Npy*^{-/-} vs. *Npy*^{+/+}, MSG vs. SAL), diet (AL vs. CR) and their interaction. Tests were performed using Sigma Stat with statistical significance set at *P* < 0.05. All pairwise comparisons were made using Bonferroni t-tests in Sigma Stat and StatView using *P* < 0.0083 as significant except where otherwise noted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the Intramural Research Program of the National Institute on Aging (NIH), Fondo Investigaciones Sanitarias (ML: PS09/01880), Ministerio de Educacion y Ciencia (CD: BFU2008; ML: RYC-2007-00211), the European Union (CD and ML: Health-F2-2008-223713: Reprobesity), and Xunta de Galicia (ML: 10PXIB208164PR). CIBER de Fisiopatología de la Obesidad y Nutrición is an initiative of ISCIII. We would also like to acknowledge the excellent technical assistance of Dawn Nines, Dawn Phillips-Boyer and Justine Lucas.

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Fig. 1.

Body weight and food intake in *ad libitum*-fed mice and mice on calorie restriction. (A) Body weight over time in the B6 mice show weight gain was attenuated by CR regardless of genotype or treatment. Arrows indicate onset of carcinogen treatment starting with tumor initiation (DMBA) at 11 wk on the diets and followed by tumor promotion (TPA) starting at 13 wk on the diets. (B) Body weight over time in the 129S groups. See also Fig. S1. for body composition analysis of the groups. (C) B6 food intake over time. AL food intake was monitored and the CR animals were fed daily weighed amounts that were reduced to 70% of AL intake gradually with full CR achieved after week 6. (D) 129S food intake over time. See also Fig. S2 for rate of food consumption in CR mice. Data are represented as the mean $\pm$ SD.

Fig. 2.

The neuropeptide response to CR is attenuated in the ARC and NPY knockout models (A) *In situ* hybridization for NPY in the ARC of B6 mice shows relative NPY expression and its localization in the brains and ARC (inset) of the different groups. (B) *In situ* hybridization for NPY in the ARC of 129S brains. (C) NPY expression increased with CR in the SAL mice and, while expression is barely detectable in the MSG-treated mice, it is not increased with CR ($n \geq 6$). (D) NPY staining in the 129S brains also shows increased NPY expression after CR in wild type mice. NPY was not detected in any of the AL or CR knockout mice ($n \geq 6$). See also Fig. S3 for further characterization of neuropeptide expression in and around the ARC. (E) Serum levels of NPY in B6 mice was not affected by MSG treatment but was significantly reduced by CR. (F) In 129S mice, serum NPY was also reduced by CR in wild type mice while NPY levels were nearly imperceptible in knockout mice. Data are represented as the mean $\pm$ SD. * = $P < 0.0083$ from AL within genotype/treatment group. † = $P < 0.0083$ from genotype or treatment within diet. ND = not detected, 3V = third ventricle.

Fig. 3.

CR is ineffective against tumorigenesis after ARC disruption or NPY knockout. (A) The course of tumor development in B6 mice shows that all groups began to present with tumors between 12 to 15 wk following DMBA treatment. The groups differ in rate of tumor development, and a comparison of the AUC as an indicator of overall tumor development (graphed in the insert) shows tumorigenesis is less in the SAL CR group compared with SAL ALs, but was increased in the MSG CR group compared with MSG ALs ($n = 6$). (B) Progression of tumor incidence over time in 129S mice shows tumor onset began between 9 to 11 wk after DMBA treatment. Tumorigenesis overall (insert) is decreased by CR in the wild types but is unaffected by diet in the knockouts ($n = 11-13$). (C) Average tumor count per mouse showed no significant differences among B6 groups ($n = 6$). (D) The average number of tumors per individual in 129S mice was significantly reduced by CR in the wild type group, but there was no difference made by diet in the knockout group ($n = 10-12$). Data are represented as the mean $\pm$ SD. * = $P < 0.0083$ from AL within genotype/treatment group.

Fig. 4.

ARC and NPY knockout mice do not show increased circulating adiponectin levels in response to CR. (A) Circulating glucose in B6 mice is reduced by CR in SAL control mice but not in MSG treated mice ($n = 6$). (B) Glucose is reduced by CR in 129S mice regardless of NPY status ($n = 5-6$). See also Fig. S4 for insulin levels. (C) Serum leptin is increased in MSG AL mice compared with SAL AL controls and is reduced by CR regardless of ARC condition ($n = 5-6$). (D) Leptin is reduced in the 129S mice regardless of NPY condition ($n = 4-6$). (E) An increase in adiponectin was seen both with CR and MSG treatment compared with SAL AL ($n = 9-12$). CR did not increase adiponectin levels in the MSG mice. (F) In 129S mice, CR increased adiponectin levels only in wild type mice ($n = 10-12$). Data are

represented as the mean $\pm$ SD. * = $P < 0.0083$ from AL within genotype/treatment group. [†]
= $P < 0.0083$ from genotype or treatment within diet.