Defining the Transcriptional Targets of Leptin Reveals a Role for *Atf3* in Leptin Action

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Diabetes 2018;67:1093–1104 | https://doi.org/10.2337/db17-1395

Leptin acts via its receptor (LepRb) to modulate gene expression in hypothalamic LepRb-expressing neurons, thereby controlling energy balance and glucose homeostasis. Despite the importance of the control of gene expression in hypothalamic LepRb neurons for leptin action, the transcriptional targets of LepRb signaling have remained undefined because LepRb cells contribute a small fraction to the aggregate transcriptome of the brain regions in which they reside. We thus employed translating ribosome affinity purification followed by RNA sequencing to isolate and analyze mRNA from the hypothalamic LepRb neurons of wild-type or leptin-deficient (*Lep*$^{ob/ob}$) mice treated with vehicle or exogenous leptin. Although the expression of most of the genes encoding the neuropeptides commonly considered to represent the main targets of leptin action were altered only following chronic leptin deprivation, our analysis revealed other transcripts that were coordinately regulated by leptin under multiple treatment conditions. Among these, acute leptin treatment increased expression of the transcription factor *Atf3* in LepRb neurons. Furthermore, ablation of *Atf3* from LepRb neurons (Atf3^{LepRb}KO mice) decreased leptin efficacy and promoted positive energy balance in mice. Thus, this analysis revealed the gene targets of leptin action, including *Atf3*, which represents a cellular mediator of leptin action.

Leptin, which is produced by adipocytes in proportion to their triglyceride content, signals the repletion of body energy stores to modulate diverse determinants of energy balance and glucose homeostasis (1–3). The absence of leptin or its receptor (LepRb) results in hyperphagic obesity and predisposition to diabetes. Hypothalamic LepRb neurons play a particularly crucial role in the control and coordination of metabolic parameters including food intake, energy expenditure, and glucose homeostasis (1,4–6). Although we have defined many of the second messengers in LepRb signaling (7–12), the downstream mediators of leptin action in hypothalamic LepRb neurons remain largely undefined.

LepRb is a type I cytokine receptor of the interleukin-6 receptor family (13,14). Like other members of this family, ligand binding to LepRb stimulates the autophosphorylation and activation of the noncovalently receptor-associated Janus kinase 2 (JAK2) tyrosine kinase, which promotes the subsequent phosphorylation of multiple tyrosine residues on the intracellular tail of LepRb (15,16). Importantly, phosphorylation of LepRb Tyr_{1138} leads to the recruitment and phosphorylation of signal transducer and activator of transcription-3 (STAT3), a latent transcription factor whose phosphorylation promotes its translocation to the nucleus to mediate the control of gene expression (9,15). This pathway is crucial for leptin action, since mice lacking LepRb Tyr_{1138} or ablated for STAT3 in LepRb neurons mimic the hyperphagia and obesity of leptin-deficient (*Lep*$^{ob/ob}$) or LepRb-deficient (*Lepr*$^{db/db}$) mice (7,17–19).

Although LepRb→STAT3 signaling (and thus the regulation of gene expression by LepRb) is necessary for leptin action, the identities of most leptin-regulated target genes in LepRb neurons have remained undefined (20,21). Exceptions to this include *Socs3*, a leptin-stimulated feedback inhibitor of LepRb signaling (22). Leptin also regulates the expression of some neuropeptide-encoding genes that are found in specific subtypes of arcuate (ARC) LepRb neurons (including

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Received 17 November 2017 and accepted 27 February 2018.

This article contains Supplementary Data online at http://diabetes.diabetesjournals.org/lookup/suppl/doi:10.2337/db17-1395/-/DC1.

the anorectic *Pomc* and orexigenic *Npy* and *Agrp* genes) (1,23–25). Because disruption of LepRb in the neurons that express these genes fails to recapitulate the phenotype of LepRb-null *db/db* mice (26,27), however, these cannot represent the only salient genes controlled by leptin action. Indeed, the disruption of *Npy* or *Agrp* minimally alters energy balance in mice (28). Thus, defining the leptin-mediated control of gene expression in hypothalamic LepRb neurons will be crucial as we seek to understand the cellular mediators of leptin action.

RESEARCH DESIGN AND METHODS

Animals

Rosa26$^{eGFP\text{-}L10a}$, *Lepr*^{cre}, and *Lepr*$^{cre/cre}$;*Rosa*$^{eGFP\text{-}L10a/eGFP\text{-}L10a}$ (LepRb$^{eGFP\text{-}L10a}$) mice were generated as previously described (29–31). LepR$^{eGFP\text{-}L10a}$ mice were crossed to *Lep*$^{ob/+}$ mice purchased from The Jackson Laboratory (stock no. 000632) to obtain *Lepr*$^{cre/cre}$;*Rosa*$^{eGFP\text{-}L10a/eGFP\text{-}L10a}$;*Lep*$^{ob/+}$ mice, which were subsequently intercrossed to generate *Lepr*$^{cre/cre}$;*Rosa*$^{eGFP\text{-}L10a/eGFP\text{-}L10a}$;*Lep*$^{ob/ob}$ (LepRb$^{eGFP\text{-}L10a}$;*Lep*$^{ob/ob}$) mice for study. For the generation of Atf3^{LepRb}KO mice, *Atf3*$^{flox/+}$ mice (as described [32]) were bred to *Lepr*$^{cre/cre}$ mice for two generations to obtain *Lepr*$^{cre/cre}$;*Atf3*$^{flox/+}$ mice, which were interbred to obtain sibling *Lepr*$^{cre/cre}$;*Atf3*$^{+/+}$ (control) and *Lepr*$^{cre/cre}$;*Atf3*$^{flox/flox}$ (Atf3^{LepRb}KO) mice for study. POMC-dsRed transgenic mice have been described previously (33), as have *Agrp*^{cre} mice (The Jackson Laboratory, stock no. 012899) (34), which we bred to the *Rosa*$^{eGFP\text{-}L10a}$ background to label AgRP neurons.

All procedures involving animals were approved by the University of Michigan University Committee on the Use and Care of Animals in accordance with guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care International and the National Institutes of Health. Animals were bred at the University of Michigan and maintained in a 12-h light/12-h dark cycle with ad libitum access to food and water except as noted in experimental protocols.

Leptin Treatment and RNA Sequencing

Mixed male and female 10–13 week old LepRb$^{eGFP\text{-}L10a}$ and LepRb$^{eGFP\text{-}L10a}$;*Lep*$^{ob/ob}$ mice had food removed at the onset of the light cycle. Animals were immediately treated with metreleptin (5 mg/kg, i.p) (a generous gift from MedImmune, Inc.) or vehicle (0.9% saline) for analysis 3 or 10 h post-treatment. Hypothalami were dissected and mRNA was isolated from eGFP-tagged ribosomes, as well as from the eGFP-depleted supernatant, and subjected to RNA sequencing (RNA-seq) as previously described (31,35,36). Differential expression was determined using Cufflinks Cuffdiff analysis, with thresholds for differential expression set to fold change >1.5 or <0.66 and a false discovery rate of ≤ 0.05 (37). Lists of differentially expressed genes for the comparisons noted in the results were prepared and queried against the UniProt database for gene ontology and protein class analysis (38). These gene lists were then compared with the differentially expressed genes, and groups of genes that were both differentially regulated and expressed underwent clustered image map (https://discover.nci.nih.gov/cimminer) (39) analysis.

RNA Extraction of Whole Hypothalami and Analysis by RT-qPCR

RNA was extracted from microdissected hypothalami using TRIzol (Invitrogen) according to the manufacturer's protocol or obtained from translating ribosome affinity purification (TRAP) isolation, as above, and subsequently converted to cDNA using iScript cDNA Synthesis Kit (Bio-Rad #1708891) for use in RT-PCR. cDNA was analyzed in duplicate by real-time quantitative PCR (qPCR) on an Applied Biosystems StepOnePlus Real-Time PCR System for TBP (endogenous control) and each of the following: *Socs3, Atf3, JunB, Arid5a,* and *Etv6*. All TaqMan assays were acquired from Applied Biosystems (Foster City, CA).

Perfusion and Immunohistochemistry

For phospho-STAT3 (pSTAT3) staining, mice were fasted overnight and treated with leptin (5 mg/kg i.p.) or vehicle 60 min prior to perfusion. For ATF3 staining, animals had food removed at the onset of the light cycle; two hours later, they were treated with leptin (5 mg/kg i.p.) or vehicle for 6 h prior to perfusion. Perfusion, preparation of tissues, and immunostaining were performed as previously described (40) using primary antibodies for GFP (Aves Labs #GFP-1020, chicken, 1:1,000), ATF3 (Sigma #HPA001562, rabbit, 1:1,000), and pSTAT3 (Cell Signaling Technology #9131S, rabbit). All antibodies were reacted with species-specific Alexa Fluor 488– or 568–conjugated (Invitrogen, 1:200) secondary antibodies or processed via the avidin-biotin/diaminobenzidine (DAB) method (ABC kit, Vector Laboratories; DAB reagents, Thermo-Fisher) and imaged as previously described (41). Some DAB images were pseudocolored using Photoshop software.

Phenotyping of Atf3^{LepRb}KO Mice

Mice were weaned at 3 weeks of age and were single-housed for study beginning at 4 weeks of age. Mice were weighed weekly; food was also weighed weekly for the determination of food intake. At 12 weeks of age, animals were subjected to analysis of body composition using a Minispec (Bruker) in the University of Michigan Mouse Metabolic Phenotyping Center. Serum was sampled via tail-vein bleed at 12 weeks of age to obtain serum for the determination of leptin concentrations via ELISA (Crystal Chem) in the Chemistry Laboratory of the Clinical Core of the Michigan Diabetes Research Center. To determine the anorectic effect of leptin, food was removed from male control and Atf3^{LepRb}KO mice prior to the onset of the dark cycle on day 1; 24 h later, the mice received an injection of leptin (5 mg/kg i.p.) and food was returned. Food intake for the subsequent 24 h was determined by weighing the food. Glucose tolerance test (2 g/kg body weight, i.p.) and insulin tolerance test (1 unit/kg body weight, Humulin [Eli Lilly], i.p.) were performed after a 5-h fast that began 3 h after the start of the light cycle. Tail

vein glucose concentrations were determined using a glucometer (Accucheck).

Statistics

Statistical analysis for RNA-seq is described above. RT-qPCR data are reported as mean fold change versus vehicle. Statistical analysis of RT-qPCR data and animal phenotyping data were performed using Prism (version 6.0) software. Unpaired *t* tests were used to compare results between groups of two. $P < 0.05$ was considered statistically significant.

RESULTS

Hypothalamic Transcriptional Programs Induced by Leptin Deficiency and Leptin Treatment

Since LepRb neurons comprise only a fraction of all cells in the hypothalamus (42,43), it has been difficult to isolate LepRb neurons (or the mRNA from these cells) from the surrounding milieu. To mitigate this issue and define the transcriptome of LepRb neurons, we previously used TRAP to isolate ribosome-associated mRNA from LepRb neurons in mice (31). We have now extended this line of inquiry by analyzing gene expression changes in hypothalamic LepRb neurons in response to leptin (Fig. 1*A*). We used LepRb$^{eGFP\text{-}L10a}$ mice (31), which express a chimeric L10a ribosomal subunit fused to enhanced green fluorescent protein (eGFP) (L10-eGFP), permitting the eGFP-mediated immunoprecipitation of ribosomes (and their associated mRNAs) specifically from LepRb neurons. We produced LepRb$^{eGFP\text{-}L10a}$ mice on the wild-type background for treatment with vehicle or leptin (5 mg/kg i.p.) for analysis 3 or 10 h after injection, as well as on the *Lep*$^{ob/ob}$ background for treatment with vehicle or leptin (5 mg/kg i.p.) for analysis 10 h after injection. *Lep*$^{ob/ob}$ mice were obese at the time of analysis (8–12 weeks of age) (body weight: wild-type 22.1 ± 0.7 g, *Lep*$^{ob/ob}$ 35.7 ± 1.2 g).

Following treatment, we dissected hypothalami (4–6 per replicate), performed TRAP purification of LepRb neuron-derived mRNAs, and subjected the TRAP and supernatant mRNA fractions to RNA-seq (TRAP-seq) (3–4 replicates per condition) (Fig. 1*A*). Comparing each TRAP condition to its supernatant revealed the mRNA species enriched in hypothalamic LepRb neurons under each condition, while comparing the TRAP fraction among conditions revealed the regulation of gene expression across conditions.

Since the TRAP fraction contains small amounts of mRNA derived from non-LepRb neurons, we restricted our analysis to LepRb neuron–specific mRNAs by focusing on changes in mRNA for the transcripts found to be enriched in hypothalamic LepRb neurons (under any condition). No differences emerged between LepRb$^{eGFP\text{-}L10a}$ mice treated with vehicle for 3 and 10 h (data not shown), so we combined these data into a single vehicle-treated group for comparisons with other groups. Of the 2,704 mRNA species enriched in hypothalamic LepRb neurons under at least one condition, 364 were different in at least one comparison among conditions (Supplementary Tables 1–3). Figure 1*B* shows a heat map comparing the changes in expression for these genes across the various conditions, revealing that 10-h leptin treatment in wild-type and *Lep*$^{ob/ob}$ animals produced the most related gene expression changes, while changes in gene expression provoked by 3-h leptin treatment in control mice are the least related to the other changes.

Of the genes found to be modulated by these conditions, few demonstrated coordinate regulation (in which leptin treatment changed expression in a consistent direction across conditions and in which leptin deficiency altered gene expression oppositely) (Table 1). Only six of these mRNAs were coordinately regulated across all four conditions (increased with 3- or 10-h leptin in wild-type and with 10-h leptin in *Lep*$^{ob/ob}$ mice; decreased in *Lep*$^{ob/ob}$ compared with wild-type mice). As expected, *Socs3* represents one of these genes, consistent with the known regulation of this gene by leptin in hypothalamic LepRb neurons (22,44).

To validate the results and sensitivity of this TRAP-seq analysis, we used qPCR to analyze the expression of selected genes in TRAP and whole hypothalamic mRNA from mice treated with vehicle or leptin for 3 h (Table 2). In addition to analyzing the expression of *Socs3*, we examined the expression of *Atf3* and *Junb*, which were regulated in response to acute leptin (including at the 3-h time point) and *Arid5a* and *Etv6*, which were also highly regulated by leptin in our TRAP-seq analysis (Supplementary Table 3). Our qPCR analysis confirmed the increased expression of all of these genes in TRAP samples. Although we replicated the increased expression of *Socs3* and *Atf3* in the whole hypothalamic sample, changes in expression of the other three genes were not detectable, presumably due to the expression of these genes in other hypothalamic neurons (31). Thus, TRAP-seq more sensitively detects changes in gene expression in LepRb neurons.

Chronic Control of Neuropeptide Expression by Leptin

The neuropeptides that are expressed in LepRb neurons (e.g., *Pomc, Cartpt, Agrp*, and *Npy*), which are most commonly thought of as the transcriptional targets of leptin action (1,23–25), were absent from the list of genes that were regulated coordinately across three to four conditions. Indeed, even though the expression of most LepRb neuron-enriched neuropeptides was not altered in response to treatment with exogenous leptin in normal or *Lep*$^{ob/ob}$ animals, most were changed in the chronically leptin-deficient *Lep*$^{ob/ob}$ animals compared with wild-type mice (Table 3). Thus, the LepRb neuron-expressed neuropeptides are unlikely to represent the direct transcriptional targets of leptin action but rather must respond to intervening changes. While some of these indirect effects may be mediated by leptin action on upstream neurons (5,6), cell-autonomous transcription factors that rapidly respond to leptin could also participate.

Leptin Treatment Induces ATF3 in LepRb Neurons

Atf3 is highly enriched in LepRb neurons and is the most highly induced of the coordinately regulated genes at the 3-h time point (Table 1). *Atf3* encodes an ATF/CREB family bZIP transcription factor that incorporates into an AP-1 transcription factor complex and has known roles in metabolic

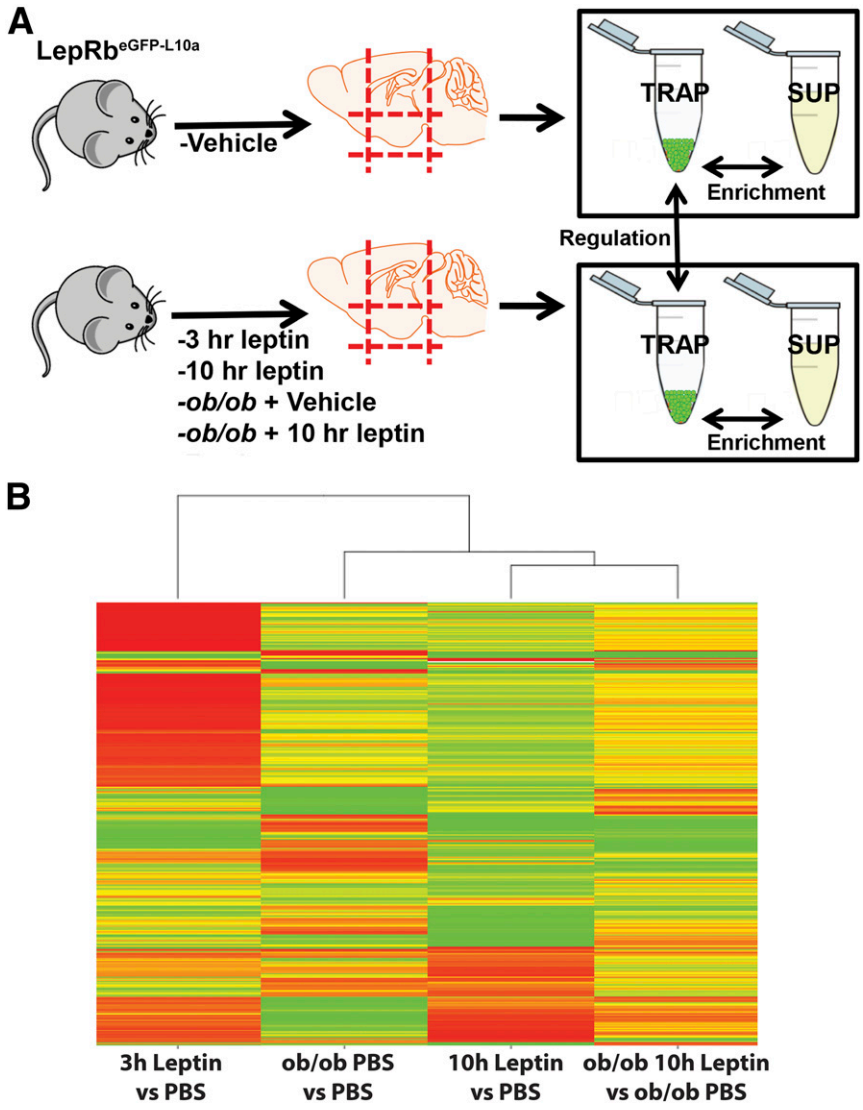

Figure 1—TRAP-seq analysis to identify leptin-regulated genes in hypothalamic LepRb neurons. *A*: Experimental design: LepRb$^{eGFP-L10a}$ mice were subjected to a variety of perturbations in leptin levels before hypothalami were dissected and mRNA-containing ribosomes from LepRb neurons were TRAP purified. TRAP and supernatant mRNAs were subjected to RNA-seq; comparisons of TRAP and supernatant fractions revealed the identity of genes enriched in LepRb neurons under each condition, and comparing TRAP material among conditions revealed the regulation of these enriched genes by leptin. *B*: A heat map demonstrating changes in gene expression in LepRb neuron-derived TRAP samples across conditions. Fold change scale is from 4.1-fold increased (green) to –9.7-fold decreased (red). Euclidean distance among the conditions (maximum 50) is shown along the top. hr, hour; SUP, supernatant.

control (45). To confirm the induction of *Atf3* by leptin in LepRb neurons, we treated normal mice with exogenous leptin (5 mg/kg i.p.) or vehicle and perfused them 6 h later for immunohistochemical (IHC) analysis of ATF3 protein in the hypothalamus (Fig. 2). This revealed that leptin increased ATF3 immunoreactivity (-IR) in the hypothalamus in a pattern reminiscent of the distribution of LepRb neurons. Indeed, performing this analysis in LepRb$^{eGFP-L10a}$ mice revealed the colocalization of ATF3-IR with eGFP-expressing LepRb neurons, confirming the rapid induction by leptin of ATF3 protein in hypothalamic LepRb neurons. Although >90% of ATF3-IR neurons colocalized with eGFP, only 5–10% of eGFP-containing neurons contained ATF3-IR. Despite the low percentage of LepRb neurons that contained ATF3-IR, however, leptin stimulated ATF3-IR in both POMC and AgRP neurons of the ARC (Supplementary Fig. 1). Thus, leptin stimulated the rapid induction of ATF3 in subsets of LepRb-expressing cell populations in the hypothalamic ARC and dorsomedial hypothalamic nucleus (DMH)—two areas that play important roles in the control of energy balance by leptin (1). We did not detect leptin-stimulated ATF3-IR in other brain regions.

Atf3 in LepRb Neurons Contributes to the Control of Energy Balance by Leptin

To determine the role for *Atf3* in LepRb neurons for leptin action, we crossed the conditional *Atf3*^{flox} allele (32) onto the *Lepr*^{cre} background (43). Intercrossing *Lepr*$^{cre/cre}$;*Atf3*$^{flox/+}$ mice

Table 1—Genes coordinately regulated by leptin across multiple conditions in hypothalamic LepRb neurons

Gene	Expression (FPKM), WT PBS	Enrichment (fold), WT PBS	Regulation (fold) WT 3-h Lep vs. WT PBS	WT 10-h Lep vs. WT PBS	Ob PBS vs. WT PBS	Ob 10-h Lep vs. Ob PBS
Serpina3i	1.08	**11.67**	**1.89**	**2.48**	**0.13**	**10.46**
Socs3	3.15	**2.89**	**5.60**	**3.14**	**0.25**	**10.00**
Serpina3h	3.55	**5.83**	**2.97**	**6.15**	**0.32**	**7.33**
Traf3ip3	1.15	**2.83**	**1.61**	**2.36**	**0.53**	**1.91**
Irgm2	0.79	0.90	**1.56**	**1.77**	**0.59**	**1.71**
Prokr2	1.12	1.26	**1.94**	**2.29**	**0.61**	**1.51**
Serpina3c	0.76	**7.70**	1.04	**2.77**	**0.20**	**9.92**
Serpina3n	90.24	**8.14**	1.04	**2.06**	**0.26**	**5.06**
Irf9	9.23	**2.05**	1.38	**1.83**	**0.38**	**3.01**
Bcl3	1.34	**2.42**	1.48	**1.75**	**0.43**	**1.54**
Vwa5a	7.08	**2.33**	1.23	**2.01**	**0.52**	**1.63**
Serpina3m	2.93	**7.67**	1.38	**3.92**	**0.52**	**2.79**
Stat3	37.86	**2.48**	1.44	**1.62**	**0.52**	**2.33**
Asb4	69.15	**7.10**	1.30	**1.74**	**0.61**	**1.64**
Vwce	0.20	1.27	1.01	**0.59**	**2.53**	**0.61**
Rasl11a	12.01	0.96	**1.80**	**1.57**	0.74	**1.83**
Junb	31.52	**2.02**	**1.76**	**1.91**	0.97	**1.67**
Atf3	2.81	**4.35**	**5.82**	**4.58**	1.02	**2.39**
Ly6a	4.95	1.40	**0.57**	**0.45**	**1.67**	0.79
Vwf	0.45	**2.52**	**0.52**	**0.23**	**1.69**	0.76
Acer2	0.86	1.20	**0.66**	**0.44**	**1.72**	0.67
Drd1a	2.30	0.74	**1.63**	0.72	**0.66**	**1.66**
1700024P16Rik	0.11	0.78	**0.59**	1.44	**8.42**	**0.43**

Shown are the LepRb-enriched transcripts that were significantly different for at least three of the comparisons listed and for which increased leptin promoted changes in the same direction (and/or leptin deficiency promoted opposite changes). Expression (in fragments per kilobase per million, FPKM) and enrichment (fold relative to supernatant) are shown for the wild-type vehicle-treated (WT PBS) condition. Note that while not all transcripts shown were enriched in this condition, all were enriched in LepRb neurons in at least one condition. Also shown is the fold change in expression across the listed conditions/comparisons. Lep = leptin, 5 mg/kg i.p. for the indicated time; Ob = *Lep*$^{ob/ob}$ mice. Bolded values are statistically significant between conditions.

generated sibling *Lepr*$^{cre/cre}$;*Atf3*$^{+/+}$ (control) and *Lepr*$^{cre/cre}$;*Atf3*$^{flox/flox}$ (Atf3^{LepRb}KO mice expected to be null for *Atf3* specifically in LepRb neurons) for analysis (Fig. 3*A*). To confirm the ablation of *Atf3* from LepRb neurons in the Atf3^{LepRb}KO mice, we treated control and Atf3^{LepRb}KO mice with leptin (5 mg/kg i.p.) and perfused them 6 h later for the IHC detection of ATF3 (Fig. 3*B* and *C*). This analysis revealed the expected LepRb-like distribution of ATF3 in control mice, while ATF3-IR was absent from the hypothalamus of Atf3^{LepRb}KO mice, consistent with the expected ablation of *Atf3* from LepRb neurons. In contrast, leptin treatment (5 mg/kg i.p., 1 h) promoted the expected induction of tyrosine-phosphorylated STAT3 (pSTAT3)-IR in both control and Atf3^{LepRb}KO mice (Fig. 3*D* and *E*). Thus, the ablation of *Atf3* from LepRb neurons in Atf3^{LepRb}KO mice does not interfere with the leptin-mediated induction of pSTAT3 by leptin.

There was no increase in body weight, food intake, fat mass, or circulating leptin concentrations in male Atf3^{LepRb}KO mice relative to controls (Fig. 4*A–D*). In contrast, female Atf3^{LepRb}KO mice exhibited a (nonsignificant) trend toward increased body weight and circulating leptin concentrations, and body composition analysis revealed a significant increase in adipose mass in the female Atf3^{LepRb}KO mice (Fig. 4*E–G*). Thus, *Atf3* in LepRb neurons contributes to the long-term control of adiposity in females. We examined the potential for adiposity-independent changes in glucose or insulin tolerance in male Atf3^{LepRb}KO mice (Supplementary Fig. 2); these parameters of glucose homeostasis were not different than in controls.

Since *Atf3* expression was not altered by the chronic lack of leptin in *Lep*$^{ob/ob}$ mice, but rather was increased in response to hours-long treatment with exogenous leptin (Table 1), we sought to understand the potential role for *Atf3* in the acute response to leptin. We examined the ability of exogenous leptin (5 mg/kg i.p.) to suppress feeding following a 24-h fast; because body weight, adiposity, and leptin were not different between control and Atf3^{LepRb}KO males, we

Table 2—Increased sensitivity for detecting changes in LepRb neuron gene expression in TRAP relative to whole hypothalamic samples

Gene	Fold change, TRAP	Fold change, whole hypothalamus
Socs3	**12.51**	**6.02**
Atf3	**18.90**	**2.40**
JunB	**4.49**	1.29
Arid5a	**10.67**	1.10
Etv6	**3.48**	1.18

We performed qPCR to measure changes in gene expression following 3 h of leptin treatment (5 mg/kg i.p.) compared with vehicle in LepRb neuron–derived TRAP material or RNA derived from whole hypothalamic lysates. Bolded numbers represent statistically significant changes in gene expression (unpaired *t* test, $P < 0.05$).

used male mice in this assay to avoid the potentially confounding effect of increased adiposity in female mice (Fig. 4*I*). Although leptin suppressed refeeding following a fast in control mice, there was no suppression of food intake by leptin in the Atf3^{LepRb}KO mice. Thus, in addition to its contribution to long-term energy balance in females, *Atf3* in LepRb neurons is required for the acute anorectic response to leptin.

DISCUSSION

We examined the regulation of the LepRb transcriptome in response to leptin deficiency and treatment with exogenous leptin, revealing genes cell-autonomously regulated by acute and chronic leptin and which likely mediate the physiologic response to leptin. Among these, the transcription factor *Atf3* increases in expression in LepRb neurons following acute treatment with leptin, and we demonstrated a role for *Atf3* in LepRb neurons for the anorectic response to leptin.

Table 3—Changes in the expression of neuropeptide-encoding genes across conditions in LepRb neurons

	Change in gene expression (fold)			
Gene	WT 3-h Lep vs. WT PBS	WT 10-h Lep vs. WT PBS	Ob PBS vs. WT PBS	Ob 10-h Lep vs. Ob PBS
Agrp	0.76	1.19	**5.55**	0.94
Apoa1	1.22	1.22	**2.25**	1.00
Ghrh	0.74	0.94	**2.66**	0.96
Nmb	0.90	0.99	**4.14**	0.77
Npy	0.85	1.29	**4.17**	0.89
Rbp4	0.84	0.81	**1.92**	0.90
Nts	0.87	1.36	**0.41**	1.10
Cartpt	0.97	1.12	**0.28**	0.81
Edn3	0.64	0.82	**1.85**	0.77
Gal	0.93	**1.60**	1.01	0.98
Kiss1	1.14	**3.29**	**0.46**	0.97
Ucn	1.11	**0.28**	**0.35**	**0.50**
Pomc	1.01	**1.56**	**0.16**	1.35

Shown are the fold changes in LepRb-enriched transcripts encoding neuropeptides. Lep = leptin, 5 mg/kg i.p. for the indicated time; Ob = *Lep*$^{ob/ob}$ mice. Bolded values are statistically significant between conditions.

Cell-specific TRAP is the preferred method of analysis for neuronal cells that respond poorly to cellular dispersion and sorting techniques; since mRNA is rapidly extracted during TRAP, this method minimizes alterations in gene expression that might occur during lengthy dispersion processes. As for other methods, however, low levels of material from surrounding tissue contaminate the mRNA isolated by TRAP. Indeed, some regulated genes (e.g., *Hcrt*, *Mch*) in our TRAP samples were de-enriched in LepRb neurons and are known to be derived from cells that do not express LepRb (Supplementary Tables 1–2) (31,46). We thus focused our analysis on transcripts that were enriched in LepRb neurons at baseline or that became enriched in response to one of our treatment paradigms, using the standard 1.5-fold enrichment level as our cutoff to ensure that all genes identified represent bona fide targets of leptin action in LepRb neurons. Using less stringent cutoffs for enrichment (e.g., 1.0-fold) would identify additional genes cell-autonomously regulated by leptin in hypothalamic LepRb neurons but would introduce a significant number of false positives (genes that are regulated by leptin in non-LepRb cells).

One of our major focuses was the identification of LepRb neuron-expressed genes coordinately regulated by leptin across multiple conditions (i.e., changed in the same direction following 3- or 10-h leptin treatment on any background and oppositely changed in leptin-deficient animals), since these genes have a high likelihood of representing important transcriptional targets of leptin action. The list of genes coordinately regulated across three or more conditions was small (24); only six were coordinately regulated across all four conditions. In addition to *Socs3* (which is known to be cell-autonomously increased by leptin [22]), several other genes and classes of genes stand out for their potential roles in leptin action: nine extracellular serine protease inhibitors (six members of the SerpinA3 clade, plus *Vwa5a*, *Vwce*, and *Vwf*) were coordinately regulated under three or four conditions. Thus, defining the roles for these genes in leptin action represents a high priority going forward. Additional genes of interest include *Asb4*, for which nearby polymorphisms segregate with increased obesity in human genome-wide association studies (47) and which is highly enriched and regulated by leptin and leptin deficiency in hypothalamic LepRb neurons.

Unsurprisingly, given that LepRb mediates JAK2/STAT3-dependent signaling, a number of the genes most highly and coordinately regulated by leptin include known targets of/participants in cytokine signaling, including *Socs3*, *Trafip3*, *Irgm2*, *Irf9*, and *Stat3*. Roles for *Socs3* and *Stat3* in LepRb signaling have been defined previously (10,19,48), but it will be important to determine roles for these other modulators of cytokine signaling in leptin action.

The preponderance (19/24) of genes coordinately regulated by leptin under three or more conditions increased in

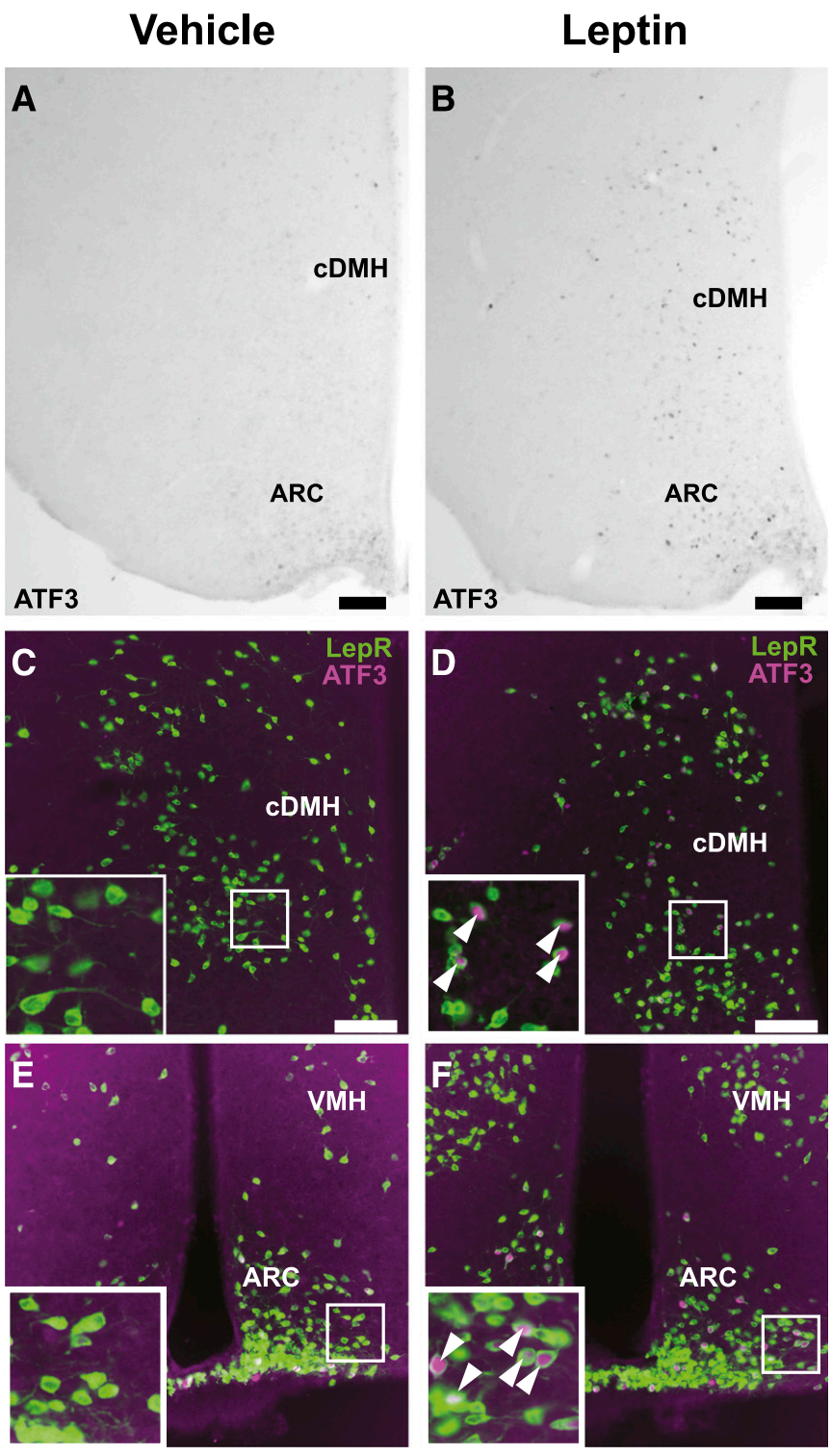

Figure 2—ATF3 is induced specifically in LepRb neurons following acute leptin stimulation. LepRb$^{eGFP\text{-}L10a}$ mice were treated i.p. with vehicle or leptin (5 mg/kg i.p.) for 6 h. Brains were harvested, sectioned, and stained for ATF3 and/or GFP. Images are representative of 3–4 mice per treatment group. *A* and *B*: IHC detection of ATF3-IR (black nuclei) in the hypothalamus of control (*A*) and leptin-treated (*B*) mice. *C–F*: Dual IHC/immunofluorescence for ATF3 and eGFP in the DMH (*C* and *D*) and ARC (*E* and *F*) of control (*C* and *E*) and leptin-treated (*D* and *F*) LepRb$^{eGFP\text{-}L10a}$ mice. White arrowheads indicate colocalized neurons. Scale bars = 100 μmol/L. cDMH, caudal DMH; VMH, ventromedial hypothalamus.

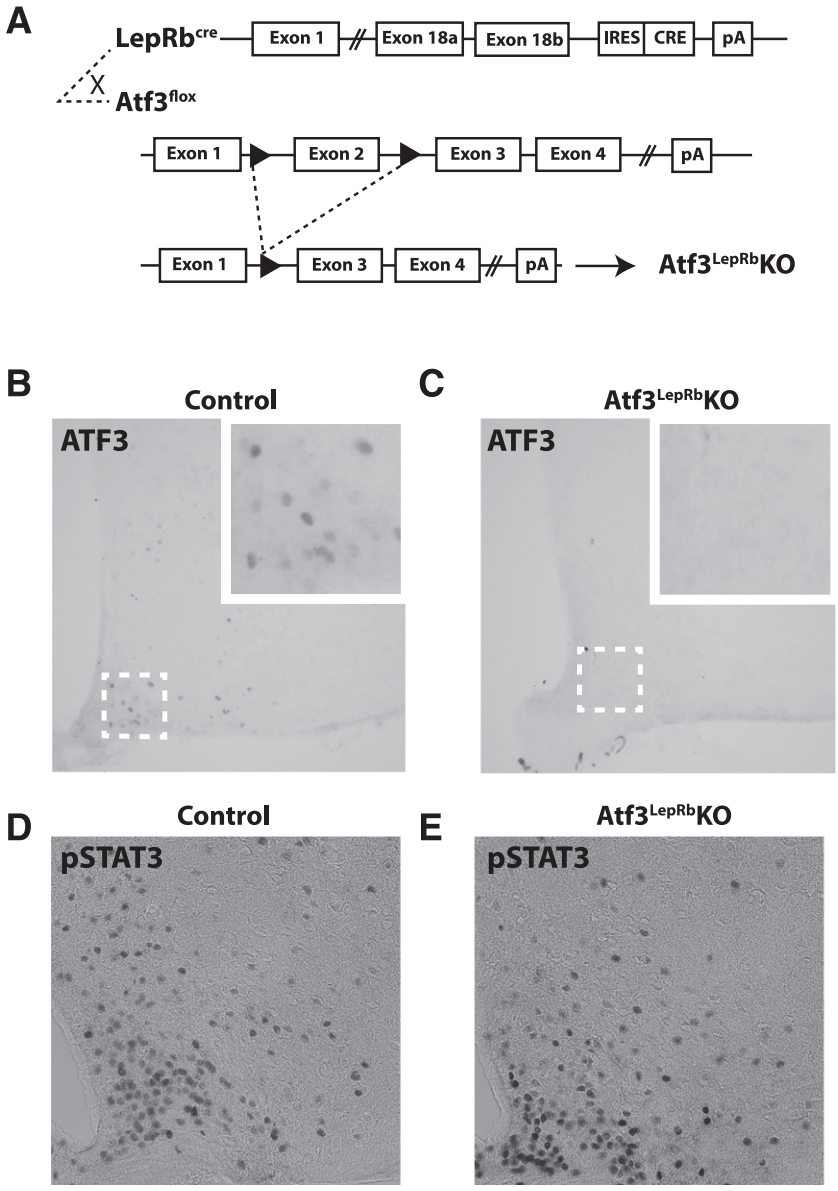

Figure 3—Ablation of ATF3, but not pSTAT3, induction by leptin in Atf3^{LepRb}KO mice. *A*: We crossed *Lepr*^{cre} mice onto the *Atf3*^{flox} background to generate *Lepr*$^{cre/cre}$*;Atf3*$^{flox/flox}$ (Atf3^{LepRb}KO) mice and littermate controls. *B* and *C*: Control (*B*) and Atf3^{LepRb}KO (*C*) mice were fasted for 4 h prior to treatment with leptin (5 mg/kg i.p.) followed by perfusion 6 h later and processing for the IHC detection of ATF3 (black nuclei). *D* and *E*: Control (*D*) and Atf3^{LepRb}KO (*E*) mice were fasted overnight before treatment with leptin (5 mg/kg i.p.) 1 h before perfusion and processing for the IHC detection of pSTAT3 (black nuclei). *B–E*: Digital zoom of the boxed area is shown in the upper right corner.

expression with increasing leptin action, consistent with the notion that STAT3, a major mediator of leptin action, generally increases gene expression. In contrast, the majority of genes regulated by acute leptin action in wild-type mice decreased with leptin treatment; this was especially true for the genes changed only in the samples from mice treated with leptin for 3 h, where 152 of the 158 genes changed were decreased compared with vehicle. Decreasing the expression of genes dramatically over as short a period as 3 h would require very rapid turnover of the mRNA.

It is more likely that the dramatic decrease in recovery of these many mRNA species following 3-h leptin treatment represents an artifact of our method of mRNA purification, since the expression of many genes is controlled at the translational level. For instance, the activity of the mechanistic target of rapamycin complex 1 (mTORC1) pathway increases the translation of mRNA species that encode proteins required for macromolecular (especially protein) synthesis, such as ribosomal subunits and elongation factors (49). Conversely, decreased mTORC1 signaling causes the dissociation of such mRNA species from the ribosome. The vast majority of mRNA species exhibiting decreased TRAP recovery following 3-h leptin treatment encode ribosomal subunits and other proteins involved in macromolecular synthesis, consistent with leptin decreasing mTORC1 activity in a substantial population of hypothalamic LepRb cells (50).

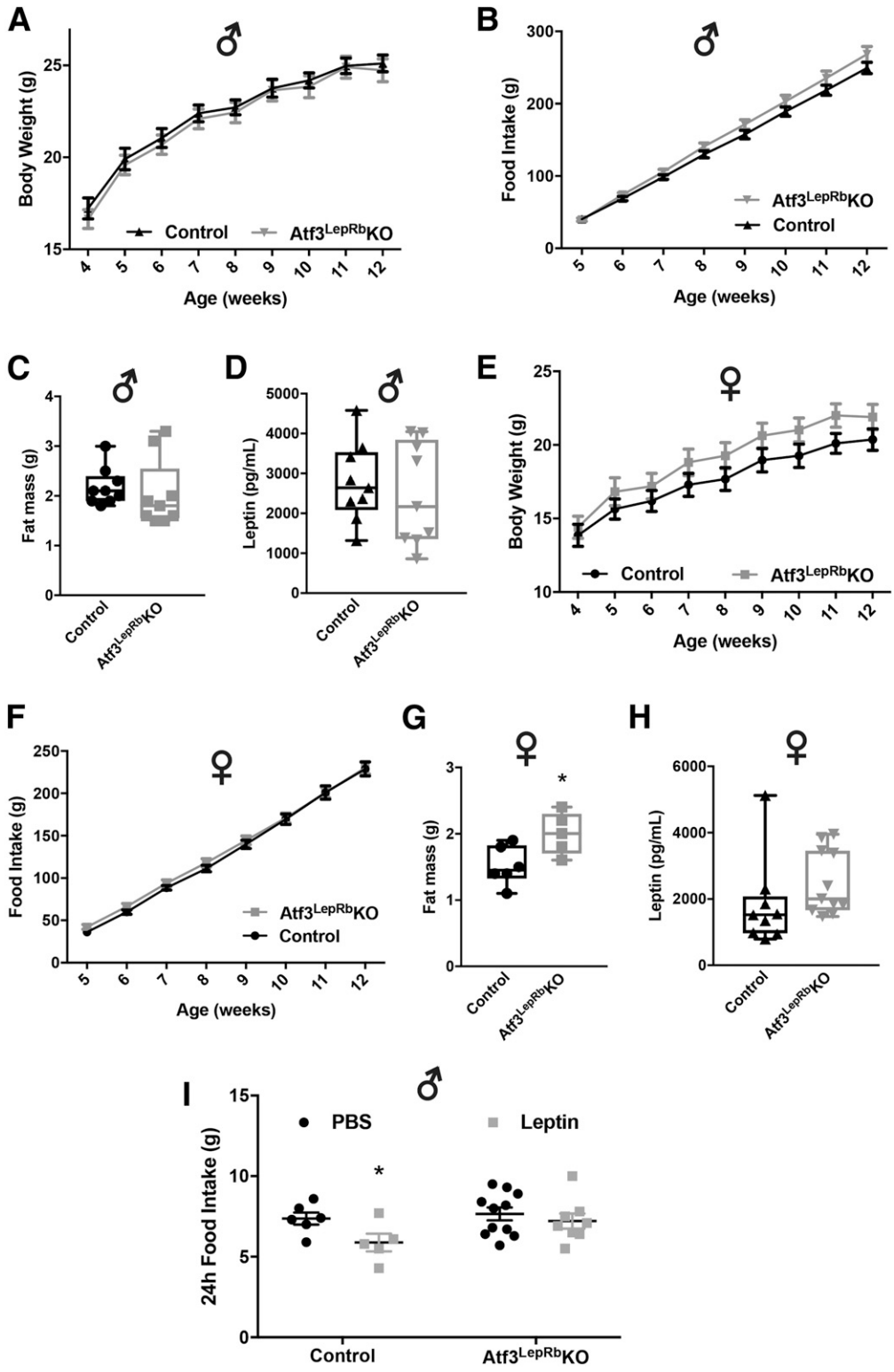

Figure 4—Control of energy balance by leptin in Atf3^{LepRb}KO mice. We examined parameters of energy balance in control and Atf3^{LepRb}KO male (*A–D*, *I*) and female (*E–H*) mice. Longitudinal measurements of body weight (*A* and *E*) and food intake (*B* and *F*) are shown, along with measurements of adipose mass (*C* and *G*) and leptin (*F* and *H*). *I*: Male control and Atf3^{LepRb}KO mice were fasted for 24 h and then treated with vehicle (PBS) or leptin (5 mg/kg i.p.) before the return of food at the onset of the dark cycle. Food intake over the subsequent 24 h is shown. *$P <$ 0.05 by unpaired *t* test.

It is possible that some of the other presumptive changes in gene expression that we have identified in our analysis also represent genes whose expression is controlled at the translational rather than the transcriptional level. Although we confirmed that the mRNA expression of *Socs3* and *Atf3* increased in the hypothalamus following 3-h leptin treatment, the fold increase in whole hypothalamus was much lower than in TRAP samples, and we could not detect the increased expression of *JunB*, *Arid5a*, or *Etv6* in whole hypothalamic mRNA. This failure to detect changes in whole hypothalamic mRNA could result from masking of the signal by the expression of these genes in non-LepRb neurons (indeed, their fold enrichment is much lower than that of *Socs3* and *Atf3*), but it could also reflect an increase in protein translation that would not be detected by qPCR of whole hypothalamic mRNA. In either case, these are genes whose protein expression is predicted to change in LepRb neurons in response to leptin.

Our analysis also suggests the potential for a cascade of gene expression effects downstream of LepRb signaling. Not only did our analysis define sets of genes whose expression changes following acute, but not chronic, alterations in leptin concentrations (i.e., changed with exogenous leptin but not different in $Lep^{ob/ob}$ mice), but also it identified genes that were changed in chronic leptin deficiency but not altered in response to acute treatment with exogenous leptin. Importantly, genes that are altered by chronic leptin deficiency but not by acute leptin include most LepRb neuron–expressed neuropeptides. Thus, the control of most of these neuropeptide genes must require long-term changes in cell physiology (e.g., by leptin-stimulated transcription factors or other parameters) rather than being mediated directly by LepRb second messengers, such as STAT3. *Pomc* represents a potential exception to this rule, since it possesses known STAT3 binding sites in its promotor (51) and its expression increases following 10 h of leptin treatment in addition to being decreased in $Lep^{ob/ob}$ mice. However, the failure of 3 h of leptin to increase *Pomc* expression suggests that secondary changes, not just STAT3, may be required to control the expression of this gene.

To identify a potential such second-order mediator of leptin action, we examined the role for the leptin-stimulated transcription factor *Atf3* in leptin action. Not only does IHC-detectable ATF3 protein increase in ARC and DMH LepRb neurons following leptin treatment, but ATF3 protein appears to accumulate in the nucleus of these cells, suggesting a potential role for ATF3 in the rapid control of gene expression in these cells. We thus crossed $Lepr^{cre}$ onto the $Atf3^{flox}$ background to generate $Atf3^{LepRb}$KO mice ablated for *Atf3* expression in LepRb neurons. The body weight of female $Atf3^{LepRb}$KO mice tended to be higher than that of controls because of their significantly increased adipose mass. Furthermore, exogenous leptin failed to suppress 24-h food intake following a fast in male $Atf3^{LepRb}$KO mice (which displayed no alteration in body weight or adiposity at baseline that might confound such an analysis).

The finding that the ablation of *Atf3* from LepRb neurons only modestly alters adiposity in females and does not alter energy balance in males, but blunts the ability of exogenous leptin to decrease food intake, suggests that *Atf3* in LepRb neurons may primarily participate in the response to elevated leptin. Indeed, we found that *Atf3* mRNA expression in LepRb neurons is not altered in fasted animals (data not shown). Together with the unchanged *Atf3* expression in *ob/ob* mice, these data suggest that ATF3 does not mediate the response to negative energy balance but rather contributes to the response to elevated leptin. Also consistent with the primary role for ATF3 in the response to elevated leptin, the hyperphagic response to fasting is not altered in $Atf3^{LepRb}$KO mice relative to controls; only the suppression of hyperphagia by leptin is diminished.

The small percentage of LepRb neurons that express ATF3 suggests a potentially restricted function for ATF3 in leptin action (and could contribute to the modest phenotype of $Atf3^{LepRb}$KO mice, especially relative to mice lacking STAT3 in LepRb neurons [19]). ATF3 and STAT3 recognize distinct DNA sequences and thus control the expression of different sets of genes; interestingly, both contribute to the response to neuronal injury (e.g., following axotomy) and contribute to neurite outgrowth (52), suggesting a potential role for ATF3 in these processes.

While leptin stimulates pSTAT3 (and its direct target, *Socs3*) in most LepRb neurons (22,31,44) and *Atf3* expression is associated with signaling by interleukin-6 family members (which all activate pSTAT3) following axotomy, we observed no alteration in *Atf3* expression in mice lacking *Stat3* in LepRb neurons (data not shown).

Overall, by examining the responses of the LepRb transcriptome to exogenous leptin and genetic leptin deficiency, we have identified a set of genes that are likely to play important roles in leptin action, including in the control of energy balance and other metabolic parameters. Even though nontranscriptional/nontranslational LepRb signals could also contribute to important aspects of leptin action, at least some of these genes are likely to play important roles in energy balance. One of these genes, *Atf3*, is induced by exogenous leptin administration and appears to participate primarily in the anorectic response to exogenous leptin, rather than in the control of body weight at baseline (at least in male mice). Determining the roles for other genes that we have identified whose expression is coordinately regulated by leptin across multiple conditions will be crucial for our understanding of how the control of gene expression by leptin mediates physiologic leptin action.

Acknowledgments. The authors thank Tsonwin Hai for generously providing $Atf3^{flox}$ mice. The authors thank James Trevaskis and MedImmune, Inc. for the generous gift of leptin and members of the Myers and Olson laboratories for helpful discussions.

Funding. Research support was provided by the Michigan Diabetes Research Center (National Institutes of Health grant P30-DK020572, including the Molecular Genetics, Animal Phenotyping, and Clinical Cores) and the Michigan Mouse Metabolic Phenotyping Center, the American Diabetes Association (M.G.M., W.C., and T.B.), the Marilyn H. Vincent Foundation (M.G.M.), and the National Institute of Diabetes and Digestive and Kidney Diseases (M.G.M.: DK056731; M.B.A.: DK097861; W.P.: GM007315; T.B.: DK101357; K.S.: DK088752).

Duality of Interest. No potential conflicts of interest relevant to this article were reported.

Author Contributions. M.B.A., W.P., D.P.O., and M.G.M. designed experiments, researched and analyzed data, and wrote and edited the manuscript. A.M., C.P., K.S., T.B., W.C., and A.R. researched and analyzed data and proofread the manuscript. M.G.M. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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