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Single-nucleus RNA sequencing of the hypothalamic arcuate nucleus of C57BL/6J mice after prolonged diet-induced obesity

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Abstract

Prolonged obesity is associated with blunted feeding and thermogenic autonomic responses to leptin, but cardiovascular responses to leptin are maintained. This state of “selective leptin resistance” (SLR) is therefore proposed to contribute to the pathogenesis and maintenance of obesity-associated hypertension. Cells of the arcuate nucleus of the hypothalamus (ARC) detect leptin, and although the cellular and molecular mechanisms remain unclear, altered ARC biology is hypothesized to contribute to SLR. Male C57BL/6J mice were fed a high fat diet (HFD) or chow from 8–18 weeks of age, as this paradigm models SLR. Nuclei were then isolated from ARC for single-nucleus RNA sequencing (snRNA-seq). HFD caused expected gains in adiposity and circulating leptin. Twenty-three unique cell-type clusters were identified, and Ingenuity Pathway Analysis (IPA) was used to explore changes in gene expression patterns due to chronic HFD

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Disclosures

None.

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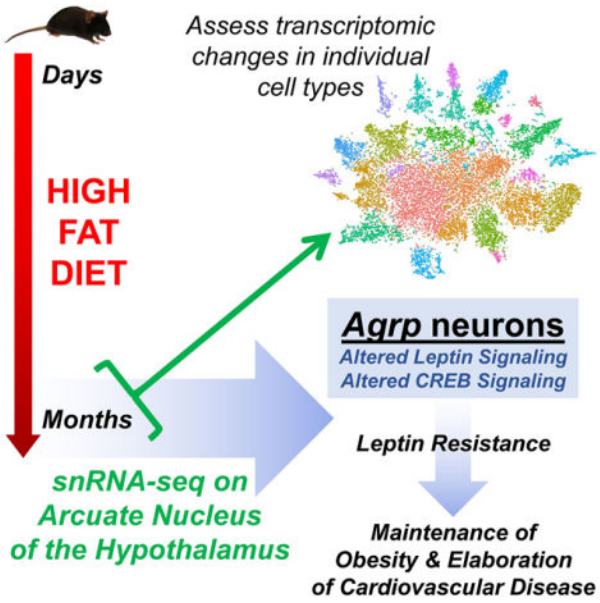
within each cluster. Notably, gene expression signatures related to leptin signaling exhibited suppression predominantly in neurons identified as the Agouti-related peptide (*Agrp*) subtype. IPA results were also consistent with alterations in cAMP response element-binding protein (CREB) signaling in *Agrp* neurons after HFD, and reduced phosphorylated CREB was confirmed in ARC after prolonged HFD by capillary electrophoresis-based Western blotting. These findings support the concept that prolonged HFD-induced obesity is associated with selective changes in *Agrp* neuron biology, possibly secondary to altered CREB signaling.

Graphical Abstract



Keywords

Obesity; Metabolism; Leptin; Arcuate Nucleus; Hypothalamus

Introduction

Obesity represents a major risk factor for the development of hypertension, and the leptin and melanocortin systems are implicated in this process.[1] Although leptin normally acts to inhibit energy intake and increase energy output, obesity is associated with hyperleptinemia and attenuated energy balance effects of leptin. Simultaneously, the stimulatory effects of leptin upon autonomic projections innervating cardiovascular tissues is maintained during obesity. This resistance to the metabolic effects and maintenance of cardiovascular effects of leptin (i.e. selective leptin resistance, SLR) has been proposed to contribute to the development and maintenance of obesity and obesity-associated cardiovascular disease.[2]

Leptin is known to act at its receptor (LEPR) in the arcuate nucleus of the hypothalamus (ARC) to stimulate an array of second-messenger signaling cascades; these mechanisms result in the subsequent activation of multiple neural networks throughout the hypothalamus and beyond to ultimately promote melanocortin signaling and autonomic activity to many

effector tissues (recently reviewed[3]). Importantly, although many mechanisms of LEPR signaling that contribute to the control of energy balance have been mapped, the changes in these networks that mediate leptin resistance during prolonged obesity remain unclear. We hypothesize that changes in second-messenger signaling cascades within specific cell types of the ARC, such as proopiomelanocortin (*Pomc*)- or Agouti-related peptide (*Agrp*)-expressing neurons, underlie the development of SLR.

Previously, we demonstrated that ten weeks of a moderate high fat diet (HFD; OpenSource D12451; 45% kcal from fat / 35% kcal from carbohydrate) feeding in C57BL/6J male mice caused diet-induced obesity (DIO) and SLR.[4] Mice fed HFD from 8 to 18 weeks of age exhibited attenuated autonomic activation and feeding behaviors in response to leptin compared to mice maintained on standard chow (Teklad 7913; 18% kcal from fat / 59% kcal from carbohydrate) for the same timeframe. In the current study, we utilized single nucleus (sn)RNA-seq to dissect the unique transcriptional changes within individual cell types of the ARC that are induced in this model of prolonged HFD feeding, to identify molecular perturbations that may underlie changes in leptin sensitivity.

Methods

All methods are included in the associated supplemental methods document, and all data and materials that support the findings of this study are available from the corresponding authors upon reasonable request. All studies were approved by the Institutional Animal Care and Use Committee at the University of Iowa. Briefly, male C57BL/6J mice were maintained on a chow diet (Teklad 7913) or fed HFD or from 8–18 weeks of age before nuclei from the ARC were isolated and analyzed by snRNA-seq using the 10X Genomics platform. Sequencing libraries were constructed using the Chromium Single Cell 3’ GEM, Library & Gel Bead Kit v3 and sequenced on an Illumina HiSeq 4000 with 150 basepair (bp) paired-end reads. Resulting FASTQ sequencing files are publicly available at PRJNA604055. Data were analyzed using Cell Ranger v3.0.1 (10X Genomics) and the R package Seurat v3.0. Differentially expressed genes within resulting nuclei clusters were used for canonical pathway and upstream pathway analyses using the Ingenuity Pathway Analysis (IPA) software package.[5] In a separate cohort of mice, CREB phosphorylation was assessed in ARC punches using the Simple Wes system (Protein Simple, San Jose, CA) as previously described.[6]

Results

HFD increases adiposity and circulating leptin

Male C57BL/6J mice (Jackson Laboratories, #000664) were randomly assigned to diet treatment. Body masses and adiposity were confirmed to not differ between groups at 6 weeks of age (Fig 1A). Mice were switched to 45% HFD (OpenSource D12451) at 8 weeks of age or maintained on standard chow (Teklad 7913) for the remainder of the study. As expected, HFD caused increased body mass, adiposity, and plasma leptin levels compared to chow-fed mice at 18 weeks of age (Fig 1B). At 10 weeks of age and again at 18 weeks of age, food intake was assessed by daily measures of food hopper masses. Caloric intake was significantly increased at 10 weeks of age, but this increase was attenuated at 18 weeks of

age, despite the increased body mass (Fig 1C). Whereas feeding efficiency (a rough, inverse metric of energy expenditure) was not different between HFD- and chow-fed mice at 10 weeks of age, it was significantly increased in the HFD-fed group at 18 weeks of age. Such results support the concept that total energy expenditure relative to weight gain is reduced in the HFD-fed group despite increased plasma leptin levels. Thus, energy output mechanisms appear to exhibit resistance to the effects of increased endogenous plasma leptin levels after ten weeks of exposure to HFD; these results are consistent with our previous demonstration that thermogenic autonomic and feeding behavior responses to acute exogenous leptin administration are attenuated after ten weeks of HFD.[4]

snRNA-seq identifies 23 cell types in ARC

At 18 weeks of age, each mouse was euthanized, the hypothalamus was dissected, and nuclei from the ARC were isolated and utilized for snRNA-seq (Tables S1 & S2). Sequence alignment, quality control (QC) assessment and feature-barcode generation were performed using the Cell Ranger software suite (10x Genomics). The QC metrics of this dataset were of similar quality to other recent snRNA-seq studies[7, 8] (Table S3, and Figures S1 & S2). After filtering out low-quality nuclei, followed by combining nuclei from all six samples, unbiased clustering analyses via the R package Seurat[9] identified twenty-three unique clusters of nuclei (Fig 2A). Each cluster was populated by nuclei from both chow- and HFD-fed animals (Fig 2B) and defined by the top uniquely expressed gene(s) in that cluster (Fig 2C). The cell types represented by each cluster were then identified based on comparison to three previous studies examining the transcriptomes of individual cell types within the ARC of mice.[10–12] Clusters without a previously-defined cell type were identified by the two most abundantly expressed genes associated specifically with that cluster. As expected, major cell types that are sensitive to leptin and known to be present within the ARC were identified, including cells characterized by high levels of expression of *Pomc* (cluster 5) or *Agrp* (cluster 15).

Cluster-specific alterations in gene expression with HFD

The impact of chronic HFD on the transcriptome of each nuclear cluster was determined by performing differential gene expression analyses (FDR $q < 0.1$, $|\ln FC| \geq 0.15$, HFD v Chow) using Seurat (Supplemental Data File 2). With these cutoff values, HFD was associated with profound changes in gene expression ranging from 60 differentially expressed genes in cluster 0, to 1300 genes in cluster 18 (median across all clusters = 328, mean across all clusters = 418) (Fig 2D). Volcano plots were constructed to visualize gene expression changes within clusters with the greatest number of differentially expressed genes (clusters 2, 8, and 18), clusters of canonically leptin-sensitive cell types including cluster 5 (*Pomc* neurons) and cluster 15 (*Agrp/Npy* neurons), and inflammatory cells such as cluster 16 (microglia) (Fig 2E). Notably, despite elevated endogenous plasma leptin levels within the HFD group (Fig 1B), several well-recognized leptin targets were differentially expressed in these clusters in directions opposite that which is expected in response to leptin. For example, within cluster 5 (*Pomc* neurons), *Pomc* expression was significantly suppressed by prolonged HFD feeding, and within cluster 15 (*Agrp*/*Npy* neurons) the expression of *Agrp* was significantly increased by prolonged HFD feeding (Fig 2E).

Leptin pathway analysis identifies robust changes in Agrp neuron after prolonged HFD

To more comprehensively examine cell type-specific changes in response to leptin signaling after prolonged HFD, we used Ingenuity Pathway Analysis (IPA) to probe each cluster for enrichment of the "*Leptin Signaling in Obesity*" gene set. This analysis method reports both a p-value and a z-value. The p-value is calculated by Fisher's exact test and reflects confidence that genes in the selected gene set (i.e. "*Leptin Signaling in Obesity*") from the selected cluster are responding to the stimulus (i.e. HFD) as a group, regardless of the directionality of the response of each gene. In contrast, the z-value is an assessment of the match between the expected direction vs the observed gene expression responses, integrated across all genes in the gene set. It reflects the overall direction of response to the stimulus, such that a positive value ($z>0$) indicates that the gene set is responding in the predicted direction, which may be up- or down-regulated depending on the expected responses of individual genes within the set. A negative z-value ($z<0$) instead indicates that the genes in the set are generally responding in the opposite direction than would be predicted. A zero z-value ($z=0$) indicates that although the expression levels of individual genes may be significantly changed within the set, that cumulatively there is no pattern in the directionality of response of the group of genes; some genes are responding as predicted and others are responding in the opposite direction as would be predicted.

Surprisingly, despite increased plasma leptin levels in the HFD-fed group (Fig 1B), no cluster exhibited a statistically significant ($p<0.05$) positive ($z>0$) response for this set of leptin-responsive genes (Table 1, and Supplemental File 3). More surprisingly, of the five clusters assigned a significant ($p<0.05$) p-value, only three were awarded a non-zero z-score; indeed, the three clusters exhibiting significant p-values were awarded negative z-scores. Cluster 8, whose cell type was not clearly identified but was characterized by strong expression of *Foxb1* and *Rprm*, has significant enrichment of leptin-responsive genes but there was little pattern in the directionality of the response of the genes as a group. Cluster 15, the *Agrp*/*Npy* neuronal cluster, and cluster 18, one of three oligodendrocyte clusters, were each awarded a more impressive z-score of −1.633. Of these two, however, enrichment was only statistically significant in the *Agrp/Npy* cluster (cluster 15). The determination that canonical leptin-responsive genes exhibit expression patterns opposite of what is expected given elevated plasma leptin, combined with the selective negative z-score for the leptin-responsive gene set specifically in cluster 15 but not cluster 5, support multiple interim conclusions. First, molecular evidence for leptin resistance is present within the ARC after 10 weeks of HFD feeding, thereby confirming alterations in ARC biology during prolonged obesity. Second, the most pronounced reduction or reversal in responsiveness to leptin after this prolonged HFD feeding is observed in the *Agrp*/*Npy* neuron, which strongly contrasts with the traditional field-wide focus upon the *Pomc* neuron subtype but agrees with a handful of recent high-profile studies.[13, 14]

Long-term HFD feeding interrupts CREB signaling in the Agrp neuron

A previous study examined the effects of short-term (1 week) feeding of an ultra-HFD / low-carbohydrate (D12492: 60% kcal from fat / 20% kcal from carbohydrate) versus low-fat / high-carbohydrate (D12450B, 10% kcal from fat / 70% kcal from carbohydrate) diet upon the transcriptomes of cells within the ARC.[11] To dissociate the effects of short-term versus

prolonged exposure to HFD, we compared the list of differentially expressed genes within the current *Agrp*/*Npy* neuronal cluster (cluster 15) to genes that were differentially expressed in analogous *Agrp*/*Npy* clusters from the dataset from Campbell (Fig 3A, and Tables S4–S7). We determined that 529 genes were differentially expressed in this cell type with only short-term HFD feeding, and 47 genes were differentially expressed in similar ways with both short-term and prolonged HFD feeding. In contrast, 683 genes were differentially expressed only after prolonged HFD feeding, and 34 genes that were differentially expressed during short-term HFD feeding exhibited the opposite expression pattern after long-term HFD feeding. The resulting 717 genes that exhibited unique patterns of expression with prolonged HFD feeding were then analyzed by IPA to identify canonical pathways that are uniquely altered within *Agrp*/*Npy* neurons after prolonged HFD feeding. Given our biased focus upon this cell cluster, it was unsurprising to note significant negative z-scores for pathways associated with leptin signaling (such as *Leptin Signaling in Obesity*, z-score = −1.633, and *JAK/STAT Signaling*, z-score = −1.890). Interestingly, the *cAMP Response Element-Binding Protein (CREB) Signaling in Neurons* pathway was identified as having the most consistent and robust alteration in expression pattern, and this pattern was negative (z-score = −2.714) (Table 2, and Supplemental File 3). Similarly, the identification of candidate upstream regulators by the IPA algorithm supports likely inhibition of Protein Kinase A (PKA) in cluster 15 (z-score = −2.208) (Supplemental File 4).

CREB phosphorylation is reduced in ARC after prolonged HFD

Finally, we confirmed reductions in CREB phosphorylation within the ARC using capillary electrophoresis-based Western blotting. ARC tissue from chow- and HFD-fed animals was isolated, homogenized, and analyzed for phosphorylated (pCREB) and total (tCREB) cAMP Response Element Binding Protein and β-actin content (Fig 3B and Figure S3). As anticipated given the above sequencing data, ARC samples from HFD-fed mice were characterized by a reduced pCREB/tCREB ratio (Fig 3C). Together these data support the working hypothesis that prolonged HFD feeding results in disrupted PKA/CREB signaling within the ARC, and more specifically, within *Agrp* neurons.

Discussion

Our group previously established that 10 weeks of HFD feeding was sufficient to model SLR in mice, as this simple intervention resulted in blunted thermogenic brown adipose sympathetic nervous activity responses to leptin while renal sympathetic nervous responses to leptin were maintained.[4] While disparate data from our group and others supports the involvement of various second-messenger systems within the hypothalamus in SLR, snRNAseq-based dissection of the transcriptomic changes induced within cell types of the ARC in this diet-induced model provides a simple and unbiased method to tease apart the cellular and molecular mechanisms that contribute to SLR. Given the overwhelming focus of the field upon the biology of the *Pomc* neuron in mediating the physiological and pathophysiological responses to leptin, it was surprising to discover a much more robust signature for leptin dysfunction in *Agrp* neurons after prolonged obesity. These results lead to the conclusion that molecular evidence of SLR is present at the level of the ARC, independent of whether other synergistic changes are discovered in second-order regions of

the hypothalamus or within the brainstem. Further, these results support the iconoclastic conclusion that the *Agrp* neuron may contribute a much more important role in the development of SLR than other cell types of the ARC. Further, data presented herein provide new, independent, unbiased identification of altered CREB signaling – specifically within the *Agrp* neuron – as a potential mediator of SLR.

Other experimental approaches have supported the concept that changes in *Agrp* neuron biology contribute to the pathogenesis of SLR, and identified potential genetic mediators. For example, leptin modulates the electrical activity of *Agrp* neurons, and diet-induced obesity is associated with persistent activation of these cells.[15] Activation of c-Jun N-terminal kinase 1 in *Agrp* neurons is sufficient to induce leptin resistance,[16] and inhibition of PKA activity[17] or ablation of Forkhead Box O1[18] in these cells prevents leptin resistance. Conditional deletion of *Lepr* from *Agrp* neurons also attenuates sympathetic nerve activity to interscapular brown and subcutaneous white tissues, and accelerates weight gain.[19] Thus, identification of altered CREB, 14-3-3, JAK/STAT, and leptin signaling in *Agrp* neurons after prolonged HFD in the current study (Table 2) provides independent support for these molecular mechanisms, specifically within *Agrp* neurons, in the pathogenesis of SLR.

Multiple studies have also demonstrated that leptin resistance is correlated with changes in the control of *Agrp* expression within the ARC of C57BL/6J mice. Short-term HFD (1- to 7-weeks) causes suppression of Agrp mRNA despite 4- to 6-fold elevations in endogenous plasma leptin.[20, 21] In contrast, longer-term HFD exposure (8- to 18-weeks) is associated with loss of this suppression despite maintenance of elevated plasma leptin.[21, 22] Further, 20-weeks HFD feeding has been shown to abolish ARC *Agrp* expression, food intake, and body mass responses to acute exogenous leptin in diet-sensitive C57BL/6J mice.[23] Importantly, all of these studies analyzed Agrp expression in the complete ARC, and did not analyze expression in individual cell types. Future studies to discern changes in responses of *Agrp* (and other genes) to acute leptin delivery within individual cell types after prolonged diet-induced obesity are logical, given the results of the current study.

A large body of evidence supports a major role for the *Pomc*-expressing neuron in the control of sympathetic nervous system activity to both cardiovascular and metabolic targets, and the signaling cascades and neural projections that mediate these effects are largely defined (recently reviewed in detail[24, 25]). In contrast, mechanisms contributing to the control of *Agrp* expression and the activity of *Agrp*-expressing neurons are not deeply characterized at this time, however, a role for CREB has been suggested. For example, manipulation of phosphodiesterase-3B resulted in reciprocal changes in *Agrp* expression within mouse hypothalamic mHypoE-46 cells.[26] Further, mice lacking the RIIβ regulatory subunit of PKA exhibit altered leptin signaling and resistance to diet-induced obesity, and inhibition of PKA activity within *Agrp* neurons partially recapitulated this effect.[17] CREB signaling has also been implicated in the induction of NOR1 in response to leptin, which additionally acts to modulate the expression of *Agrp* within the ARC.[27] Studies of mouse hypothalamic GT1–7 cells have demonstrated a complex interaction between CREB and ERK signaling in the transcriptional control of *Agrp,*[28] and insulin has also been shown to act via Extracellular signaling Related Kinase (ERK) within mHypoE-46 cells to control *Agrp* expression.[29] Importantly, we previously demonstrated that intracerebroventricular

delivery of ERK inhibitors PD98059 or U0126, but not the phosphoinositide 3-kinase (PI3K) inhibitor LY294002, attenuated leptin-induced sympathetic nervous activity responses to thermogenic brown adipose while having no effect on renal, lumbar, or adrenal sympathetic nerve responses to leptin.[30] Interestingly, in other cell types it has been demonstrated that the PD98059 and U0126 ERK inhibitors are also known to indirectly interfere with PKA/CREB signaling,[31, 32] while the PI3K inhibitor LY294002 stimulates CREB signaling.[33] Finally, angiotensin II type 1A receptors (AT_{1A}) are known to activate second-messenger signaling cascades involving both CREB and ERK, and we previously demonstrated a critical role for AT_{1A}, localized to a subset of *Agrp*-expressing neurons of the ARC, in the control of *Agrp* gene expression, brown adipose sympathetic nerve activity, and resting metabolic rate in response to various stimuli.[34, 35] These findings reinforce the working hypothesis that a complex mechanism involving CREB and ERK (but apparently not PI3K) contributes to the control of *Agrp*. Future work to comprehensively characterize the transcriptional control of *Agrp* and the functional control of *Agrp* neuron activity is clearly required to understand the development of SLR.

Finally, increasing evidence supports the concept that glial (astrocytes, microglia, oligodendrocyte, etc.) cell dysfunctions and hypothalamic inflammation may contribute to obesity and leptin resistance.[36–38] While no significant changes in canonical leptin signaling after prolonged HFD were observed in glial cell types in the current study (Table 1), further exploration of IPA outputs for these various cell types (Supplemental Files 2–4) may inform these mechanisms. For example, interleukin-3, −6, −17A, and −23 signaling pathways were significantly changed ($p<0.05$, $z\leq-1$) in Cluster 18, and such cytokines have been implicated in the control of leptin sensitivity and *Agrp* expression.[39, 40] Thus, a role for diet-induced changes in the activities of other (non-*Agrp*) cell types in the development of SLR may additionally be supported by the current study.

Limitations

This study must be interpreted in light of its limitations. First, this study is limited by the inherent sequencing depth issues that plague all single nucleus and single cell RNA-seq studies; changes in expression of low-abundance transcripts are not detectable. This complication limits our ability to analyze the distinct transcriptomes of subtypes of each major cell type (eg – subtypes of *Agrp* neurons), and also limits analysis of changes in low-abundance transcripts (eg – G protein coupled receptors, and leptin receptor). Second, the study is limited by a relatively small number of replicates. Consensus on methods to calculate power and sample size has not been reached with regard to single nucleus RNA-seq studies, but we suspect that the current study is underpowered to detect smaller changes in gene expression within and across clusters. The current sample size was selected for pragmatic reasons including cost and the goal of restricting sequencing to a small (6 to 8) number of samples to permit simultaneous sequencing of all samples in a single lane, which reduces experimental error. Third, and again for pragmatic reasons, only one sex was examined in the current study. Males were chosen for study because of their greater propensity for weight gain on a 45% HFD, plus our previous demonstration that ten weeks of 45% HFD is sufficient to model leptin resistance in male C57BL/6J mice.[4] It has been clearly established that sex differences exist with regard to *Agrp* neuron biology,[41–43] and

thus the identification of changes specifically within *Agrp* neurons in the current study provides a critical rationale to pursue future studies of gene expression changes in the ARC after prolonged HFD in both sexes. Fourth, our comparison of the effects of long-term versus short-term HFD feeding took advantage of the previous study from Campbell et al.[11] In their study, the authors examined gene expression in cells of the ARC between mice that were fed an ultra-HFD (60% kcal from fat) compared to a low fat / high carbohydrate (10% kcal from fat, 70% from carbohydrates) diet for just 1-week. In the current study, mice were fed a HFD (45% kcal from fat) diet for ten weeks, or maintained on a standard chow. It is likely that the difference in diets (both "HFD" and "control" diets) between studies will therefore account for some of the variability between datasets, and thus contribute to the relatively low overlap of the lists of differentially expressed genes at 1- vs 10-weeks (Fig 2A). As the cost of snRNA-seq studies decreases, it will be appropriate to replicate time-course studies in a much more controlled manner to more comprehensively identify differences in the transcriptomes of individual cell types of the ARC after short- vs long-term HFD feeding and diet-induced obesity. Fifth, in the current study we utilized the single nucleus (snRNA-seq) instead of a single cell (scRNA-seq) approach. Because scRNA-seq requires a large fraction of viable cells, snRNA-seq has become the preferred method to analyze transcriptomes of banked or frozen tissues. Most importantly, several studies have compared the outcomes of simultaneous analyses by each method and a high congruence of results has been observed in various tissue types including mouse visual cortex,[44] and mouse kidney,[45] and human induced pluripotent stem cells.[46] Nonetheless, future validation of the snRNA-seq approach specifically within the ARC is warranted. Sixth, we utilized the C57BL/6J mouse for these studies, and it is known that these animals exhibit spontaneous individual sensitivity or resistance to weight gain and other cardiometabolic phenotypes in response to HFD.[23] Thus, some within-group variability in the current study may result from our grouping of animals that individually may or may not have exhibited sensitivity to the HFD stimulus. Finally, we utilized an analysis approach that focused on gene network analysis, which reduces reliance upon analysis of low-abundance transcripts. As a result, some biologically-significant changes in expression of individual genes within each cell type are likely to be missed. It is important to therefore appreciate that the current study provides positive implication of alterations in generalized leptin and CREB signaling within the *Agrp* neuron of the ARC during prolonged diet-induced obesity – but should not be taken as evidence against the importance of other mechanisms that are likely active in other cell types such as the Pomc neuron.

Perspectives

The findings of this study lead to several exciting conclusions. First, prolonged HFD feeding is associated with unique gene expression patterns that are qualitatively and quantitatively distinct from the patterns observed with short-term HFD exposure. Second, we conclude that long-term HFD feeding is associated with molecular evidence of leptin resistance at the level of the ARC, the primary central nervous system detection site for circulating leptin. Third, this decoupling of leptin signaling is most pronounced in specific cell types of the ARC, such as the *Agrp*/*Npy* neuron – but not the substantially more well-characterized *Pomc* neuron. Fourth, unbiased exploration of the dysfunctions within the *Agrp*/*Npy* neuron after

prolonged HFD feeding point to dysfunction of CREB signaling pathways. Thus, it follows that selective leptin resistance may involve dysfunctional CREB-mediated control of *Agrp* neuronal function, and subsequent disruption of the portion of the melanocortin signaling system that specifically contributes to the control of energy balance. Clarification of the mechanisms by which CREB contributes to *Agrp*/*Npy* neuronal function in health and disease is warranted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Novelty and Significance

What Is New?

- Single nucleus RNAsequencing (snRNAseq) was used to examine transcriptomes of individual cell types of the mouse arcuate nucleus after prolonged diet-induced obesity
- Unlike short-term dietary interventions, prolonged high fat diet caused selective changes in leptin signaling in the Agouti-related peptide (*Agrp*) neuron subtype
- Changes in leptin signaling in the *Agrp* neuron after prolonged diet-induced obesity are paralleled by changes in CREB signaling

What Is Relevant?

- Selective leptin resistance is proposed to contribute to the pathogenesis and maintenance of obesity-associated hypertension, but the cellular & molecular mechanisms remain unclear
- The iconoclastic findings presented here support a dominant role for altered biology of the *Agrp* neuron, not the much more well-characterized proopiomelanocortin (*Pomc*) neuron, in selective leptin resistance

Summary

Understanding the cellular and molecular changes that occur with prolonged obesity is critical to understand and address selective leptin resistance and thereby obesity-associated cardiovascular disease.

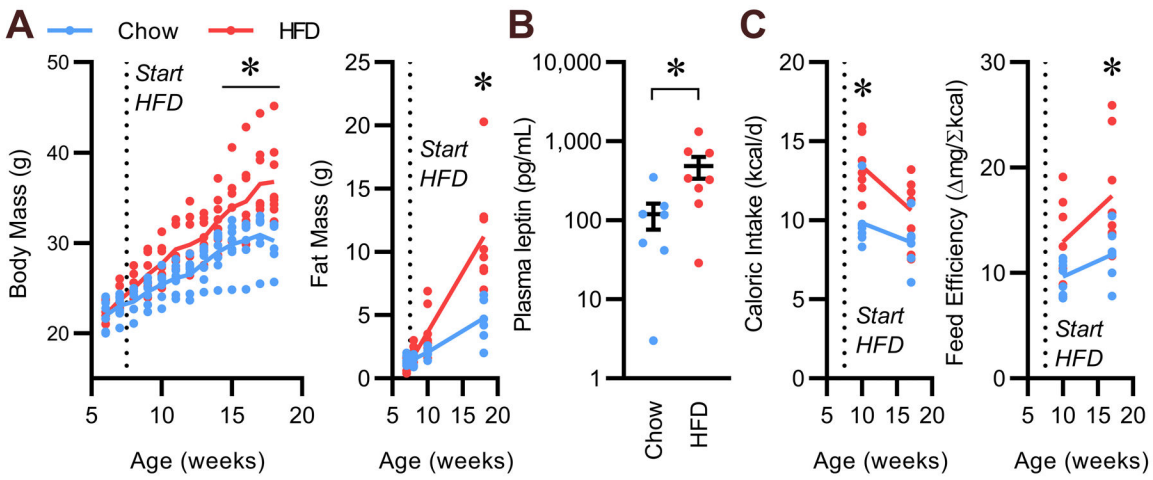

Figure 1. Prolonged HFD feeding increases body mass and plasma leptin.

(A) Body masses and fat masses determined by time-domain NMR, versus age in mice fed HFD (45% kcal from fat) versus chow from 8 – 18 weeks of age. (B) Plasma leptin at 18 weeks of age. (C) Caloric intake and feeding efficiency after 2 and 10 weeks of dietary intervention. n=7 chow and n=8 HFD; trendlines represent mean; summary data presented as mean ± SEM; dots represent individual animals; *p<0.05 by Tukey multiple-comparisons procedure (A, C) or two-tailed Student's t-test after Log_{10} transform (B).

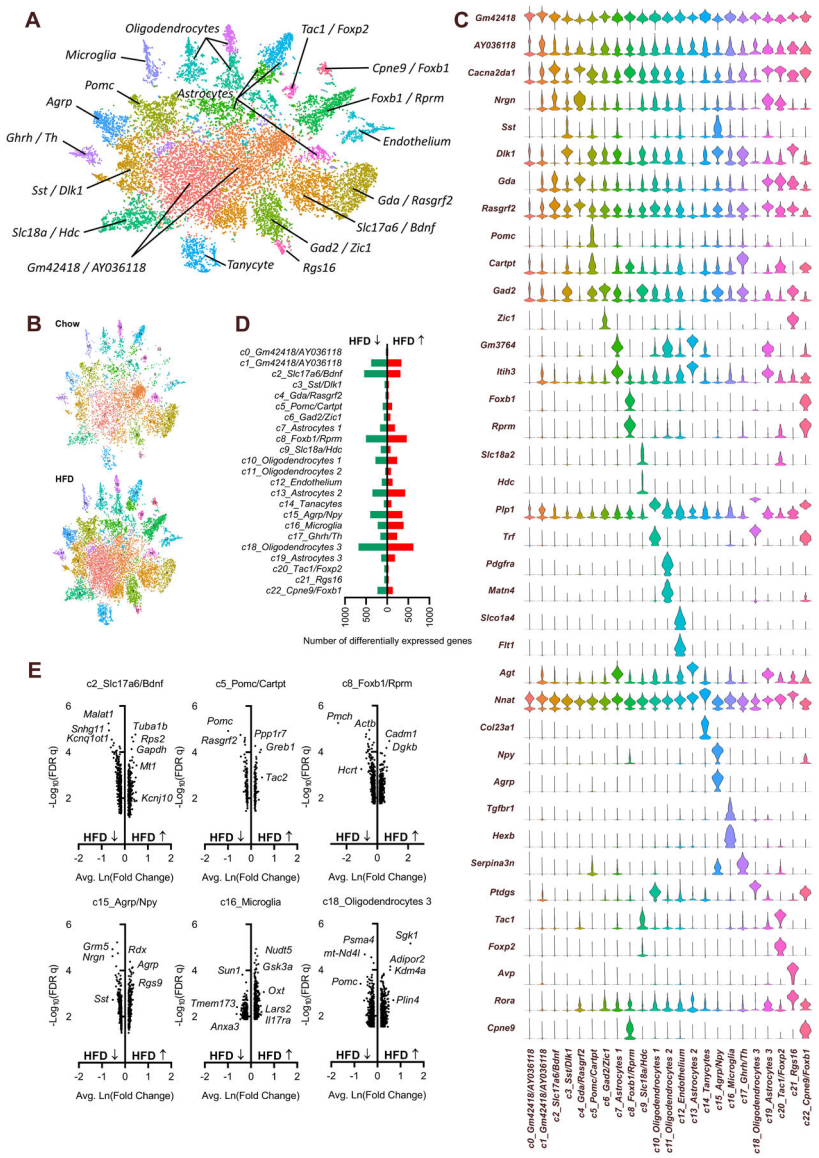

Figure 2. Prolonged HFD feeding causes differential expression of various genes in individual cell types of the ARC.

(A) t-distributed stochastic neighbor embedding (TSNE) plot of cell clusters, identified by Seurat. (B) TSNE plot of cell clusters, split by diet. (C) Expression of top genes that uniquely identify individual clusters. (D) Differential gene expression counts, HFD vs Chow, by cluster. (E) Volcano plots of differentially-expressed genes, HFD vs Chow, in selected clusters. Related information also included in Tables S1–S3 and Figures S1–S2.

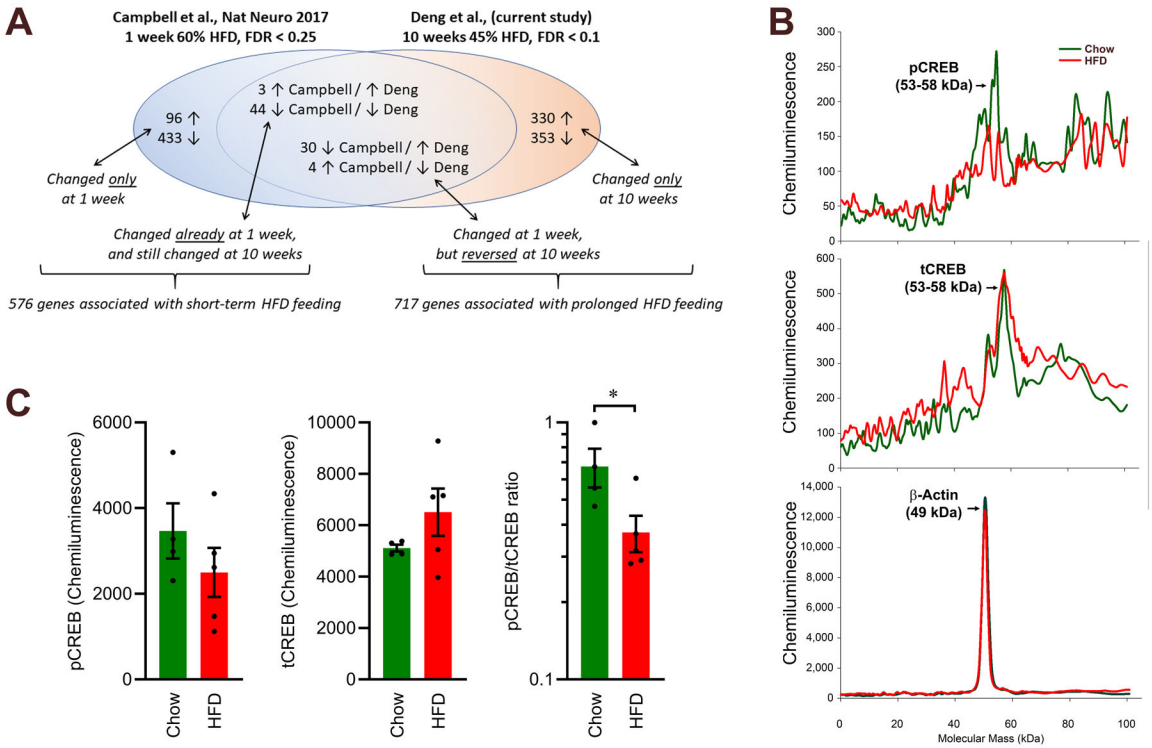

Figure 3. Comparison of differentially expressed gene lists within *Agrp*/*Npy* neuron clusters between short-term and prolonged HFD exposures, and altered CREB signaling in the ARC after prolonged HFD feeding.

(A) Genes that were differentially expressed in *Agrp*/*Npy* cells from Campbell et al. (1 week ultra-HFD vs low-HFD) and the current study (10 weeks HFD vs chow) were compared to identify patterns of gene expression that are unique to short-term versus prolonged HFD exposures. (B) Example chemiluminescent profile of capillary electrophoresis-based Western blots for pCREB, tCREB, and β-actin in samples from ARC tissue. (C) Quantification of area under the curve from chemiluminescence profiles. Chow n=4, HFD n=5. *p<0.05 by two-tailed Student's t-test after Log_{10} transform (C). Related information also included in Tables S4–S7.

Hypertension. Author manuscript; available in PMC 2021 August 01.

Table 1.

Comparison of IPA "Leptin Signaling in Obesity" gene expression signature enrichment between cells assigned to each cluster, from chow- vs HFD-fed animals.

Cluster	Cell type	Genes within cluster that match "*Leptin Signaling in Obesity*" gene expression signature & exhibit significant changes in expression with HFD	Z-score	P-value
0	Gm42418/AY036118	*Pomc*	*(none)*	0.197
1	Gm42418/AY036118	*Plcb4, Pik3c2a, Map2k2, Akt3*	*(none)*	0.288
2	Slc17a6/Bdnf	*Pik3c2a, Fgfr1, Pomc*	*(none)*	1.000
3	Sst/Dlk1	*Npy, Agrp*	*(none)*	0.048
4	Gda/Rasgrf2	*Pomc*	*(none)*	0.238
5	Pomc/Cartpt	*Agrp, Pomc*	*(none)*	0.201
6	Gad2/Zic1	*(none)*	*(none)*	*(none)*
7	Astrocytes 1	*Fgfr3, Prkacb, Plce1, Pomc*	*(none)*	0.044
8	Foxb1/Rprm	*Npy, Plcb4, Mapk1, Fgfr1, Plcb1, Pomc, Stat3, Adcy8*	−0.447	0.031
9	unkSlc18a/Hdc	*(none)*	*(none)*	*(none)*
10	Oligodendrocytes 1	*Pik3r3, Gab1, Fgfr1, Fgfr2, Plcb1, Agrp, Pomc*	0.000	0.003
11	Oligodendrocytes 2	*Pik3ca*	*(none)*	0.413
12	Endothelial Cells	*Pde3a, Pomc*	*(none)*	0.252
13	Astrocytes 2	*Mapk3, Pomc*	*(none)*	1.000
14	Tanycytes	*(none)*	*(none)*	*(none)*
15	Agrp/Npy	*Prkar2b, Ptpn11, Fgfr1, Plcb1, Agrp, Pomc, Stat3, Map2k1*	−1.633	0.009
16	Microglia	*Prkar2b, Pik3r2*	*(none)*	1.000
17	Ghrh/Th	*Plcb4, Prkar2b, Adcy1*	*(none)*	0.188
18	Oligodendrocytes 3	*Gab2, Akt1, Prkar2b, Map2k2, Pik3r1, Pomc, Jak2, Pik3r2*	−1.633	0.122
19	Astrocytes 3	*Prkar2a*	*(none)*	1.000
20	Tac1/Foxp2	*Lepr*	*(none)*	0.316
21	Rgs16	*Mapk1*	*(none)*	0.324
22	Cpne9/Foxb1	*Lepr, Plcg1, Pik3r2*	*(none)*	0.154

Additional information included in Supplemental File 2.

Table 2.

Ingenuity Pathway Analysis pathway enrichment in *Agrp/Npy* cell cluster (cluster 15) after prolonged (10 wk) but not short-term (1 wk) HFD ($p<0.05$, $|z|>1.5$).

IPA Pathway	Z-score
CREB Signaling in Neurons	−2.714
Actin Cytoskeleton Signaling	−2.496
Insulin Receptor Signaling	−2.333
Huntington's Disease Signaling	−2.236
14-3-3-mediated Signaling	−2.121
FcγRIIB Signaling in B Lymphocytes	−2.000
Amyotrophic Lateral Sclerosis Signaling	−1.890
Prolactin Signaling	−1.890
JAK/Stat Signaling	−1.890
Synaptic Long Term Potentiation	−1.667
Leptin Signaling in Obesity	−1.633
ATM Signaling	1.633
Androgen Signaling	1.633
Cardiac β-adrenergic Signaling	2.121

Additional information included in Supplemental Files 3 and 4.