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WANG, Zhen, DO CARMO, Jussara M, DA SILVA, Alexandre A, BAILEY, Kandice C, ABERDEIN, Nicola <http://orcid.org/0000-0002-6147-4221>, MOAK, Sydney P and HALL, John E

ROLE OF SOCS3 IN POMC NEURONS IN METABOLIC AND CARDIOVASCULAR REGULATION

Zhen Wang[1,2], Jussara M. do Carmo[1,2], Alexandre A. da Silva[1,2], Kandice C. Bailey[1,2], Nicola Aberdein[3], Sydney P. Moak[1,2], and John E. Hall[1,2]

1-Department of Physiology and Biophysics, University of Mississippi Medical Center, Jackson, Mississippi, USA.

2-Mississippi Center for Obesity Research, University of Mississippi Medical Center, Jackson, Mississippi, USA.

3-Biomolecular Science Research Centre, Sheffield Hallam University, Sheffield, UK.

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Corresponding author:

Zhen Wang, Ph.D.

Department of Physiology & Biophysics

University of Mississippi Medical Center

2500 North State St, Jackson, MS 39216

Phone: (601) 984-1820

Fax: (601) 984-1817

1 E-mail: zwang3@umc.edu

ABSTRACT

Suppressor of cytokine signaling 3 (SOCS3) is a negative regulator of leptin signaling. We previously showed that the chronic effects of leptin on blood pressure (BP) and glucose regulation are mediated by stimulation of pro-opiomelanocortin (POMC) neurons. In this study, we examined the importance of endogenous SOCS3 in POMC neurons in control of metabolic and cardiovascular function and potential sex differences. Male and female SOCS3$^{flox/flox}$/POMC-Cre mice in which SOCS3 was selectively deleted in POMC neurons and control SOCS3$^{flox/flox}$ mice were studied during a control diet (CD) or high fat diet (HFD) and during chronic leptin infusion. On CD, male and female SOCS3$^{flox/flox}$/POMC-Cre mice were lighter in body weight despite similar food intake compared to control mice. Male SOCS3$^{flox/flox}$/POMC-Cre mice exhibited increased energy expenditure. BP and heart rate were similar in male and female SOCS3$^{flox/flox}$/POMC-Cre and control mice on CD. On a HFD, male and female SOCS3$^{flox/flox}$/POMC-Cre mice showed attenuated weight gain. Female SOCS3$^{flox/flox}$/POMC-Cre mice exhibited greater HFD-induced elevations in baseline BP and BP responses to air jet stress test compared to control mice. Chronic leptin infusion produced similar responses in all groups for food intake, body weight, oxygen consumption, blood glucose, BP and heart rate. Thus, SOCS3 deficiency in POMC neurons influences body weight regulation in CD and HFD and differentially affects BP and energy balance in a

sex specific manner, but does not amplify the dietary, glycemic or cardiovascular effects of leptin.

Key words: Blood pressure; obesity; leptin; glucose; energy expenditure; adipose

INTRODUCTION

Suppressor of cytokine signaling 3 (SOCS3) is a cytoplasmic protein that belongs to the family of inducible negative regulators of cytokine signaling and is expressed in various tissues including brain, muscle and adipose tissue. Expression of SOCS3 is induced by several cytokines such as leptin, interleukin (IL)-1, and IL-10. In the leptin receptor signaling pathway, activated SOCS3 binds to phosphorylated tyrosine residues and inhibits leptin receptors downstream activation of Janus kinase (JAK) tyrosine kinase and signal transducer and activator of transcription 3 (STAT3) (3, 6, 12, 26). These effects of SOCS3 to inhibit JAK and STAT3 are believed to negatively regulate leptin signaling and may contribute to development of resistance to leptin's anorexic and metabolic effects in obesity (2, 3, 22). Mice with central nervous system SOCS3 deficiency are resistant to diet-induced obesity (17), suggesting a role for SOCS3 in body weight regulation in obesity.

Pro-opiomelanocortin (POMC) neurons, located within the arcuate nucleus (ARC) of the hypothalamus and in the nucleus tractus solitarius (NTS) of the brainstem, are thought to be important targets for leptin's effects on appetite, blood glucose, and blood pressure (BP)

regulation (15, 16, 21). We previously showed that POMC neuronal specific deletion of leptin receptors abolished leptin's ability to increase BP and to reduce fasting insulin and glucose levels (8). However, the importance of endogenous SOCS3 in POMC neurons for regulation of BP and metabolic functions is still unclear, and there have been no detailed studies, to our knowledge, on potential sex differences in metabolic and cardiovascular regulation by SOCS3 in POMC neurons.

The main goal of the current study was to examine the impact of SOCS3 deficiency in POMC neurons on metabolic and cardiovascular regulation in male and female mice fed a regular control diet (CD) or a high fat diet (HFD). We also determined whether POMC neuronal SOCS3 deficiency amplifies the dietary, metabolic and cardiovascular effects of chronic hyperleptinemia.

Our results indicate that SOCS3 deficiency in POMC neurons influences weight regulation in CD and HFD and differentially affects BP and energy balance in a sex specific manner, but does not amplify the dietary, glycemic or cardiovascular effects of chronic leptin infusion.

MATERIALS AND METHODS

Animals

The experimental procedures described in this study followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the University of Mississippi Medical Center. SOCS3$^{flox/flox}$ mice (generously provided by Dr. George Booz, University of Mississippi Medical Center) have loxP sites flanking exon 2. These mice were crossed with heterozygotic POMC-Cre mice (generously provided by Dr. Joel Elmquist, University of Texas Southwestern Medical Center) that express Cre-recombinase specifically in POMC neurons. Mice that were homozygous for SOCS3$^{flox/flox}$ and expressed Cre-recombinase in POMC neurons were labeled SOCS3$^{flox/flox}$/POMC-Cre, and littermate homozygous SOCS3$^{flox/flox}$ mice not expressing Cre-recombinase were used as controls. SOCS3$^{flox/flox}$/POMC-Cre and SOCS3$^{flox/flox}$ mice are on a mixed C57BL/6 and 129S background. All the studies in female mice were carried out in random cycling females.

Validation of SOCS3 deficiency in SOCS3$^{flox/flox}$/POMC-Cre mice

Validation of specific inactivation of SOCS3 in POMC neurons in SOCS3 $^{flox/flox}$/POMC-Cre mice has been previously reported (19). To further confirm that SOCS3 was inactivated in POMC neurons of SOCS3 $^{flox/flox}$/POMC-Cre mice, immunohistochemistry was performed to examine phosphorylated-STAT3 (p-STAT3), the primary pathway by which

SOCS3 influences leptin signaling(1). Studies were conducted in SOCS3$^{flox/flox}$ and SOCS3$^{flox/flox}$/POMC-Cre mice injected with leptin (5 mg/kg, i.p.) or saline 45 minutes before euthanasia and brain collection. Immunohistochemistry staining was performed using p-STAT3 antibodies (Cell Signaling, Danvers, MA) in frozen coronal brain sections (30 μm thick) to visualize the expression level of p-STAT3 in the arcuate nucleus of the hypothalamus. qRT-PCR was also performed to further examine SOCS3 and protein-tyrosine phosphatase 1B (PTP1B) mRNA expression levels in brain cortex and hypothalamus of male and female SOCS3$^{flox/flox}$/POMC-Cre and control mice. SOCS3$^{flox/flox}$ (n=4-6/group per sex) and SOCS3$^{flox/flox}$/POMC-Cre mice (n=4-6/group per sex) were euthanized, the brains were quickly removed, and the hypothalamus and cortex were isolated on an ice-cold platform. Tissue samples were frozen immediately by immersion in liquid nitrogen and stored at -80^{o}C. Total RNA was extracted using RNase Mini Kit (QIAGEN, Germantown, MD) according to the manufacturer's protocol and quantified by spectrophotometry. Total RNA was reverse transcribed by SuperScript VILO cDNA synthesis kit (Thermo Fisher Scientific, Waltham, MA). qRT-PCR was performed on 1 ng of RNA using a StepOne Plus qRT-PCR system with PowerUP SYBR green master mix (Thermo Fisher Scientific). The following primer pair was used to amplify mouse SOCS3: 5'-GGACCAAGAACCTACGCATCCA-3' (forward) and 5'-CACCAGCTTGAGTACACAGTCG-3' (reverse) PTP1B: 5'-CGCCATGGAGATGGAGAAGG-3' (forward) and 5'-

GTCGAATGTCCTGGTAAATAGCC-3' (reverse). Mouse 18S rRNA was used as an internal control to normalize the expression levels of the target genes and delta-delta CT method was used to calculate the fold changes of target genes in $SOCS3^{flox/flox}$/POMC-Cre mice compared to sex matched $SOCS3^{flox/flox}$ control mice.

Experimental protocols

Between 6 and 17 weeks of age, male and female $SOCS3^{flox/flox}$ (n=9/group per sex) and $SOCS3^{flox/flox}$/POMC-Cre (n=9/group per sex) mice were individually housed and fed a CD (Envigo Teklad Custom Diets, Madison WI. CA-170955, 4.0 kcal/g, 66% Kcal from carbohydrate; 16% Kcal from fat; 18% Kcal from protein with 0.24-0.25% Na^+ and 1% K^+). Food intake and body weight were measured bi-weekly and weekly changes in body composition were analyzed using magnetic resonance imaging (4-in-1 EchoMRI-900TM, Echo Medical System, Houston, TX). When the mice were 18 weeks old, a fasting/refeeding protocol and food intake responses to an acute leptin injection were performed. At 20 weeks of age, oxygen consumption and fasting blood glucose, leptin and insulin concentrations were measured during baseline and during a 7-day leptin infusion. A glucose tolerance test (GTT) was performed at 23 weeks of age.

Separate groups of 23-week-old male and female $SOCS3^{flox/flox}$ and $SOCS3^{flox/flox}$/POMC-Cre mice (n=11/group per sex) were implanted with radiotelemetry probes to measure BP and heart rate (HR). These mice were fed a HFD (Envigo Teklad

Diets, Madison, WI. TD-08811, 4.7 Kcal/g, 45% from fat) for 6 weeks. Food intake, body weight, BP and HR were measured twice each week. At the end of the 6 weeks of HFD, GTT, air jet stress test, fasting blood glucose, leptin and insulin concentrations were examined.

Fasting/refeeding protocol and food intake responses to acute leptin injection

At 18 weeks of age, groups of male and female SOCS3$^{flox/flox}$ (n=6/group per sex) and SOCS3 $^{flox/flox}$/POMC-Cre mice (n=6/group per sex) were examined for daily food intake and body weight for 3 consecutive days as a baseline followed by a 24-hour fast. After the fasting period, the mice were given food ad libitum for 3 days, while daily food consumption was recorded during this refeeding period.

After one week to recover from the fasting/refeeding protocol, the mice were injected with leptin (5 mg/kg, i.p.) or saline vehicle (0.2 mL) 1 hour before lights out (5:00 pm) and food intake was measured at 2, 4, 15, and 24 hours post-injection. A within-subjects design was used in this study. Changes in food intake following leptin injections were compared to saline injection in the same animals.

Oxygen consumption measurements during chronic leptin infusion and after HFD

Male and female SOCS3$^{flox/flox}$ (n=7/group per sex) and SOCS3$^{flox/flox}$/POMC-Cre mice (n=7/group per sex) were placed individually in metabolic cages (Promethion metabolic and behavioral system, Sable Systems International, Las Vegas, NV) to measure body weight,

food intake, feeding bouts (number of food intake events, defined as eating from food hopper by a significant difference before and after the eating event), energy expenditure (EE), respiratory quotient (RQ), and motor activity. Mice were acclimatized to the new environment for 3 days followed by 5 consecutive days of baseline recordings. Then, the mice were anesthetized with isoflurane and an osmotic minipump (model 1007D, Alzet, Cupertino, CA) was implanted i.p. to deliver leptin (4 µg/kg/min) for 7 consecutive days. Metabolic data were recorded during leptin infusion followed by a 5-day post-leptin recovery period. Blood samples (100 µL) were collected via a tail snip after 6 hours of fasting (8:00 am to 2:00 pm) on day 5 of the baseline period, on day 7 of leptin infusion, and at the end of the recovery period to measure plasma glucose, leptin and insulin concentrations.

Metabolic phenotypes including EE, RQ, and motor activity were measured for 7 consecutive days in separate groups of 20-23 week-old $SOCS3^{flox/flox}$ and $SOCS3^{flox/flox}$/POMC-Cre male and female mice fed a HFD for 5 weeks (n=4-6/group per sex).

Oral Glucose Tolerance Test (GTT)

D-glucose (3 mg/kg of lean tissue plus 1 mg/kg of fat mass) was administered by gavage after a 5-h fast in 23 week-old male and female $SOCS3^{flox/flox}$ (n=7/group per sex) and $SOCS3^{flox/flox}$/POMC-Cre (n=10/group per sex). Blood samples were collected by tail snip and blood glucose was measured using glucose strips (ReliOn) at baseline, 15, 30, 60, 90, and 120 minutes after glucose administration. An oral GTT test was also performed in

30-week-old male and female mice of each genotype that were fed a HFD for 6 weeks (n=7/group per sex).

Acute air-jet stress test

Male and female SOCS3$^{flox/flox}$ (n=5/group per sex) and SOCS3$^{flox/flox}$/POMC-Cre (n=6/group per sex) mice at 30 weeks of age were fed a HFD for 6 weeks and placed in special cages used for air-jet stress testing. Briefly, after a 2-hour period of acclimation to the cage, BP and HR were continuously measured by telemetry for 30 minutes before the air-jet stress test was applied. The air-jet stress test consisted of 2-second pulses of air delivered every 5 seconds during 5 consecutive minutes aimed at the forehead of the mice at an approximate distance of 5 cm using a 14 gauge needle opening at the front of the tube connected to compressed air. After the 5-minute air-jet stress, BP and HR continued to be measured for an additional 30-minute recovery period. Changes in BP and HR responses during the air-jet stress and recovery period were calculated compared to the baseline period (average of the last 10 minutes of baseline period before air-jet stress was initiated). We also calculated the areas under curves (AUCs) of the MAP during the air-jet stress and recovery periods using the following parameters: average change in MAP for each minute during the 5-minute air-jet stress test and for every 5 minutes during the 30-minute recovery period.

Liver weight and fat/lean composition

Male and female SOCS3$^{flox/flox}$ (n=6/group per sex) and SOCS3$^{flox/flox}$/POMC-Cre mice (n=6/group per sex) fed a HFD for 8 weeks were sacrificed at 31 weeks of age. Whole livers were harvested and weighed and then analyzed for fat and lean mass content using EchoMRI analyzer.

MAP and HR responses to chronic leptin infusion in mice fed a CD

Subgroups of 23 week old male and female SOCS3$^{flox/flox}$ (n=4/group per sex) and SOCS3$^{flox/flox}$/POMC-Cre mice (n=5/group per sex) were implanted with telemeters. Briefly, mice were anesthetized with 2% isoflurane and a telemetry probe (TA11PA-C10, Data Science International, St. Paul, MN) was implanted in the left carotid artery and advanced into the aorta for measurement of BP and HR, 24 hours/day, using computerized methods (8). Ten days after recovery from surgery, five days of stable baseline BP and HR measurements were recorded. Then, the mice were anesthetized with isoflurane and an osmotic minipump (model 1002, ALZET) was implanted i.p. to deliver leptin (4 µg/kg/min) for 14 days, followed by a 5-day recovery period. BP and HR were measured during the leptin infusion and recovery periods. Blood samples (100 µL) were collected via a tail snip after 6 hours of fasting (8:00 am to 2:00 pm) during the control period (day 5), on day 13 of leptin infusion, and at the end of the recovery period to measure plasma glucose, leptin and insulin concentrations.

Plasma Hormones and Glucose Measurements

Fasting plasma leptin and insulin concentrations were measured with ELISA kits (R&D Systems and Crystal Chem Inc., respectively), and plasma glucose concentrations were determined using a glucose analyzer (Beckman Coulter, CA). During GTTs, blood glucose levels were measured using glucose meter and glucose strips (ReliOn).

Statistical analyses

Data are expressed as means ± SEMs. Significant differences between two groups were determined by unpaired Student's t-test. Significant differences between multiple groups were determined by one-way ANOVA followed by Tukey multiple comparisons tests. Significant differences between two groups over time were determined by two-way ANOVA with repeated measures. Bonferroni post-hoc multiple comparisons were used to compare the means between two different groups at the same time point after two-way ANOVA test. Statistical significance was accepted at a level of $P<0.05$.

RESULTS

SOCS3$^{flox/flox}$/POMC-Cre mice had reduced SOCS3 mRNA expression in the arcuate nucleus and amplified effect of leptin to increase p-STAT3 expression

Hypothalamus p-STAT3 staining after i.p. saline injection was similar in SOCS3$^{flox/flox}$/POMC-Cre mice and SOCS3$^{flox/flox}$ control mice (**Fig. 1, A and D**). However after acute i.p. leptin injection, positive staining for p-STAT3, a major downstream signaling

pathway activated by leptin, was enhanced in the arcuate nucleus of the hypothalamus in SOCS3$^{flox/flox}$/POMC-Cre mice (**Fig. 1, E and F**) compared to SOCS3$^{flox/flox}$ control mice (**Fig. 1, B and C**).

The qRT-PCR analysis showed that expression levels of SOCS3 mRNA in the hypothalamus of SOCS3$^{flox/flox}$/POMC-Cre mice was significantly lower compared to control mice in both male and female (41 % less in males and 58 % less in female respectively, **Fig. 1, G and I**). However, SOCS3 expression in brain cortex was not significantly different between groups. We also measured the mRNA expression levels of PTP1B, which is another leptin signaling negative regulator expressed in POMC neurons. In both hypothalamus and cortex of SOCS3$^{flox/flox}$/POMC-Cre and SOCS3$^{flox/flox}$ control mice, expression levels of PTP1B were similar between groups in both sexes (**Fig. 1, H and J**).

SOCS3 deficiency in POMC neurons reduced body weight and fat mass in mice fed a CD

Both male and female SOCS3$^{flox/flox}$/POMC-Cre mice had significantly lower body weight compared to sex-matched control mice from 8 to 17 weeks of age. We also observed sex differences for weight gain between male and female SOCS3 $^{flox/flox}$/POMC-Cre mice when compared to their respective controls each week. For instance, while the differences in body weight in males between groups were significant from 8 to 11 weeks of age; in female SOCS3 $^{flox/flox}$/POMC-Cre mice, the differences in body weight became significant from 16 to

17 weeks with female SOCS3 $^{flox/flox}$/POMC-Cre mice having a lower body weight (**Fig. 2, A and D**).

EchoMRI scans showed that the lower body weight in male SOCS3 $^{flox/flox}$/POMC-Cre mice was due mostly to reduced body fat content compared to controls (**Fig. 2, B and C**), whereas female SOCS3 $^{flox/flox}$/POMC-Cre mice exhibited reduced fat and lean mass compared to control mice (**Fig. 2, E and F**).

SOCS3 deficiency in POMC neurons did not alter food intake, refeeding response to prolonged fasting, or the acute anorexic action of leptin in mice fed a CD

Average daily food intake from 8 to 17 weeks of age was not significantly different in SOCS3$^{flox/flox}$/POMC-Cre and control mice (**Fig. 3, A and D**). These results suggest that the lighter body weight of SOCS3$^{flox/flox}$/POMC-Cre mice may not be caused mainly by reduced food intake. To further examine whether food intake regulation was altered in SOCS3$^{flox/flox}$/POMC-Cre mice, a test of 24 hours fasting followed by a 3-day refeeding period was performed. Compared to baseline before fasting, food intake increased significantly during the refeeding period in all groups (**Fig. 3, B and E**). The 3-day cumulative food intake during the refeeding period was not significantly different between genotypes for male or female mice. Despite similar daily food intake (**Fig. 4, A and C**), male and female SOCS3$^{flox/flox}$/POMC-Cre mice exhibited different eating patterns compared to controls with reduced nighttime feeding bouts (**Fig. 4, B and D**).

In addition to examining the impact of SOCS3 inactivation in POMC neurons on food intake regulation under normal conditions and after prolonged fasting, we also investigated if reduced SOCS3 expression in POMC neurons potentiates the acute anorexic effect of leptin. Acute leptin injections, however, caused similar reductions in food intake in SOCS3$^{flox/flox}$/POMC-Cre mice and controls of both sexes (**Fig. 3, C and F**). These observations indicate that POMC neuronal SOCS3 deficiency has minimal impact on food intake regulation and on leptin's acute anorexic effect.

SOCS3 deficiency in POMC neurons increased energy expenditure in male mice and reduced plasma insulin and RQ in female mice fed a CD

Compared to control mice, male SOCS3$^{flox/flox}$/POMC-Cre mice had significantly higher (~22%) daytime and nighttime energy expenditure when normalized for body weight, while no differences were observed in female mice (**Fig. 5, A and D**). Female SOCS3 $^{flox/flox}$/POMC-Cre mice exhibited reduced nighttime RQs and motor activity (**Fig. 5, E and F**) compared to female control mice (0.85±0.02 vs. 0.90±0.01 and 104±3 vs 169±3 m/12 hrs, respectively), while no significant differences were found in male mice (**Fig. 5, B and C**).

No differences in baseline fasting plasma concentrations of leptin, insulin, and glucose were observed between male control and SOCS3$^{flox/flox}$/POMC-Cre when fed a CD. However, the fasting plasma insulin concentration in female SOCS3$^{flox/flox}$/POMC-Cre mice was significantly lower than in controls (**Table 1, 2**). In male and female SOCS3$^{flox/flox}$/POMC-

Cre mice, the glucose tolerance and AUC during an oral GTT were similar to that measured in sex matched controls (**Fig. 5, G to J**).

SOCS3 deficiency in POMC neurons did not alter leptin's anorexic and glucose or insulin lowering effects in mice fed a CD

To examine whether SOCS3 deficiency in POMC neurons amplifies the chronic effects of leptin on metabolic function and glucose regulation in mice fed a CD, we infused leptin for 7 days at a rate calculated to raise plasma leptin concentrations to those observed in obesity. As expected based on our previous studies (8, 11), leptin treatment reduced fasting plasma insulin and glucose concentrations in all groups of mice (**Table 1, 2**). Contrary to what we anticipated, although leptin treatment reduced glucose levels in both genotypes, the reduction in insulin levels was statistically significant only in control mice (**Table 1, 2**).

Leptin infusion for 7 days had similar effects to reduce food intake and to cause small but not significant reductions in body weight in male and female control and SOCS3$^{flox/flox}$/POMC-Cre mice (**Table 1, 2**). Leptin infusion did not significantly alter energy expenditure in any of the groups but significantly reduced RQ in control female mice (**Table 1, 2**). Motor activity was reduced in both control and SOCS3$^{flox/flox}$/POMC-Cre mice during leptin infusion and this reduction was significantly greater in SOCS3$^{flox/flox}$/POMC-Cre mice compared to controls for both male and female mice (**Table 1, 2**).

SOCS3 deficiency in POMC neurons attenuated HFD-induced weight gain without altering liver weight or liver fat content

Previous studies have shown that CNS SOCS3 deletion confers resistance to diet-induced obesity. Therefore, we fed SOCS3 $^{flox/flox}$/POMC-Cre and control mice a HFD for 6 weeks to test if selective inactivation of SOCS3 in POMC neurons would attenuate weight gain. Compared to controls, male and female SOCS3$^{flox/flox}$/POMC-Cre mice showed significantly attenuated weight gain (35% and 37% lower body weight than controls, respectively, after 6 weeks on a HFD) (**Fig. 6, A and D**). In males, daily caloric intake for HFD was higher than in CD in both SOCS3$^{flox/flox}$/POMC-Cre and control mice, with no significant differences between groups (**Fig. 6, B**). In female SOCS3$^{flox/flox}$/POMC-Cre mice, daily caloric intake was lower than in CD and was significantly reduced compared to control mice after switching to HFD (**Fig. 6, E**).

After 8 weeks on HFD, the mice were euthanized and liver was weighed and fat content measured. Despite differences in weight gain on HFD, liver weight and liver fat content were not significantly different in SOCS3$^{flox/flox}$/POMC-Cre mice compared to SOCS3$^{flox/flox}$ control mice (**Fig. 6, C and F**).

To further examine whether reduced body weight in POMC SOCS3 deficient mice after HFD was due to increased leptin signaling sensitivity, we performed metabolic studies to examine EE, RQ and motor activity after 5 weeks of HFD in POMC SOCS3 deficient mice

and control mice of both sexes. Energy expenditure per gram of body weight, RQ, and motor activity (**Fig. 7, A, B and C**) were slightly but not significantly increased in male SOCS3$^{flox/flox}$/POMC-Cre mice compared to sex-matched control mice. In female SOCS3$^{flox/flox}$/POMC-Cre mice, EE was also slightly increased (**Fig. 7, D)** and there was a significant increase in RQ accompanied by significantly lower motor activity compared to control mice (**Fig. 7, E and F**).

SOCS3 deficiency in POMC neurons attenuated HFD-induced fasting hyperinsulinemia and hyperglycemia without affecting glucose tolerance

Six weeks of HFD significantly increased plasma leptin levels similarly in all groups of male and female mice compared to CD (**Fig. 8, A and D**) although female mice exhibited lower plasma leptin levels on both diets compared to male mice. However, SOCS3 deficiency in POMC neurons markedly attenuated HFD-induced hyperinsulinemia and hyperglycemia compared to control mice. Insulin levels in male and female SOCS3$^{flox/flox}$/POMC-Cre mice were significantly lower (1.0±0.2 vs 2.2±0.6 ng/ml in males, and 0.6±0.1 vs 1.1±0.2 ng/ml in females) compared to control mice after 6 weeks of HFD (**Fig. 8, B and E**). Glucose levels were also significantly lower in female SOCS3 $^{flox/flox}$/POMC-Cre mice compared to female control mice after HFD (**Fig. 8, F**).

Although SOCS3 deficiency in POMC neurons protected against HFD-induced hyperinsulinemia and hyperglycemia under fasting conditions, it did not alter glucose tolerance during an oral GTT when compared to control mice (**Fig. 8, G and H**).

SOCS3 deficiency in POMC neurons potentiated HFD-induced elevation in BP and cardiac responses to acute stress in female mice

There were no significant differences in MAP and HR in male or female SOCS3 $^{flox/flox}$/POMC-Cre and SOCS3$^{flox/flox}$ control mice at baseline or during 6 weeks of HFD (**Fig. 9, A and D; C and F**). However, when we calculated the change in BP before and after HFD, MAP increased similarly by approximately 4 mmHg in males of both genotypes (**Fig. 9, B**). In contrast, the increases in MAP in female SOCS3 $^{flox/flox}$/POMC-Cre mice were significantly higher than in female SOCS3$^{flox/flox}$ controls during HFD. As shown in (**Fig. 9, E**), MAP in female SOCS3 $^{flox/flox}$/POMC-Cre mice increased by approximately 5 mmHg, whereas MAP did not change in female SOCS3$^{flox/flox}$ controls during HFD. Feeding a HFD for 6 weeks did not alter HR in any of the groups (**Fig. 9, C and F**).

To test whether SOCS3 deficiency in POMC neurons alters cardiovascular responses to an acute pressor stress, we performed an air-jet stress test in male and female mice after 6-weeks of HFD. The increases in MAP and HR in response to the air-jet stress were similar in males from both genotypes (**Fig. 10, A and B**). Female control mice exhibited a lower MAP and HR responses compared to male control mice. However, female

SOCS3$^{flox/flox}$/POMC-Cre mice showed significantly higher BP and HR responses compared to female control mice (32±3 vs 20±4 mmHg and 193±10 vs 101±10 bpm, respectively) (**Fig. 10, A and B)**. AUCs of MAP during and post stress were significantly higher in female SOCS3$^{flox/flox}$/POMC-Cre mice compared to female controls (**Fig. 10, D)**, whereas no difference between male SOCS3$^{flox/flox}$/POMC-Cre mice compared to male controls were observed (**Fig. 10, C**).

SOCS3 deficiency in POMC neurons did not alter the dietary and cardiovascular responses to chronic leptin infusion in mice fed a CD

We also examined if the dietary and cardiovascular responses to a 14-day leptin infusion were altered in mice fed a CD. Body weight (**Fig. 11, A and D**) and daily food intake (**Fig. 11, B and E**) responses to chronic leptin treatment were similar in male and female mice of both genotypes with a significant reduction in cumulative food consumption (**Fig. 11, C to F**) during leptin infusion associated with approximately a 8% and 10% weight loss in males and females, respectively. Despite the weight loss, MAP in male and female mice of all groups did not fall; in fact, male and female SOCS3$^{flox/flox}$/POMC-Cre mice showed a transient increase in MAP during the first 5 days of leptin treatment (**Fig. 12, A and C**), however, the increases were not significant. The changes of HR during leptin treatment were similar between SOCS3$^{flox/flox}$/POMC-Cre mice and control mice (**Fig. 12, B and D**). These

1 results suggest that deficiency of SOCS3 in POMC neurons does not markedly amplify the

2 chronic effects of leptin on BP and HR.

DISCUSSION

The main question addressed in this study was whether SOCS3 deficiency in POMC neurons had a significant impact on metabolic and cardiovascular regulation in male and female mice fed a CD or HFD. There are three major findings in this study: first, we demonstrated that SOCS3 deficiency in POMC neurons reduced body weight and fat mass without significant changes in daily food intake, fasting blood glucose and leptin concentrations in mice fed a CD. In POMC SOCS3 deficient mice fed a HFD, their body weight gain was also significantly attenuated and was associated with reduced caloric intake. Second, we found important sex differences in the metabolic and cardiovascular responses of POMC SOCS3 deficiency in mice fed a CD or HFD. Third, POMC SOCS3 deficiency, surprisingly, did not amplify the chronic effects of leptin to reduce food intake, improve glucose regulation, or to raise BP and HR. Thus, our results indicate that SOCS3 in POMC neurons regulates body weight and energy balance as well as the BP responses to a HFD in a sex specific manner. However, we found no evidence that SOCS3 levels in POMC neurons alter the food intake, BP and HR responses to chronic leptin infusions that raised plasma levels to those observed in severe obesity.

Role of SOCS3 in POMC neurons in regulating energy balance and body weight

Our data showed that when fed a CD or HFD, male and female SOCS3$^{flox/flox}$/POMC-Cre mice were lighter with less fat mass, and gained less weight compared to age matched control mice. These observations confirm that SOCS3 deficiency in POMC neurons confers resistance to diet-induced obesity (DIO) as reported in previous studies (2, 22) and demonstrate that SOCS3 levels in POMC neurons contribute to regulation of body weight and adiposity under normal conditions or during a HFD.

The smaller weight gain observed in POMC SOCS3 deficient mice fed a HFD was due, at least in part, to lower caloric intake compared to control mice, which was more apparent in female mice. Metabolic phenotype measurements after HFD showed that EE and RQ were slightly elevated in both male and female POMC SOCS3 deficient mice compared to controls after 5 weeks of HFD. We also found significant reductions in plasma insulin and glucose concentrations in POMC SOCS3 deficient mice compared to control mice after a HFD. However, these changes were not observed in mice fed a control diet. These results are consistent with increased leptin sensitivity in POMC SOCS3 deficient mice but only when challenged with HFD.

Although SOCS3 in POMC neurons may not play a major role in food intake regulation during a regular feeding regimen, it may contribute to regulation of caloric intake during a HFD. However, SOCS3 in POMC neurons does not appear to evoke sensitization

to the acute or chronic anorexic effects of leptin and does not alter the food intake response to refeeding after prolonged fasting. These observations are consistent with our previous findings showing nearly intact anorexic responses to chronic leptin infusion in mice lacking leptin receptors in POMC neurons (8) and suggest that other neurons are more important in mediating the effects of leptin to reduce food intake. Our meal pattern studies showed that total food intake was not significantly different in SOCS3$^{flox/flox}$/POMC-Cre mice compared to control male or female mice. However, SOCS3$^{flox/flox}$/POMC-Cre mice had significantly fewer meal bouts suggesting larger meal sizes in SOCS3$^{flox/flox}$/POMC-Cre mice compared to control mice. Hypothalamic neurons are recognized to control meal size as well as overall food intake, and leptin signaling in the arcuate nucleus has been reported to regulate meal size (14). Results from our study suggest that SOCS3 in POMC neuron may also have an important role in modulating leptin's effect on meal size.

Another potential explanation for the lower body weight and attenuated weight gain of POMC SOCS3 deficient mice on CD or HFD is that they had elevated energy expenditure compared to control mice. In the present study, we measured oxygen consumption, an indirect assessment of energy expenditure, and found that male SOCS3$^{flox/flox}$/POMC-Cre mice exhibited greater oxygen consumption than male control mice. Female POMC SOCS3 deficient mice, although showing similar oxygen consumption as in female control mice, exhibited lower RQ values which may be interpreted as greater utilization of fat as the main

fuel source, thus contributing to reduced fat mass when compared to female controls. We observed significantly reduced motor activity with similar energy expenditure before and after chronic leptin infusion in POMC SOCS3 deficient mice. Although leptin reduced motor activity, it did not significantly alter total energy expenditure, perhaps due to leptin's known counterbalancing effects to increase sympathetic activity which tends to increase metabolic rate. The net effect was unaltered energy expenditure. Taken together, these results indicate that SOCS3 in POMC neurons contributes to body weight regulation via a combined effect on appetite and energy balance and plays an important role in DIO.

Role of SOCS3 in POMC neurons in glucose regulation

Kievit et al (19) previously showed improved GTT and reduced plasma glucose and insulin concentrations in male POMC SOCS3 deficient mice compared to control mice fed regular diet or HFD. In contrast, we did not observe significant differences in GTT, fasting blood glucose and insulin, or plasma leptin concentration between male $SOCS3^{flox/flox}$/POMC-Cre and control mice when fed a CD. This discrepancy may be due to different ages of the animals and different diet compositions. We did, however, observe lower fasting plasma insulin levels in $SOCS3^{flox/flox}$/POMC-Cre mice than in control mice after feeding them a HFD for 6 weeks. These results suggest that SOCS3 in POMC neurons may have a significant

role in glucose and insulin regulation but SOCS3 deficiency cannot completely prevent the development of HFD-induced glucose intolerance.

Role of POMC neuronal SOCS3 in blood pressure regulation

DIO in both humans and rodents is associated with an increased risk of hypertension (15, 16). Although the underlying molecular, cellular and hormonal mechanisms of obesity-induced hypertension are not fully understood, previous studies from our lab and others suggest that elevated leptin levels may raise BP by increasing sympathetic nervous system (SNS) activity in male rats and mice (5, 15, 23). Leptin has been reported to increase BP by activation of leptin receptors in the dorsomedial hypothalamus (DMH) (20). However, we previously showed that leptin receptor in POMC neurons mediate much of the chronic effects of leptin to raise BP in male mice (8-11). For example, leptin receptor deficiency specifically on POMC neurons completely abolished the rise in BP that occurred in control mice during 7 days of leptin infusion (8).

Because SOCS3 is a negative regulator of leptin receptors activation, we hypothesized that SOCS3 deficiency in POMC neurons would increase BP in obese mice fed a HFD. However, we found this was observed only in female POMC SOCS3 deficient mice when comparing BP changes before and after HFD, whereas male mice of both groups exhibited similar BP. We also observed enhanced BP and HR responses to an acute air jet

stress in female but not male POMC SOCS3 deficient mice when compared to sex-matched controls, suggesting the existence of sex differences in the role of POMC SOCS3 in cardiovascular regulation. (18).

Although the precise mechanisms for the sex differences in BP regulation observed in the present study are still unclear, it is possible that POMC SOCS3 deficient female mice are more sensitive to HFD-induced increases in aldosterone production and/or mineralocorticoid receptor (MR) activation (13). Studies by Huby el al.(18) suggest that leptin may increase BP in females via increases in aldosterone secretion and activation of MR. However, the role of aldosterone and MR activation in mediating the BP effects of leptin in female mice was not tested in the present study.

Alternatively, POMC SOCS3 deletion in female mice may be associated with HFD-induced SNS activation as we previously observed in male rodents (5). This possibility would be consistent with our finding that female mice with POMC SOCS3 deficiency have greater BP responses to acute stress. However, the mechanisms that mediate the greater BP responses to a HFD and acute stress are still uncertain and will require further investigation.

Chronic leptin infusion in male rodents has been reported to reduce food intake and raises MAP and HR mainly via activation of the SNS (15). However, we did not observe significant differences in BP or HR responses to chronic leptin infusion in control mice or in POMC SOCS3 deficient mice. These surprising results do not support our hypothesis that

SOCS3 deficiency in POMC neurons substantially enhances the BP and HR effects of leptin. One possible explanation for these findings is the reduced body weight associated with SOCS3 deficiency in POMC neurons may offset the effects of leptin on BP. Further experiments utilizing weight-matched mice may be useful in determining whether POMC SOCS3 deficiency alters BP regulation independently of changes in body weight.

Another possible explanation for the lack of enhanced BP responses to leptin is that SOCS3 signaling in POMC neurons may modulate the effects of additional factors (e.g. PTP1B (4), another leptin signaling inhibitor) that either inactivate POMC neurons or attenuate the effects of leptin on SNS activity, BP and HR regulation. It is also possible SOCS3 deficiency does not substantially enhance the activity of the downstream pathways associated with leptin receptor mediated SNS activation and BP regulation by POMC neurons. Although SOCS3 in known to inhibit STAT3 phosphorylation, it is not clear whether SOCS3 attenuates other leptin signaling pathways (e.g. SHP2) possibly important in BP regulation as we previously showed SHP2 in POMC neurons also contributes to leptin's actions on BP, energy balance, and glucose regulation (9).

Sex difference in regulation of glucose, energy balance and blood pressure by SOCS3 in POMC neurons

Accounting for sex differences in the design of experimental studies and interpretation of results is increasingly recognized as an important step in developing translational approaches to prevention and treatment of human diseases. As discussed previously, an important finding of our study is there were sex differences in some of the metabolic and cardiovascular effects of POMC SOCS3 deficient mice compared with control mice. For example, we found higher energy expenditure only in male POMC SOCS3 deficient mice compared to controls and a reduced motor activity and RQ was observed in female POMC SOCS3 deficient mice fed a CD. Also female mice with POMC neuronal SOCS3 deficiency had increased MAP and BP response to air jet stress compared with female control mice fed a chronic HFD. In contrast, BP in male POMC SOCS3 deficient and control mice did not differ when they were fed a HFD. Quantitative differences in SOCS3 expression and/or deletion in males and females might explain some of the sex differences observed in our study but are unlikely to fully account for qualitative differences. Different expression levels of NPY/AGRP and POMC in the hypothalamus of male and female mice may contribute to the observed phenotype differences (7, 25) but this was not tested in the present study. Although our studies were not designed to investigate the mechanisms responsible for sex differences in SOCS3 signaling and POMC neuronal control of metabolism and BP, they further emphasize the need to account for sex as a biological variable in future studies of CNS regulation of metabolic and cardiovascular function (24).

Perspectives and Significance:

Our study indicates that SOCS3 deficiency in POMC neurons attenuates weight gain and adiposity, and increases energy expenditure in males while reducing RQ in female mice. We also found that POMC neuronal SOCS3 deficiency in female mice increased BP as well as the HR and BP stress responses associated with a HFD despite less weight gain. Our findings, therefore, suggest that SOCS3 in POMC neurons has an important role in regulating body weight, daily energy balance and BP in a sex specific manner. Future studies are needed to assess mechanisms responsible for sex differences in SOCS3 signaling and POMC neuronal control of metabolic and cardiovascular function.

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DISCLOSURES

No conflicts of interest, financial or otherwise are declared by the authors.

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Figure Legends

Figure 1. Immunohistochemistry of pSTAT3 staining in the ARC of SOCS3$^{flox/flox}$ and SOCS3$^{flox/flox}$/POMC-Cre mice injected i.p. with saline (**A, D**) or leptin (5 mg/kg, **B, C, E, F**). SOCS3 (**G, I**) and PTP1B (**H, J**) mRNA levels were quantified by qRT-PCR in hypothalamus of SOCS3$^{flox/flox}$/POMC-Cre and SOCS3$^{flox/flox}$ control mice. (n=4-6/group per sex, *p<0.05 compared to SOCS3$^{flox/flox}$ mice at the same brain area by t-test)

Figure 2. Body weight and body fat/lean mass from 8 to 17 week of ages in male and female mice on CD. **A.** Body weight, **B.** Body fat mass, and **C**. Body lean mass in male SOCS3$^{flox/flox}$/POMC-Cre and SOCS3$^{flox/flox}$ control mice. **D.** Body weight, **E.** Body fat mass, and **F.** Body lean mass in female SOCS3$^{flox/flox}$/POMC-Cre and SOCS3$^{flox/flox}$ control mice. (n=9 /group per sex, * P<0.05 between groups by 2-way ANOVA, # P<0.05 between groups at the same time point by Bonferroni post-hoc test)

Figure 3. Average daily food intake, fasting-refeeding responses, and food intake responses to acute leptin injection in male and female mice on CD. **A**. Average daily food intake from 8 to 17 weeks of age in male SOCS3$^{flox/flox}$/POMC-Cre and SOCS3$^{flox/flox}$ control mice, **B.** Daily food intake before and after fasting, and 24, 48 and 72 hours after refeeding in males, **C.**

Change of food intake compared to the saline control at 2, 4, 15 and 24 hours after acute leptin injection in males. **D.** Average daily food intake from 8 to 17 weeks of age in female SOCS3$^{flox/flox}$/POMC-Cre and SOCS3$^{flox/flox}$ control mice, **E.** Daily food intake before and after fasting, and 24, 48 and 72 hours after fasting in females, **F.** Change of food intake compared to the saline control at 2, 4, 15 and 24 hours after acute leptin injection in females. Three-day cumulative food intake in male and female SOCS3$^{flox/flox}$/POMC-Cre and SOCS3$^{flox/flox}$ control mice on CD after 24 hours of fasting. (n=6/group per sex, * P<0.05 compared to baseline by Bonferroni post-hoc test in SOCS3$^{flox/flox}$/POMC-Cre and SOCS3$^{flox/flox}$ control mice)

Figure 4. Daytime and nighttime food intake and feeding bouts at baseline in male and female mice on CD. **A.** Food intake during daytime and nighttime in male SOCS3$^{flox/flox}$/POMC-Cre and SOCS3$^{flox/flox}$ control mice. **B.** Feeding bouts during daytime and nighttime in male mice. **C.** Food intake during daytime and nighttime in female mice. **D.** Feeding bouts during daytime and nighttime in females. (n=7/group per sex, * P<0.05 between groups by 2-way ANOVA, # P<0.05 between groups at the same time point by Bonferroni post-hoc test)

Figure 5. Daytime and nighttime energy expenditure, respiratory quotient, and motor activity measurement at baseline and glucose tolerance tests in male and female mice on CD. **A.** Daytime and nighttime energy expenditure normalized for body weight in male SOCS3$^{flox/flox}$/POMC-Cre and SOCS3$^{flox/flox}$ control mice. **B**. Daytime and nighttime respiratory quotient in male mice. **C.** Daytime and nighttime motor activity in male mice. **D.** Daytime and nighttime energy expenditure normalized for body weight in female SOCS3$^{flox/flox}$/POMC-Cre and SOCS3$^{flox/flox}$ control mice. **E**. Daytime and nighttime respiratory quotient in female mice. **F.** Daytime and nighttime motor activity in female mice. **G.** Blood glucose of male SOCS3$^{flox/flox}$/POMC-Cre and SOCS3$^{flox/flox}$ control mice measured over 120 minutes post-glucose gavage in an oral GTT. **H.** Blood glucose AUC for male SOCS3$^{flox/flox}$/POMC-Cre and SOCS3$^{flox/flox}$ control mice. **I.** Blood glucose of female SOCS3$^{flox/flox}$/POMC-Cre and SOCS3$^{flox/flox}$ control mice measured over 120 minutes post-glucose gavage in an oral GTT. **J.** Blood glucose AUC for female SOCS3$^{flox/flox}$/POMC-Cre and SOCS3$^{flox/flox}$ control mice. (n=7/group per sex, * P<0.05, between groups by 2-way ANOVA and # P<0.05 between groups at the same time point by Bonferroni post-hoc test)

Figure 6. Body weight gain, calorie intake, liver/body weight ratio and liver fat content in male and female mice on HFD. **A.** Body weight gain during 6 weeks of HFD in male SOCS3$^{flox/flox}$/POMC-Cre and SOCS3$^{flox/flox}$ control mice. **B.** Average daily calorie intake

during 6 weeks of HFD in male mice. **C.** Ratio of liver weight to total body weight and liver fat weight after 8 weeks of HFD in male mice. **D.** Body weight gain during 6 weeks of HFD in female SOCS3$^{flox/flox}$/POMC-Cre and SOCS3$^{flox/flox}$ control mice. **E.** Average calorie intake during 6 weeks of HFD in female mice. **F.** Ratio of liver weight to total body weight and liver fat weight after 8 weeks of HFD in female mice. (n=11/group per sex, * *P*<0.05, between groups by 2-way ANOVA; # *P*<0.05, compared between groups at the same time point by Bonferroni post-hoc test)

Figure 7. Daytime and nighttime energy expenditure, respiratory quotient, and motor activity measurements in male and female mice after 5 weeks of HFD. **A.** Daytime and nighttime energy expenditure normalized for body weight in male SOCS3$^{flox/flox}$/POMC-Cre and SOCS3$^{flox/flox}$ control mice. **B**. Daytime and nighttime respiratory quotient in male mice. **C.** Daytime and nighttime motor activity in male mice. **D.** Daytime and nighttime energy expenditure normalized for body weight in female SOCS3$^{flox/flox}$/POMC-Cre and SOCS3$^{flox/flox}$ control mice. **E**. Daytime and nighttime respiratory quotient in female mice. **F.** Daytime and nighttime motor activity in female mice. (n=4-6/group per sex. * *P*<0.05, between groups by 2-way ANOVA; # *P*<0.05, compared between groups at the same time point by Bonferroni post-hoc test)

Figure 8. Comparison of plasma leptin, insulin and fasting glucose concentrations and glucose tolerance tests in male and female mice fed CD or HFD**. A.** Plasma leptin concentrations in male SOCS3$^{flox/flox}$/POMC-Cre and SOCS3$^{flox/flox}$ control mice fed CD or HFD. **B.** Plasma insulin concentrations in male mice fed CD or HFD. **C.** Fasting Glucose concentrations of male mice fed CD or HFD. **D.** Plasma leptin concentrations of female SOCS3$^{flox/flox}$/POMC-Cre and SOCS3$^{flox/flox}$ control mice fed CD or HFD. **E.** plasma insulin levels on CD and HFD in females. **F.** Fasting Glucose concentrations in female mice fed CD or HFD. **G.** Blood glucose in male SOCS3$^{flox/flox}$/POMC-Cre and SOCS3$^{flox/flox}$ control mice measured over 120 minutes post-glucose gavage in an oral GTT. **H.** Blood glucose in female SOCS3$^{flox/flox}$/POMC-Cre and SOCS3$^{flox/flox}$ control mice measured over 120 minutes post-glucose gavage in an oral GTT. (n=6/group per sex, * P<0.05, compared to the same genotype on a CD by Tukey multiple comparisons test. # P<0.05, compared to SOCS3$^{flox/flox}$ mice on a HFD by Tukey multiple comparisons test.)

Figure 9. MAP, changes of MAP compared to the baseline, and HR in male and female mice on HFD. **A.** Daily average MAP at baseline and 2, 4 and 6 weeks of HFD in male SOCS3$^{flox/flox}$/POMC-Cre and SOCS3$^{flox/flox}$ control mice. **B.** Changes of MAP compared to baseline at 2, 4 and 6 weeks of HFD in male mice. **C.** HR at baseline and 2, 4 and 6 weeks of HFD in male mice. **D.** Daily average MAP during baseline and 2, 4 and 6 weeks of HFD in

female SOCS3$^{flox/flox}$/POMC-Cre and SOCS3$^{flox/flox}$ control mice. **E.** Changes of MAP compared to baseline at 2, 4 and 6 weeks of HFD in female mice. **F.** HR at baseline and 2, 4 and 6 weeks of HFD in female mice. (n=7/group per sex, * *P*<0.05 between groups by 2-way ANOVA)

Figure 10. Blood pressure and HR during air jet stress test in male and female mice on HFD. **A.** Maximum MAP increases during air jet stress compared to baseline in male and female SOCS3$^{flox/flox}$/POMC-Cre and SOCS3$^{flox/flox}$ control mice. **B.** Maximum HR increases during air jet stress compared to baseline in male and female SOCS3$^{flox/flox}$/POMC-Cre and SOCS3$^{flox/flox}$ control mice. **C.** MAP AUC for male SOCS3$^{flox/flox}$/POMC-Cre and SOCS3$^{flox/flox}$ control mice during 5 minutes air jet stress and 30 minutes recovery after air jet. **D.** MAP AUC for female SOCS3$^{flox/flox}$/POMC-Cre and SOCS3$^{flox/flox}$ control mice during 5 minutes air jet stress and 30 minutes recovery after air jet. (n=5-6/group per sex, * *P*<0.05 compared to female SOCS3$^{flox/flox}$ mice by unpaired Student's t-test)

Figure 11. Changes in body weight and food intake of male and female mice during 14 days of leptin infusion (4 μg/kg/min). **A.** Body weight during baseline, leptin infusion, and recovery in male SOCS3$^{flox/flox}$/POMC-Cre and SOCS3$^{flox/flox}$ control mice. **B.** Daily food intake during baseline, leptin infusion and recovery in male mice. **C.** Cumulative food intake during

baseline, leptin infusion and recovery in male mice. **D.** Body weight during baseline, leptin infusion, and recovery in female SOCS3$^{flox/flox}$/POMC-Cre and SOCS3$^{flox/flox}$ control mice. **E.** Daily food intake changes during baseline, leptin infusion and recovery in female mice. **F.** Cumulative food intake during baseline, leptin infusion and recovery in female mice.

Figure 12. Changes in MAP and HR of male and female mice during 14 days of leptin infusion (4 µg/kg/min). **A.** MAP during baseline, leptin infusion, and recovery periods in male SOCS3$^{flox/flox}$/POMC-Cre and SOCS3$^{flox/flox}$ control mice. **B.** HR during baseline, leptin infusion, and recovery in male mice. **C.** MAP during baseline, leptin infusion, and recovery in female SOCS3$^{flox/flox}$/POMC-Cre and SOCS3$^{flox/flox}$ control mice. **D.** HR during baseline, leptin infusion, and recovery in female mice. (n=4-5/grou/sex)

Table 1. Changes of metabolic parameters in male SOCS3$^{flox/flox}$/POMC-Cre and SOCS3$^{flox/flox}$ control mice during 7 days of leptin infusion at 18 weeks of age

Parameter (Male)	SOCS3$^{flox/flox}$ Control	SOCS3$^{flox/flox}$/POMC-Cre	SOCS3$^{flox/flox}$ Control + Leptin	SOCS3$^{flox/flox}$/POMC-Cre + Leptin
Body Weight, g	32.2±2.3	27.7±0.8 #	**29.5±1.9**	**26.7±0.8**
Food Intake, g/day	2.6±0.3	2.9±0.4	**1.9±0.3**	**2.5±0.3**
Plasma leptin, ng/ml	10.0±2.0	8.5±2.3	**27.1±3.4 ***	**31.6±3.0 ***
Plasma insulin, ng/ml	0.46±0.10	0.42±0.09	**0.21±0.08 ***	**0.32±0.07**
Plasma glucose, mg/dL	137±10	131±7	**112±7 ***	**108±7 ***
Energy Expenditure, Kcal/day/g BW	0.21±0.01	0.25±0.01 #	**0.22±0.01**	**0.24±0.01**
RQ (VCO_2/VO_2)	0.84±0.03	0.83±0.01	**0.81±0.02**	**0.81±0.01**
Motor Activity, m/day	134±14	165±24	**114±13**	**87±10 *#**

Data are expressed as means ± SEMs and calculated as the average of last 4 days during control and leptin infusion period (shaded). * *P<0.05* compared to baseline before leptin infusion; # *P<0.05* compared to SOCS3$^{flox/flox}$ control mice.

Table 2. Changes of metabolic parameters in female SOCS3$^{flox/flox}$/POMC-Cre and SOCS3$^{flox/flox}$ control mice during 7 days of leptin infusion at 18 weeks of age

Parameter (Female)	SOCS3$^{flox/flox}$ Control	SOCS3$^{flox/flox}$/POMC-Cre	SOCS3$^{flox/flox}$ Control + Leptin	SOCS3$^{flox/flox}$/POMC-Cre + Leptin
Body Weight, g	23.0±0.9	21.4±0.6 #	**21.0±0.6**	**20.3±0.6**
Food Intake, g/day	3.1±0.4	3.1±0.4	**2.7±0.2**	**2.9±0.4**
Plasma leptin, ng/ml	4.5±1.1	4.2±1.3	**30.6±8.5 ***	**43.9±9.7 ***
Plasma insulin, ng/ml	0.44±0.10	0.22±0.05 #	**0.14±0.02 ***	**0.16±0.05**
Plasma glucose, mg/dL	131±9	119±5	**98±7 ***	**101±8 ***
Energy Expenditure, Kcal/day/g BW	0.33±0.03	0.36±0.02	**0.33±0.04**	**0.37±0.01**
RQ (Vco_2/Vo_2)	0.86±0.01	0.82±0.01 #	**0.83±0.01 ***	**0.82±0.01**
Motor Activity, m/day	212±13	134±13 #	**161±14 ***	**80±8 *#**

Data are expressed as means ± SEMs and calculated as the average of last 4 days during control (normal font) and leptin infusion period (shaded). * *P<0.05* compared to baseline before leptin infusion; # *P<0.05* compared to $SOCS3^{flox/flox}$ control mice.