Original article

MOLECULAR METABOLISM

Inactivation of SOCS3 in leptin receptor-expressing cells protects mice from diet-induced insulin resistance but does not prevent obesity[a]

João A.B. Pedroso [1], Daniella C. Buonfiglio [1], Lais I. Cardinali [1], Isadora C. Furigo [1], Angela M. Ramos-Lobo [1], Julio Tirapegui [2], Carol F. Elias [3,4,5], Jose Donato Jr. [1,*]

ABSTRACT

Therapies that improve leptin sensitivity have potential as an alternative treatment approach against obesity and related comorbidities. We investigated the effects of *Socs3* gene ablation in different mouse models to understand the role of SOCS3 in the regulation of leptin sensitivity, diet-induced obesity (DIO) and glucose homeostasis. Neuronal deletion of SOCS3 partially prevented DIO and improved glucose homeostasis. Inactivation of SOCS3 only in LepR-expressing cells protected against leptin resistance induced by HFD, but did not prevent DIO. However, inactivation of SOCS3 in LepR-expressing cells protected mice from diet-induced insulin resistance by increasing hypothalamic expression of K_{atp} channel subunits and c-Fos expression in POMC neurons. In summary, the regulation of leptin signaling by SOCS3 orchestrates diet-induced changes on glycemic control. These findings help to understand the molecular mechanisms linking obesity and type 2 diabetes, and highlight the potential of SOCS3 inhibitors as a promising therapeutic approach for the treatment of diabetes.

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Keywords Suppressor of cytokine signaling-3; Type 2 diabetes mellitus; Leptin resistance; Hypothalamus; POMC; High-fat diet

1. INTRODUCTION

The obesity epidemic has become a major health threat worldwide. Obese individuals are at high risk to develop several types of metabolic dysfunction, including type 2 diabetes mellitus (T2DM). Although definitive therapies to treat obesity are currently unavailable, significant progress has been made recently in uncovering the molecular mechanisms involved in the etiology of obesity [1]. The discovery of proteins that inhibit signaling pathways recruited by leptin and insulin was of paramount importance in understanding key molecular features of obesity and T2DM [1—4].

Leptin resistance is a hallmark of obesity [1—3]. For example, diet-induced obese animals as well as most obese humans exhibit high circulating levels of leptin and reduced responsiveness to exogenous leptin [5—8]. Consequently, leptin treatment failed as an efficient pharmacological approach to treat obesity [9,10]. However, therapies aiming to improve leptin sensitivity have become the focus in developing alternative approaches to prevent and treat obesity and related comorbidities [1,2]. Thus, it is of great interest to identify proteins that could serve as putative targets of leptin-sensitizing therapies.

Several studies have shown that the intracellular protein known as suppressor of cytokine signaling-3 (SOCS3) inhibits leptin signaling. Additionally, SOCS3 expression is induced by leptin receptor (LepR) activation, indicating that SOCS3 is part of a negative feedback loop of leptin signaling pathway [11—13]. Furthermore, SOCS3 expression is increased in the hypothalamus of obese animals [11—15]. Therefore, SOCS3 could be a promising target of leptin-sensitizing therapies. Studies using genetically engineered mice revealed the role of SOCS3 in predisposing mice to diet-induced obesity (DIO). It was shown that haploinsufficiency of the *Socs3* gene increased leptin sensitivity and partially prevented DIO [16]. The brain is the primary target of leptin to regulate the energy balance [17,18]; thus, it would be expected that central ablation of SOCS3 may be sufficient to prevent DIO. Studies confirmed that neuronal deletion of SOCS3 improved leptin sensitivity and conferred resistance to DIO [19]. However, a recent study showed modest effects in preventing DIO after neuronal deletion of SOCS3

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[1]Department of Physiology and Biophysics, Institute of Biomedical Sciences, University of São Paulo, São Paulo, SP, Brazil [2]Department of Food Science and Experimental Nutrition, Faculty of Pharmaceutical Sciences, University of São Paulo, São Paulo, Brazil [3]Department of Molecular and Integrative Physiology, University of Michigan, Ann Arbor, MI, USA [4]Department of Obstetrics and Gynecology, University of Michigan, Ann Arbor, MI, USA [5]Department of Internal Medicine, Division of Hypothalamic Research, University of Texas Southwestern Medical Center, Dallas, TX, USA

*Corresponding author. Av. Prof. Lineu Prestes, 1524, São Paulo, SP 05508-000, Brazil. Tel.: +55 11 3091 0929; fax: +55 11 3091 7285.

E-mail: jdonato@icb.usp.br (J. Donato).

Abbreviations: AP, area postrema; ARH, arcuate nucleus of the hypothalamus; DIO, diet-induced obesity; DMV, dorsal motor nucleus of the vagus; GTT, glucose tolerance test; HFD, high-fat diet; ITT, insulin tolerance test; KO, knockout; LepR, leptin receptor; NTS, nucleus of the solitary tract; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; POMC, proopiomelanocortin; PTPs, protein-tyrosine phosphatases; SOCS3, suppressor of cytokine signaling-3; T2DM, type 2 diabetes mellitus; VMH, ventromedial nucleus of the hypothalamus

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despite a phenotype of increased leptin sensitivity [20]. The Nestin-Cre mouse has been a widely used model to induce genetic deletions in the brain, including the *Socs3* gene [19,20]. However, some studies observed alterations in pituitary hormone levels, body weight, adiposity and predisposition to DIO in mice carrying the Nestin-Cre transgene [20–22]. This phenotype could represent an important confounder in studying the role of SOCS3 in the regulation of body weight. Therefore, the objective of the present study was to investigate the effects of genetic ablation of the *Socs3* gene in different mouse models to elucidate the role played by SOCS3 in the regulation of leptin sensitivity, DIO and glucose homeostasis.

2. MATERIAL AND METHODS

2.1. Animals

We studied only male mice. The experimental mice were maintained under standard conditions of light (12 h light/dark cycle), temperature (23 ± 2 °C) and relative humidity ($55 \pm 15\%$). All animal procedures were approved by the Ethics Committee on the Use of Animals of the Institute of Biomedical Sciences at the University of São Paulo or by the University of Texas Institutional Animal Care and Use Committee.

2.2. Generation of the conditional knockout (KO) mice

To induce neuronal deletion of the *Socs3* gene, we bred the Nestin-Cre strain (B6.Cg-Tg(Nes-cre)1Kln/J, Jackson Laboratories) with mice carrying loxP-flanked *Socs3* alleles (SOCS3-floxed mouse, B6; 129S4-Socs3^{tm1Ayos}/J, Jackson Laboratories). Mice carrying neuronal deletion of SOCS3 (Nestin SOCS3 KO) were homozygous for the loxP-flanked *Socs3* allele and hemizygous for the Nestin-Cre transgene, whereas their control group was composed of homozygous animals for the loxP-flanked *Socs3* allele. The ablation of SOCS3 in LepR-expressing cells was attained by breeding the LepR-IRES-Cre strain (B6.129-Lepr$^{tm2(cre)Rck}$/J, Jackson Laboratories) with the SOCS3-floxed mouse. In this case, the KO group (LepR SOCS3 KO) was composed of animals homozygous for the loxP-flanked *Socs3* allele and for the LepR-Cre allele. The respective control group was composed of homozygous animals for the LepR-Cre allele. All mouse strains were backcrossed at least 4 times to C57BL/6 background before initiating the breeding. We only used littermates as control group. Additionally, a group of Nestin-Cre and LepR-Cre mice was bred with wild-type C57BL/6 mice to produce animals heterozygous for each mutation as well as wild-type (control) littermates. These groups were studied independently to assess if the Cre alleles produce changes in the body weight and adiposity regardless of any genetic deletion. Mice were weaned at 3–4 weeks of age, and the genomic DNA was extracted from tail tip for genotyping through PCR (Sigma).

2.3. Body weight, food intake, body adiposity and serum leptin levels

After weaning, the mice received a low-fat regular rodent chow diet (2.99 kcal/g; 9.4% calories from fat; Quimtia, Brazil). To study the predisposition of mice to develop DIO, adult mice received a high-fat diet (HFD, 5.31 kcal/g, 58% calories from fat; PragSoluções, Brazil) for 10–16 weeks, and their body weight was recorded weekly. Daily food intake was measured for 5–7 consecutive days in mice previously adapted to single housing. We measured the mass of the perigonadal (PE), subcutaneous (SC) and retroperitoneal (RP) fat pads to determine the adiposity of the animals. The body composition of LepR-Cre mice was determined by an EchoMRI-100 quantitative NMR machine at the University of Texas Southwestern Medical Center (Dallas, TX, USA). The serum leptin concentration was measured by ELISA (Crystal Chem).

2.4. Leptin sensitivity

Chronic leptin sensitivity was measured by subcutaneously implanting micro-osmotic pumps (Alzet) filled with recombinant mouse leptin (infusion rate of 0.5 μg leptin/h; A.F. Parlow, NHPP, NIDDK, USA). We assessed the food intake and weight gain of the mice for 5 days prior to surgery (basal period) as well as for 14 post-operative days. Each mouse acted as its own control, and we compared their food intake both before and during leptin administration.

2.5. Glucose homeostasis

Serum glucose levels were measured during the fed state (4 h fasting) and after fasting overnight. We used ELISA to measure the serum concentrations of insulin (Crystal Chem) and glucagon (Sigma) in mice that were fasted for 4 h. A glucose tolerance test (GTT; 1 g glucose/kg, i.p.) and an insulin tolerance test (ITT; 2 IU insulin/kg, i.p.) were performed in mice that were fasted for 4 h. The sensitivity to insulin in different tissues was assessed by infusing (i.p.) 5 IU insulin/kg and euthanizing the mice 15 min after infusion. The liver and gastrocnemius/soleus muscle were processed for Western blotting as previously described [23]. The following primary antibodies (1:1000) were used: anti-phospho insulin receptor (pIR-Tyr$^{1162/1163}$, Santa Cruz), anti-phospho AKT (pAKT-Ser^{473}, Cell Signaling) and anti-GAPDH (Santa Cruz). Proteins were visualized and analyzed using the Li-COR Odyssey system (Li-COR), and band intensities were normalized to GAPDH expression.

2.6. Relative gene expression

Control and LepR SOCS3 KO mice were euthanized after 4 h fasting and their hypothalami were quickly dissected for relative gene expression (qPCR) analysis. RNA extraction, reverse transcription and real-time polymerase chain reaction were performed as previously described [23]. The data were normalized by the β-actin expression and reported as the fold-change from the control group value.

2.7. c-Fos expression

After 16 weeks on the HFD, control ($n = 4$) and LepR SOCS3 KO ($n = 5$) mice were perfused with 10% buffered formalin and their brains processed to detect c-Fos expression as previously described [23]. We counted the number of c-Fos positive cells in one side of a representative rostrocaudal level of the arcuate nucleus of the hypothalamus (ARH), ventromedial nucleus of the hypothalamus (VMH), nucleus of the solitary tract (NTS), dorsal motor nucleus of the vagus (DMV) and area postrema (AP). In addition, we assessed the percentage of cells co-expressing c-Fos and β-endorphin (1:5000; Phoenix Pharmaceuticals) in the ARH.

2.8. Statistical analysis

The experimental data were tested for normality and homogeneity of variances. The differences between groups were compared using an unpaired two-tailed Student's *t*-test. Data from the leptin and insulin sensitivity tests were analyzed by two-way ANOVA and the Bonferroni post-hoc test. Statistical analyses were performed using GraphPad Prism software. The results are expressed as the mean ± SEM. We considered p values less than 0.05 to be statistically significant.

3. RESULTS

3.1. Neuronal deletion of SOCS3 reduces the body weight of mice regardless of diet

Mice carrying a neuronal deletion of the *Socs3* gene (Nestin SOCS3 KO) were produced as previously described [19,20] to study the role of SOCS3 on the predisposition of mice to DIO. Nestin SOCS3 KO mice exhibited a

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lower body weight at the beginning of the study and during the 12 weeks of HFD consumption (Figure 1A). Despite the reduced body weight of Nestin SOCS3 KO mice, deletion of SOCS3 did not significantly decrease the cumulative weight gain during the experimental period compared to the control group ($p = 0.08$, Figure 1B). Additionally, the mean food intake (Figure 1C) and serum leptin levels (Figure 1D) did not show significant changes in Nestin SOCS3 KO mice compared to the control group. Upon examination of the body adiposity, the Nestin SOCS3 KO mice presented with a lighter perigonadal fat pad mass and reduced adiposity compared to the control animals (Figure 1E). We also assessed the glucose homeostasis using GTT and ITT. Nestin SOCS3 KO mice showed better glucose tolerance and higher insulin sensitivity compared to the control animals (Figure 1F—G). Because the Nestin SOCS3 KO mice exhibited a lower body weight at the beginning of the experiment, we studied a group of control and Nestin SOCS3 KO mice consuming a low-fat regular rodent diet (Figure 1H). The Nestin SOCS3 KO mice exhibited a reduced body weight during the entire experimental period (Figure 1H). However, no differences in the cumulative weight gain were observed between the groups (control: 4.8 $\pm$ 0.4 g; Nestin SOCS3 KO: 4.4 $\pm$ 0.3 g; $p = 0.39$).

3.2. The Nestin-Cre transgene reduces body weight and affects the adiposity independent of inducing genetic recombination

The Cre enzyme is able to produce genetic recombination only in DNA possessing a pair of loxP sequences. Therefore, no phenotype should be expected in the mice solely expressing Cre under the Nestin promoter. We produced a group of Nestin-Cre mice and their littermate (wild-type) controls to study if the Nestin-Cre transgene affects the energy balance regulation independently of any loxP-flanked gene. Confirming previous findings [20,22], adult Nestin-Cre mice showed a lower body weight compared to their wild-type littermates (Figure 2A). Additionally, an increased adiposity was observed in mice carrying the Nestin-Cre allele compared to control animals (Figure 2B).

3.3. Inactivation of SOCS3 in LepR-expressing cells increases leptin sensitivity of mice exposed to HFD

Because Nestin-Cre mice exhibited a metabolic phenotype and Nestin SOCS3 KO mice had a lower body weight at the beginning of the study, other models were required to study the role of SOCS3 on the predisposition to DIO without such confounders. For this purpose, we produced mice lacking SOCS3 only in LepR-expressing cells (LepR SOCS3 KO mice). Therefore, the genetic deletion was restricted to a much smaller population of neurons and affected more specifically LepR signaling. Before studying the predisposition to obesity in LepR SOCS3 KO mice, we determined if the LepR-Cre allele produces changes in energy balance regulation. Mice carrying the LepR-Cre allele showed no differences in their body weight compared to control (wild-type) littermates (Figure 2C). Additionally, the body composition of 20-week old LepR-Cre mice was similar to control animals

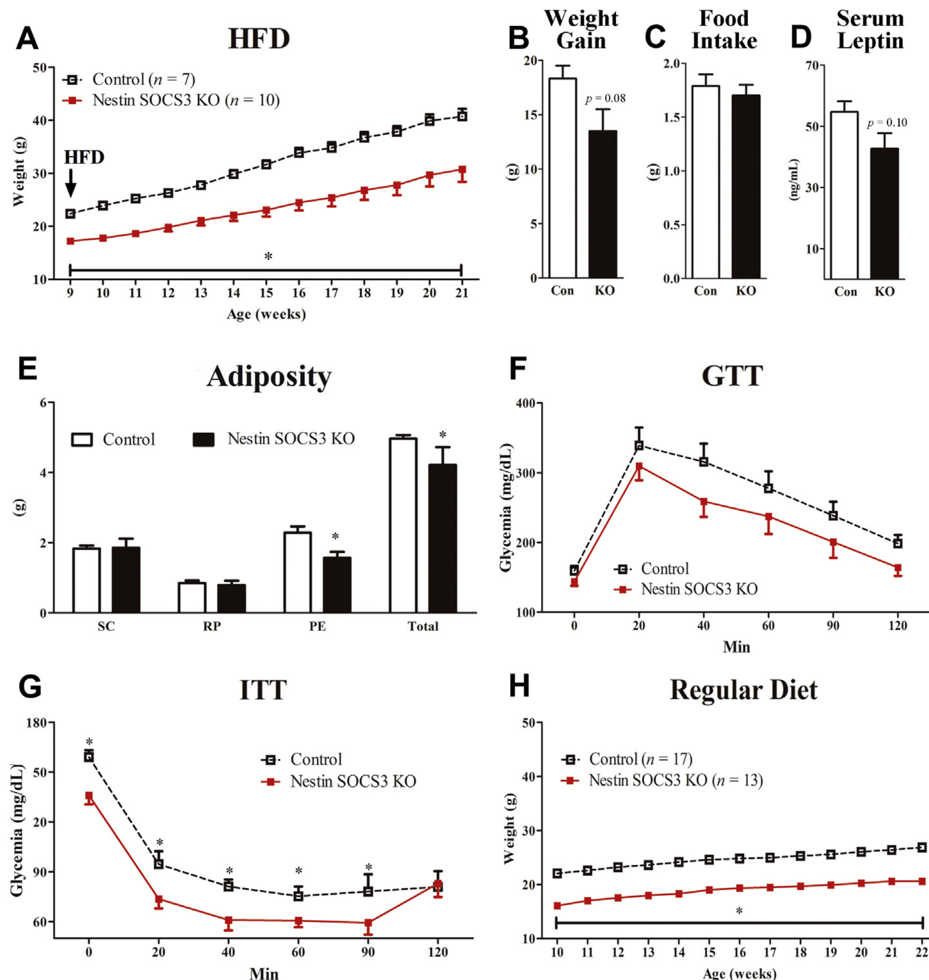

Figure 1: Neuronal deletion of SOCS3 partially prevented the accumulation of body fat and improved the glucose homeostasis of mice fed the HFD. Body weight (A), cumulative weight gain (B), food intake (C), serum leptin (D), adiposity (E), GTT (F) and ITT (G) of mice consuming HFD. A separate group was maintained on a low-fat regular rodent diet (H). $^*p < 0.05$ compared to the control group.

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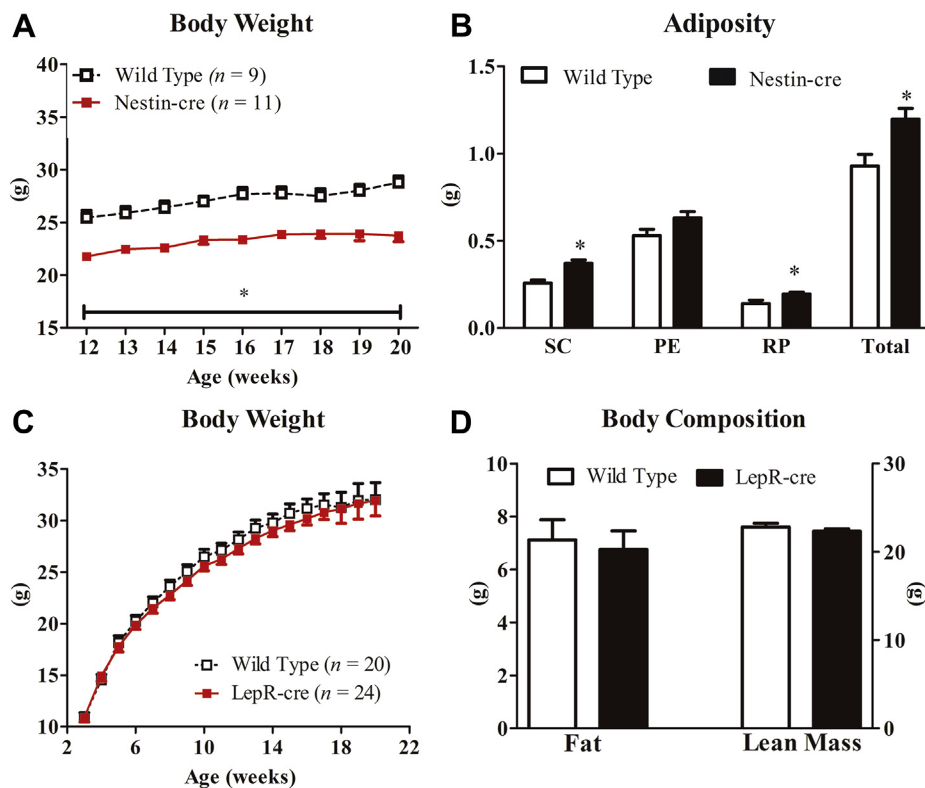

Figure 2: The Nestin-Cre transgene reduces body weight and affects the adiposity independent of inducing genetic recombination. Body weight (A) and adiposity (B) of mice carrying the Nestin-Cre transgene and their respective littermate wild-type mice. Mice carrying the LepR-Cre allele did not show any differences in body weight (C) and body composition (D) compared to littermate wild-type mice. *$p < 0.05$ compared to the wild-type group.

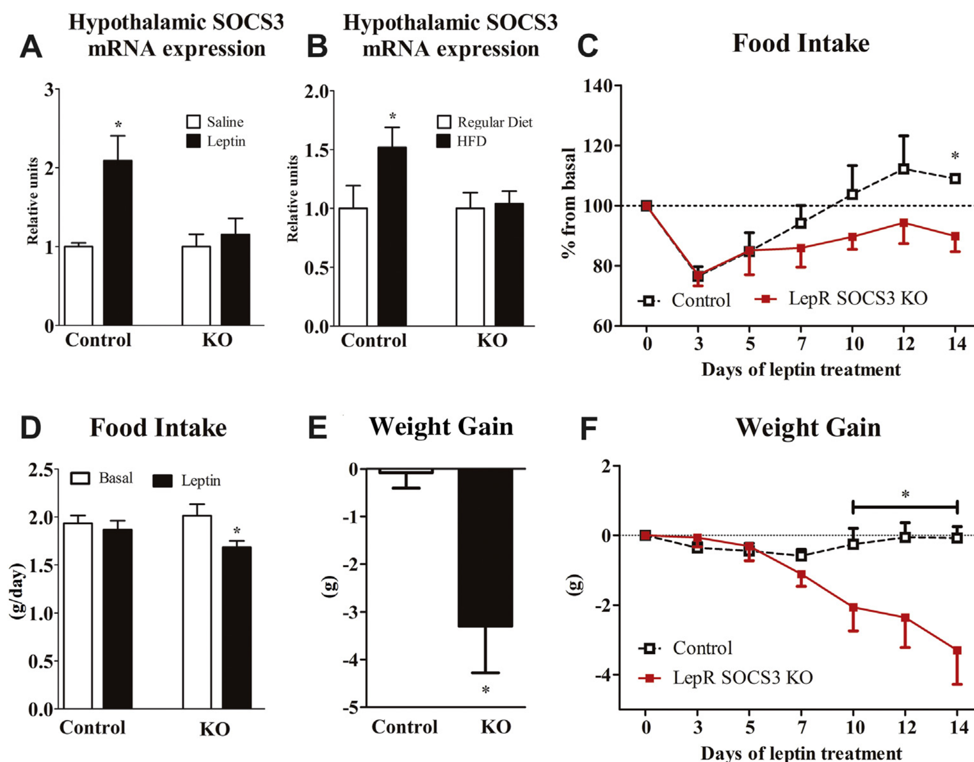

Figure 3: Inactivation of SOCS3 in LepR-expressing cells increases leptin sensitivity of mice exposed to HFD. Changes in hypothalamic SOCS3 expression after acute leptin injection (A) and in diet-induced obese mice (B). Effects of chronic leptin treatment reduced on food intake (C–D) and weight gain (E–F). *$p < 0.05$ compared to the control group.

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(Figure 2D). After confirming that the LepR-Cre allele did not affect body weight, we validated the LepR SOCS3 KO mouse by assessing the ability of an acute i.p. infusion of leptin to induce SOCS3 expression in the hypothalamus. As previously shown [11], leptin administration increased hypothalamic SOCS3 expression in the control group (Figure 3A). However, leptin infusion failed to increase the hypothalamic SOCS3 expression in LepR SOCS3 KO mice (Figure 3A). Obese animals have been shown to have higher hypothalamic SOCS3 expression [11]. Therefore, we compared hypothalamic SOCS3 mRNA expression between mice consuming the low-fat regular diet and the HFD (Figure 3B). Chronic consumption of the HFD increased hypothalamic SOCS3 mRNA expression in the control mice, but no changes in SOCS3 expression were observed in the hypothalamus of LepR SOCS3 KO mice (Figure 3B). Taken together, these results demonstrated the efficacy of SOCS3 ablation. Deletion of SOCS3 in LepR-expressing cells should release leptin signaling from the inhibitory influence of SOCS3 [15]. Therefore, leptin sensitivity is expected to be increased in LepR SOCS3 KO mice. To test leptin sensitivity in LepR SOCS3 KO mice, we implanted micro-osmotic pumps to deliver leptin in mice chronically exposed to HFD, which is a condition that causes leptin resistance. Food intake decreased in the control group but began to increase during the final days of leptin treatment (Figure 3C). Consequently, the average food intake of the control mice was not significantly affected by leptin, indicating a state of leptin resistance induced by chronic HFD consumption (Figure 3D). However, the LepR SOCS3 KO mice sustained a reduced food intake during the treatment period, resulting in lower average food intake compared to the basal period (Figure 3C—D). Leptin treatment had no effect on the

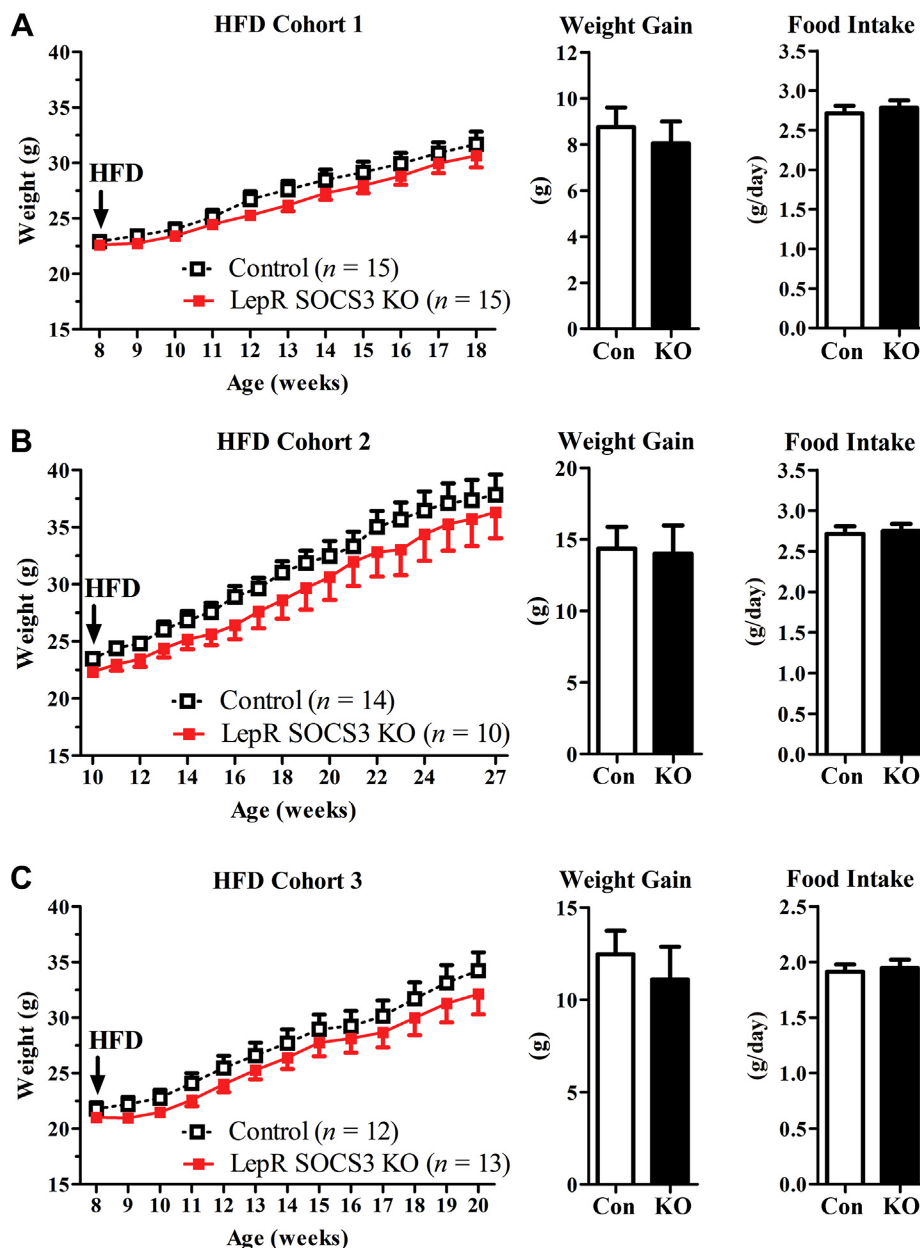

Figure 4: Inactivation of SOCS3 in LepR-expressing cells did not prevent the obesity induced by HFD. Three independent cohorts of LepR SOCS3 KO and control mice (A—C) were studied to determine the possible changes in body weight, weight gain and food intake caused by a HFD.

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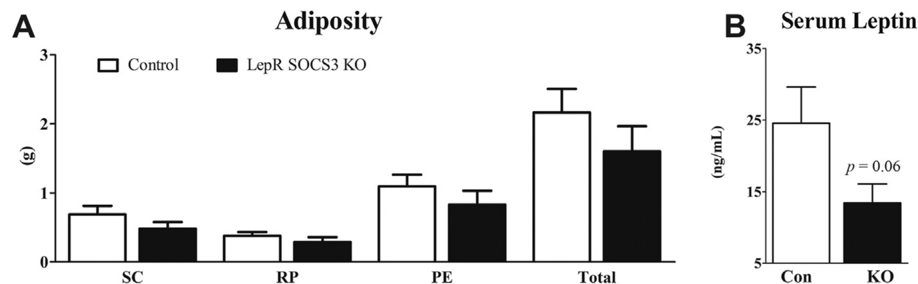

Figure 5: LepR SOCS3 KO mice did not show significant differences in the adiposity (A) and leptin levels (B) compared to control animals consuming HFD.

cumulative weight gain of control mice, whereas the LepR SOCS3 KO mice showed a sustained decrease in the weight gain during the entire course of the leptin treatment (Figure 3E–F).

3.4. Inactivation of SOCS3 in LepR-expressing cells did not prevent the obesity induced by HFD

We assessed if LepR SOCS3 KO mice are protected from DIO. For this purpose, control and LepR SOCS3 KO mice were exposed to a HFD for 10–16 weeks. No difference in the initial body weight was observed between the control and LepR SOCS3 KO mice (Figure 4). In total, we produced three independent cohorts of control and LepR SOCS3 KO mice consuming the HFD. None of these groups presented with protection against DIO (Figure 4A–C). Furthermore, food intake was not significantly affected by SOCS3 inactivation in LepR-expressing cells (Figure 4A–C). We determined the adiposity in the groups, and the LepR SOCS3 KO mice did not show significant changes compared to the control animals (Figure 5A). However, the serum leptin levels tended to be lower in the LepR SOCS3 KO mice compared to the control animals (Figure 5B), although this result was not significant ($p = 0.06$). We assessed the hypothalamic expression of genes related to leptin resistance and energy balance regulation (Figure 6). No differences between the groups were observed in the hypothalamic expression of NPY, AgRP, MCH, Orexin, PTP1B, PTPN2, PTPRε, SH2B1 and CIS (Figure 6). However, LepR SOCS3 KO mice exhibited a decreased expression of proopiomelanocortin (POMC) and SOCS1 mRNA levels and higher expression of PTPN11 mRNA compared to the control mice. Because previous studies have indicated that HFD consumption change the hypothalamic POMC expression [24,25], we compared POMC expression in mice consuming a low-fat regular rodent diet and the HFD. In accordance with the results found in earlier studies [24,25], the HFD increased the hypothalamic expression of POMC mRNA in control mice (Low-fat diet: 1.00 $\pm$ 0.12; HFD: 1.38 $\pm$ 0.08; $p = 0.01$). However, LepR SOCS3 KO mice on HFD did not show difference in the hypothalamic POMC expression compared to animals consuming the regular rodent diet (Low-fat diet: 0.74 $\pm$ 0.08; HFD: 0.99 $\pm$ 0.12; $p = 0.11$).

3.5. LepR SOCS3 KO mice are protected from insulin resistance induced by HFD

We then assessed if SOCS3 plays a role in diet-induced insulin resistance by measuring the postprandial blood glucose concentration. We observed that LepR SOCS3 KO mice exhibited lower glycemia compared to the control animals (Figure 7A). Overnight fasted mice did not present any differences in glucose levels (Figure 7B). LepR SOCS3 KO mice exhibited lower serum insulin levels compared to the control animals, suggesting that the LepR SOCS3 KO mice have increased insulin sensitivity (Figure 7C). No differences in serum glucagon levels were observed between groups ($p = 0.24$, Figure 7D). To further assess glucose homeostasis in the experimental animals, we performed GTT and ITT and observed that the glucose tolerance and insulin sensitivity were improved in LepR SOCS3 KO mice (Figure 7E–F). The liver and skeletal muscle are key tissues that control glycemia, and hypothalamic leptin signaling modulates insulin sensitivity in these tissues [1,26–30]. Acute i.p. injection of insulin induced phosphorylation of the insulin receptor (pIR) and AKT (pAKT) in the liver of control and LepR SOCS3 KO mice (Figure 7G). However, LepR SOCS3 KO mice showed an increased response compared to the control animals (Figure 7G). An improved response to insulin was also observed in the gastrocnemius/soleus muscle of the LepR SOCS3 KO mice compared to the control animals (Figure 7H). Changes in SOCS3 expression in these tissues could influence their insulin sensitivity as previous reported [31]. However, no differences in SOCS3 expression in the liver (control: 1.00 $\pm$ 0.27; LepR SOCS3 KO: 0.97 $\pm$ 0.17; $p = 0.93$) and skeletal muscle (control: 1.00 $\pm$ 0.08; LepR SOCS3 KO: 0.93 $\pm$ 0.07; $p = 0.56$) were observed between the experimental groups.

3.6. Deletion of SOCS3 from LepR-expressing cells increased hypothalamic expression of K_{atp} channel subunits and c-Fos expression in POMC neurons

Central mechanisms are likely involved in the effects of SOCS3 ablation to improve glucose homeostasis in mice consuming the HFD. Previous studies indicated that protein kinase C (PKC) signaling plays a key role in mediating the hypothalamic insulin resistance induced by fatty acids [32,33]. Among all isoforms, PKC delta and theta seem to be more important for these effects [32,33]. Therefore, we determined the hypothalamic expression of PKC delta and theta, but we did not observe significant differences between control and LepR SOCS3 KO mice (Figure 8A). The activity of K_{atp} channel in hypothalamic neurons is also essential for the maintenance of glucose homeostasis by

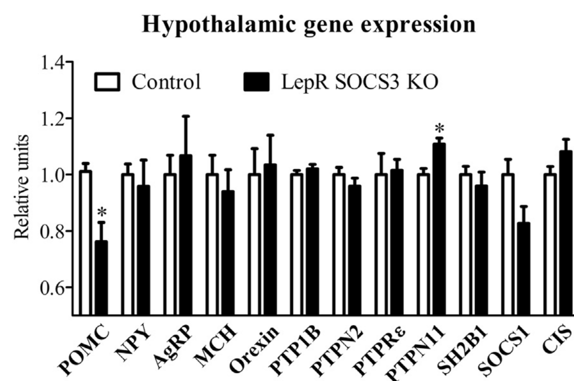

Figure 6: Hypothalamic mRNA expression of genes involved with leptin resistance and energy balance regulation. *$p < 0.05$ compared to the control group.

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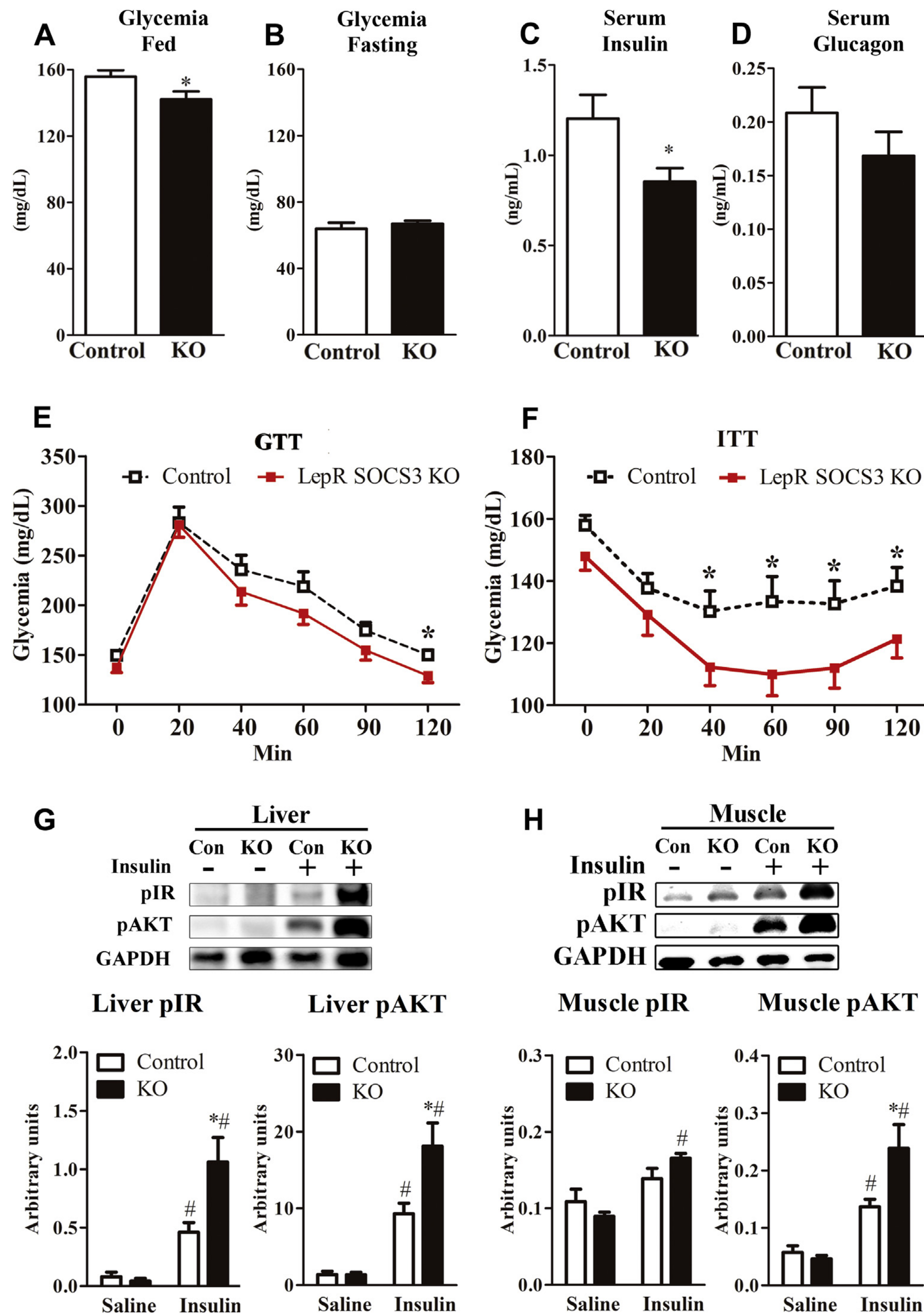

Figure 7: LepR SOCS3 KO mice are protected from insulin resistance induced by HFD. Levels of postprandial (A) and overnight fasted (B) glucose, serum insulin (C), serum glucagon (D), GTT (E) and ITT (F) in mice consuming HFD. Activation of the insulin intracellular pathway in the liver (G) and gastrocnemius/soleus muscle (H). $^{*}p < 0.05$ compared to the control group. $^{\#}p < 0.05$ compared to the respective saline-treated group.

controlling hepatic glucose production [29,34,35]. Furthermore, insulin's ability to activate K_{atp} channel in hypothalamic neurons is impaired in obese animals [36]. Therefore, we investigated whether the prevention of diet-induced insulin resistance in LepR SOCS3 KO mice is due to changes in hypothalamic expression of K_{atp} channel subunits. The LepR SOCS3 KO mice exhibited an increased hypothalamic expression of $K_{ir}6.1$ and SUR2 subunits compared to control animals (Figure 8A). No changes were observed in the expression of $K_{ir}6.2$ or SUR1 K_{atp} channel subunits (Figure 8A). Next, we investigated the expression of c-Fos, as a marker of neuronal activity, in several nuclei of the hypothalamus and brainstem that are related with the regulation of glucose homeostasis (Figure 8B). The ablation of SOCS3 in LepR-expressing cells caused a small but significant increase of c-Fos expression in the ARH ($p = 0.01$; Figure 8C–D). No changes in c-Fos expression were observed in other areas analyzed including the VMH, NTS, DMV and AP (Figure 8B). The c-Fos expression was

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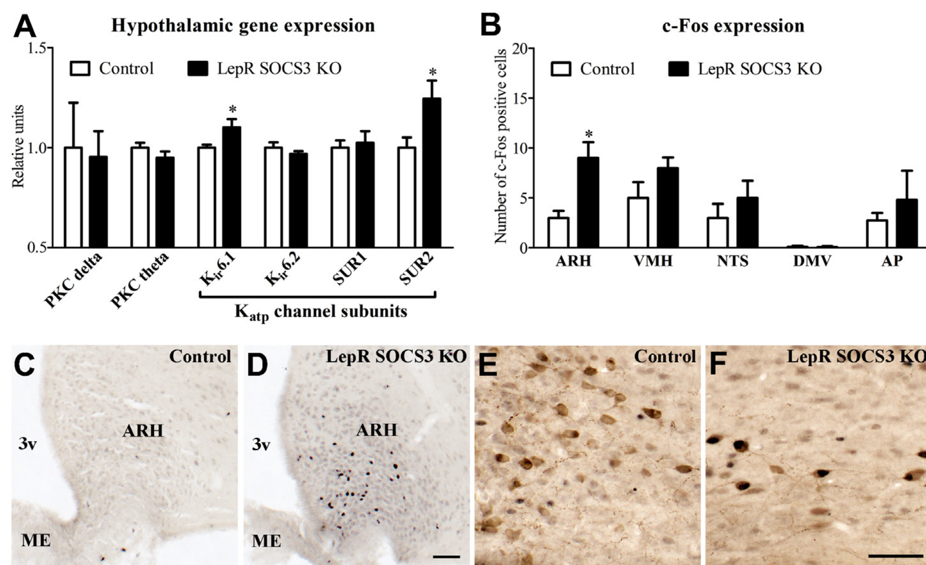

Figure 8: Deletion of SOCS3 from LepR-expressing cells increased hypothalamic expression of K_{atp} channel subunits and c-Fos expression in POMC neurons. Hypothalamic gene expression (A) and c-Fos immunoreactivity in several brain areas (B). Photomicrographs that show the c-Fos expression in the ARH of control (C) and LepR SOCS3 KO mice (D). Photomicrographs that show the co-expression between c-Fos (black nucleus) and β-endorphin (brown cytoplasm) in the ARH of control (E) and LepR SOCS3 KO mice (F). Notice that double-labeled neurons are virtually absent in the ARH of control mice (E), but are abundantly found in the ARH of LepR SOCS3 KO mice (F). *$p < 0.05$ compared to the control group. Abbreviations: 3v, third ventricle; ME, median eminence. Scale bar = 50 μm.

observed predominantly in the lateral aspects of the ARH (Figure 8D) which coincides with the distribution pattern of POMC neurons [37,38]. Thus, we investigated if c-Fos positive neurons in the ARH co-express β-endorphin, a peptide resulting from processing of POMC precursor. The LepR SOCS3 KO mice showed a higher number of c-Fos positive neurons co-expressing β-endorphin (6.6 ± 1.6 cells/section; Figure 8F) in comparison to control animals (0.3 ± 0.3 cells/section; $p = 0.029$; Figure 8E). The double-labeled neurons represented 44% of c-Fos positive cells in LepR SOCS3 KO mice compared to 4% in control mice ($p = 0.044$). In addition, 23% of β-endorphin immunoreactive neurons co-expressed c-Fos in the LepR SOCS3 KO mice compared to 3% of neurons in control mice ($p = 0.047$). No changes in the total number of β-endorphin immunoreactive neurons were observed between groups (control: 23.7 ± 6.7 cells/section; LepR SOCS3 KO: 31.2 ± 5.8 cells/section; $p = 0.444$).

4. DISCUSSION

Ob/ob and *db/db* mice are hyperphagic, massively obese and have severe insulin resistance [39,40]. Therefore, leptin signaling is essential for the appropriate regulation of food intake, body weight and glucose homeostasis [17,18,38]. A more subtle but similar phenotype is caused by chronic consumption of a HFD. These effects are believed to be secondary to the leptin resistance state that manifests in diet-induced obese mice [3]. Therefore, a better understanding of the molecular mechanisms that cause leptin resistance is of great interest for the development of new therapies to treat both obesity and T2DM [1,4]. Here we studied the role of SOCS3 in predisposing mice to DIO and diet-induced insulin resistance. We observed that neuronal deletion of SOCS3 partially prevented the accumulation of body fat and improved glucose homeostasis of mice consuming a HFD. However, the Nestin-Cre transgene alone produced important metabolic alterations, thus limiting the interpretation of the results produced with this mutant. To specifically study the effects of SOCS3 in LepR signaling, we generated a mouse model that deletes SOCS3 only in LepR-expressing cells. These mice were protected against leptin and insulin resistance caused by a HFD. However, the ablation of SOCS3 in LepR-expressing cells did not prevent the development of DIO.

Previous studies have shown that mice carrying the Nestin-Cre transgene have reduced body weight and nose—anus length but higher adiposity [20,22]. Our results confirmed these earlier findings. Nestin-Cre mice have been used in hundreds of studies to induce neuronal-specific genetic manipulations. By using Nestin-Cre to induce the neuronal ablation of SOCS3, previous studies demonstrated that SOCS3 contributes to the obese phenotype observed in mice consuming a HFD [19,20]. These findings were confirmed by studies using other mouse models [16,19,41,42]. Although we failed to observe a significant prevention in the weight gain caused by chronic consumption of a HFD, the neuronal deletion of SOCS3 significantly reduced the adiposity. This effect is highlighted by the fact that mice carrying the Nestin-Cre transgene have a higher body fat mass. Therefore, our results using the Nestin-Cre mouse confirmed previous findings indicating that neuronal inactivation of SOCS3 partially prevents the development of DIO and the insulin resistance, both of which are observed in mice consuming a HFD. However, our results also suggest that the use of Nestin-Cre mouse model may not be recommended in studies that investigate changes in energy balance regulation.

As an alternative method to study the role of SOCS3 in the metabolic alterations caused by a HFD, we produced mice that only knocked out SOCS3 in LepR-expressing cells. Conversely from the results obtained with Nestin-Cre mice, mice carrying one copy of the LepR-Cre allele did not exhibit any obvious metabolic phenotype (similar body weight, adiposity and body lean mass). Furthermore, our breeding strategy was designed to produce animals carrying the LepR-Cre allele in both control and KO groups. We observed that ablation of SOCS3 in LepR-expressing cells did not alter the body weight of animals consuming a regular rodent diet. However, the LepR SOCS3 KO mice were protected against leptin resistance induced by a HFD. Despite the improved leptin sensitivity of LepR SOCS3 KO mice, they were not protected against DIO. Other studies also found modest changes in body weight after manipulating SOCS3 expression. For example, upregulation of SOCS3

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in LepR-expressing cells did not prevent DIO [42], and a recent study that induced neuronal deletion of SOCS3 observed no effect in the prevention of weight gain and obesity in mice consuming a HFD [20]. Interestingly, manipulations of SOCS3 expression in specific populations of neurons produce significant effects on energy balance regulation. For example, deletion of SOCS3 in POMC cells partially prevented DIO [41], whereas upregulation of SOCS3 in POMC cells caused late-onset obesity [42]. POMC-expressing neurons represent an important population of cells activated by leptin which produces anorexigenic neurotransmitters that regulate energy balance and glucose homeostasis [1,25–27,38]. However, it is important to mention that only a subset of POMC neurons expresses LepR [26,27,37]. Therefore, inactivation of SOCS3 in our mouse model was restricted to the subpopulation of POMC cells that coexpresses the LepR. Differences in the cell populations affected by our genetic manipulation in comparison to other studies may be responsible for some of these divergent results.

Other intracellular proteins aside from SOCS3 are also involved in the regulation of leptin sensitivity. Several protein-tyrosine phosphatases (PTPs), adapter proteins and other components of the SOCS family showed altered expression in the hypothalamus of obese animals, and these proteins can affect leptin signaling pathways [11,15,20,43–46]. Thus, deletion of SOCS3 may be compensated by changes in the expression of other proteins that modulate leptin sensitivity. For example, the combined neural inactivation of SOCS3 and PTP1B produced synergistic and additive effects on the regulation of body energy balance [20]. However, we observed few changes in the hypothalamic expression of PTPs, adapter proteins and other SOCS family members in LepR SOCS3 KO mice. We observed increased expression of the PTPN11 transcript, which encodes the Shp2 tyrosine phosphatase. However, neuronal deletion of Shp2 in mice causes obesity and impaired leptin-induced activation of the MAPK/ERK signaling pathway [47,48]. However, other studies observed that Shp2 downregulates other LepR signaling pathways [49,50]. Interestingly, both Shp2 and SOCS3 bind to the same residue (phospho-Tyr^{985}) of LepR [13,49,50]. A mutation in the Tyr^{985} of LepR produces a lean phenotype, indicating that the phosphorylation of this residue exerts a predominantly inhibitory effect on leptin signaling [51]. The mechanism by which SOCS3 deletion in LepR-expressing cells induces changes in the hypothalamic expression of Shp2 is unknown. Additionally, LepR SOCS3 KO mice exhibited a decreased SOCS1 hypothalamic expression. Although SOCS1 inhibits insulin signaling [15], SOCS1 deficiency does not prevent either DIO or diet-induced insulin resistance in mice [52]. Previous studies have shown that mice exposed to a HFD present an increased hypothalamic expression of POMC mRNA [24,25]. We observed a decreased hypothalamic expression of POMC mRNA in LepR SOCS3 KO animals compared to control animals. In addition, the HFD consumption increased POMC expression in control animals, but it had no significant effects in LepR SOCS3 KO mice. Therefore, the inactivation of SOCS3 in LepR-expressing cells prevented the diet-induced changes in hypothalamic POMC expression.

Remarkably, inactivation of SOCS3 in LepR-expressing cells prevented diet-induced insulin resistance independently of changes in body weight. Deletion of SOCS3 from steroidogenic factor 1-positive cells also improved glucose homeostasis without affecting body weight [53]. SOCS3 inhibits both leptin and insulin signaling [15], and some neurons that are recruited by leptin are also responsive to insulin [54]. Additionally, either leptin or insulin activates the phosphatidylinositol 3-kinase (PI3K) signaling pathway [54,55]. Disrupting the PI3K pathway in specific populations of neurons that express LepR, such as POMC cells, affects glucose homeostasis [56]. Independent of insulin, the action of leptin in hypothalamic neurons has a profound impact on the regulation of glucose homeostasis [1,4,26,27]. For example, the expression of LepR only in POMC cells mildly affects the regulation of body weight in otherwise LepR-deficient mice, but POMC-specific LepR expression induces an impressive recovery in their insulin resistance [26,27]. SOCS3 expression in the liver and skeletal muscle of LepR SOCS3 KO mice did not change compared to the control animals. However, these tissues showed an increased responsiveness to insulin in LepR SOCS3 KO mice. It is well-known that central leptin signaling can modulate the insulin sensitivity in peripheral tissues [1,26–30]. In addition, the regulation of glucose homeostasis requires the activity of K_{atp} channels in hypothalamic neurons [35]. Interestingly, we observed an increased hypothalamic expression of K_{atp} channel subunits in LepR SOCS3 KO mice suggesting that central mechanisms are involved in the prevention of diet-induced insulin resistance. The activation of K_{atp} channels in the mediobasal hypothalamus lowers blood glucose levels through inhibition of hepatic gluconeogenesis by the vagus nerve [29]. Therefore, we analyzed c-Fos expression in the dorsal vagal complex as well as in the ARH and VMH which are major hypothalamic sites that regulate glucose homeostasis. The ablation of SOCS3 in LepR-expressing cells did not induce c-Fos expression in the dorsal vagal complex (NTS, DMV and AP) or in the VMH. Nonetheless, the lack of c-Fos expression does not preclude a possible involvement of these areas in the improved glycemic control observed in LepR SOCS3 KO mice. On the other hand, an increased c-Fos expression was observed in the ARH of LepR SOCS3 KO mice. POMC neurons play a pivotal role in mediating the effects of leptin in the regulation of glucose homeostasis [26,27]. Additionally, manipulations in K_{atp} channel activity in POMC neurons impair the physiological control of blood glucose [30]. Therefore, we determined whether c-Fos immunoreactive cells in the ARH are POMC-positive neurons. Remarkably, about half of c-Fos positive cells in the ARH of LepR SOCS3 KO mice co-expressed a POMC marker (β-endorphin). The co-expression of c-Fos and β-endorphin was virtually absent in the ARH of control animals. Therefore, our findings suggest that the ablation of SOCS3 in LepR-expressing cells increased the activity of POMC neurons in mice chronically exposed to a HFD. Given the recent findings that melanocortin-4 receptors expressed by sympathetic and parasympathetic preganglionic neurons regulate glucose homeostasis [57,58], our results suggest that the improved glycemic control in LepR SOCS3 KO mice is mediated by ARH POMC neurons upstream of autonomic preganglionic neuronal populations.

In summary, our results suggest that the importance of SOCS3 on the development of DIO may have been overestimated. However, the regulation of leptin signaling by SOCS3 possibly orchestrates the diet-induced changes on glucose homeostasis. These effects probably depend on POMC neurons because SOCS3 deletion prevented the diet-induced changes in hypothalamic POMC expression and increased the activity of POMC cells, at least by assessing the c-Fos expression. These findings may help to clarify the molecular mechanisms linking obesity and T2DM. Therefore, potential SOCS3 inhibitors may be developed as a promising therapy for the treatment of T2DM.

DISCLOSURE STATEMENT

The authors have nothing to disclose.

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CONFLICT OF INTEREST

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

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