Suppressor of cytokine signaling 3 knockdown in the mediobasal hypothalamus: counterintuitive effects on energy balance

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Abstract

An increase in brain suppressor of cytokine signaling 3 (SOCS3) has been implicated in the development of both leptin and insulin resistance. *Socs3* mRNA is localized throughout the brain, and it remains unclear which brain areas are involved in the effect of SOCS3 levels on energy balance. We investigated the role of SOCS3 expressed in the mediobasal hypothalamus (MBH) in the development of diet-induced obesity in adult rats. *Socs3* mRNA was down-regulated by local injection of adeno-associated viral vectors expressing a short hairpin directed against *Socs3*, after which we determined the response to high-fat high-sucrose choice diet. In contrast to neuronal *Socs3* knockout mice, rats with SOCS3 knockdown limited to the MBH showed increased body weight gain, larger amounts of white adipose tissue, and higher leptin concentrations at the end of the experiment. These effects were partly due to the decrease in locomotor activity, as 24 h food intake was comparable with controls. In addition, rats with *Socs3* knockdown in the MBH showed alterations in their meal patterns: average meal size in the light period was increased and was accompanied by a compensatory decrease in meal frequency in the dark phase. In addition, neuropeptide Y (*Npy*) mRNA levels were significantly increased in the arcuate nucleus of *Socs3* knockdown rats. Since leptin is known to stimulate *Npy* transcription in the absence of *Socs3*, these data suggest that knockdown of *Socs3* mRNA limited to the MBH increases *Npy* mRNA levels, which subsequently decreases locomotor activity and alters feeding patterns.

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Introduction

Obesity has been associated with leptin resistance in both humans and rodents. Although leptin normally reduces food intake, obese humans and rodents still overconsume calories despite increased leptin levels (Frederich *et al.* 1995, Maffei *et al.* 1995, Considine *et al.* 1996). In addition, after injection of exogenous leptin, obese humans and rodents do not respond with the decrease in food intake, which is normally observed in lean subjects and rodents (Cusin *et al.* 1996, Seeley *et al.* 1996, Halaas *et al.* 1997, Heymsfield *et al.* 1999). Several mechanisms have been proposed to mediate this leptin resistance such as impaired transport of leptin over the blood–brain barrier, impaired leptin receptor b (LEPRb) expression, and impaired LEPRb signaling (see review Morris & Rui (2009)). When leptin binds to the LEPRb, one of the pathways activated is the JAK2–STAT3 cascade (Bjorbaek *et al.* 1999). Phosphorylated STAT3 enters

the nucleus and serves as a transcription factor, increasing the transcription of several genes including suppressor of cytokine signaling 3 (*Socs3*). In turn, SOCS3 inhibits leptin signaling through binding to JAK2 and phosphorylated tyrosine 985 on the LEPRb (Bjorbaek *et al.* 1999, Bjorbaek *et al.* 2000). Thus, SOCS3 appears to act as a negative feedback regulator to prevent overactivation of leptin signaling. It has been shown that administration of leptin increases *Socs3* mRNA in lean animals and that *Socs3* mRNA levels are elevated in hypothalami of obese animals (Bjorbaek *et al.* 1998, Emilsson *et al.* 1999, Peiser *et al.* 2000, Munzberg *et al.* 2004, Tups *et al.* 2004, Krol *et al.* 2007). Together, these data suggest that SOCS3 plays an important role in the development of leptin resistance and obesity.

The importance of SOCS3 in leptin resistance and diet-induced obesity is supported by studies that examined the phenotype of mice with altered *Socs3* alleles. Although *Socs3* knockout mice die at

midgestation because of placenta failure (Roberts *et al.* 2001), heterozygous *Socs3*^{+/-} and neuronal *Socs3*-deficient animals are viable (Howard *et al.* 2004, Mori *et al.* 2004). These studies showed that *Socs3*^{+/-} and neuronal *Socs3*^{-/-} mice had normal body weight gain on a chow diet, but that they were more sensitive to leptin treatment than their wild-type littermates. Moreover, *Socs3*^{+/-} and neuronal *Socs3*^{-/-} mice were resistant to diet-induced obesity when placed on a high-fat diet (Howard *et al.* 2004, Mori *et al.* 2004).

Although deletion of genes by homologous recombination in mice has greatly contributed to our understanding of how genes affect energy balance, there are also limitations to what extent full deletion of a gene can teach us about the role of this gene in physiology, because the effect seen in knockout animals may occur due to compensation for the loss of gene expression by redundant genes, interference by selection markers, effects of the background strain, or developmental defects. Therefore, we performed complementary experiments to investigate the role of SOCS3 levels in the mediobasal hypothalamus (MBH) in the susceptibility to diet-induced obesity, via adeno-associated viral (AAV) vector-mediated knockdown of *Socs3*.

Materials and methods

Cell lines and plasmids

Human embryonic kidney (HEK) 293T cells were maintained at 37 °C with 5% CO₂ in DMEM supplemented with 10% FCS, 2 mM glutamine, 100 units/ml penicillin, 100 units/ml streptomycin, and non-essential amino acids.

pAAV-shCTRL (sh-c) and pAAV-shbase (empty, no shRNA insert (Fig. 1A)) were a kind gift from RJ Dileone (Hommel *et al.* 2006). We used bioinformatics tools on the website of Biopredsi and Invitrogen to identify possible functional siRNA/shRNA sequences against *Socs3* gene of the rat. We ordered four sets of two oligonucleotides (Stratagene, Amsterdam, The Netherlands) with SapI and XbaI overhangs (sh-1 to sh-4) (Fig. 1B). These oligonucleotides were annealed and ligated into SapI- and XbaI-digested pAAV-shbase (Fig. 1A). The shRNA was driven by a mouse U6 promoter, and after the shRNA sequence, there was a terminator sequence. In addition, this plasmid also expressed GFP under a CMV promoter hybridized to a β-actin intron. The *GFP* gene was followed by an independent terminator sequence.

The *Socs3* rat cDNA was obtained through PCR with *Pfu* polymerase on hypothalamic rat cDNA with the following primers: *Socs3*-g-F-2: 5'-AGACACAGTCTTCAGCGGG T and *Socs3*-g-R-2:

Figure 1 Constructs and overview of oligonucleotides used. Map of the AAV vector used. shRNAs were cloned between SapI and XbaI (A). Overview of the *Socs3* oligonucleotides used. The bold font indicates the *Socs3* sequence, with in between a miR-23 loop (B). The oligonucleotides were extended with SapI and XbaI overhangs (top and bottom respectively).

5'-AGAGTCCGCTTGTCATGCT. The *Socs3* PCR product was ligated in PCR-script. Subsequently, the *Socs3* gene was flanked with BamHI sites and inserted in BamHI-restricted p3xflag-renilla (p3xflag-renilla was a kind gift from M Vooijs), and this resulted in Socs3-renilla fusion plasmid. pcDNA4/TO-luc (luciferase) was a kind gift from M van der Wetering.

All constructs were verified by sequencing.

Luciferase assay

HEK293T cells in 24-well plates were transfected using polyethylenimine (PEI; Polysciences, Amsterdam, The Netherlands). Per well, 5 ng pcDNA4/TO-luc, 250 ng Socs3-renilla, and 812 ng pAAV-shRNA were transfected (molar ratio Socs3-renilla:pAAV-shRNA was 1:4).

Three days after transfection, the cells were lysed in passive lysis buffer (Promega) according to the manufacturer's protocol. Samples were assayed with a dual luciferase kit (Promega) and measured using a Victor 96-well plate reader (PerkinElmer, Groningen, The Netherlands). All values were normalized to luciferase (to correct for transfection efficiencies). Subsequently, the different pAAV-shRNA's were normalized to pAAV-shbase (empty). This was done by setting the AAV-shbase (empty) to 1. Experiments were performed four times in duplicate.

Virus production and purification

AAV production was performed with 15×15 cm dishes containing 80–90% confluent 293T cells on the day of transfection. Two hours before transfection, the 10% FCS–DMEM was replaced with 2% FCS–DMEM. The transfections were performed with PEI as described by Reed *et al.* (2006). pAAV-sh-1, pAAV-sh-2, or pAAV-sh-c was co-transfected with the helper plasmid pDP1 (Grimm *et al.* 2003; Plasmid Factory, Bielefeld, Germany) in a molar ratio of 1:1. The transfection mix remained on the cells until the next morning; then, the medium was refreshed with 2% FCS–DMEM. AAV production and purification were essentially performed as described by Zolotukhin *et al.* (2002). Briefly, 60 h after transfection, the cells were harvested in their medium, centrifuged, and washed with PBS containing 5 mM EDTA. Finally, the cells were collected in 12 ml ice cold buffer (150 mM NaCl and 50 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris), pH 8.4) and stored at –20 °C until further use. Usually, after 3 days, the cells were freeze-thawed twice, incubated for 30 min with 50 units/ml benzonase (Sigma) at 37 °C, and centrifuged. After centrifugation, the supernatant was loaded onto an iodixanol gradient (60, 40, 25, and 15% supernatant (Optiprep; Lucron Bioproducts, Sint Martens-Latem, Belgium)) in Quick-seal tubes (Beckman Coulter, Woerden,

The Netherlands). After 1 h of ultracentrifugation (350 000 *g* at 20 °C) in Ti70 rotor (Beckman Coulter), the 40% layer was extracted. This 40% layer was used for ion-exchange chromatography with 5 ml Hitrap Q HP columns (GE Healthcare, Hoevelaken, The Netherlands). The AAV-positive fractions, determined by PCR, were pooled and concentrated on Centricon Plus-20 Biomax-100 concentrator columns (Millipore, Amsterdam, The Netherlands). The titer, in genomic copies per ml (g.c./ml), was determined by qPCR with SYBR Green Mix in a LightCycler (Roche; Veldwijk *et al.* 2002). The qPCR primers were designed to detect *Gfp* and were *Gfp*-F: 5'-CACATGAAGCAGC-ACGACTT and *Gfp*-R: 5'-GAAGTTCACCTTGATGCCGT. The titer obtained was in the range of 6×10^{12} to 2×10^{13} genomic copies per ml (g.c./ml).

Animals

Male Wistar rats, weight ranging from 220 to 250 g, were purchased from Charles River (Crl-Wu, Sulzfeld, Germany). All rats were individually housed in filter-top cages in a temperature- and humidity-controlled room (temperature 21 ± 2 °C and humidity $55 \pm 5\%$) with a 12 h light:12 h darkness cycle (lights on at 0700 h). All experimental procedures were approved by the Committee for Animal Experimentation of the University of Utrecht (Utrecht, The Netherlands). From the moment of arrival, the rats received *ad libitum* chow (CRM pellets (3.31 kcal/g), Special Diet Service, Witham, Essex, UK) and water. Three weeks after injection of AAV particles into the MBH, animals were switched from chow to a high-fat high-sucrose choice (HFHS) diet. During this diet, the rats had *ad libitum* access to a 30% sucrose solution (30 g sugar in 100 ml water; 1.2 kcal/g) and saturated fat (blanc de boeuf, ossewit (9 kcal/g)) in addition to their normal chow and water. Following viral injections, rats were monitored for 50 days for effects on body weight, food intake, body core temperature, and locomotor activity. Body weight gain and food intake were measured three times a week at 1000 h (on Monday, Wednesday, and Friday). Core temperature and activity were automatically recorded via transmitters that sent digitized data to a nearby receiver. These data were recorded every 10 min using DSI software (DSI, St Paul, MN, USA). Food hoppers were automatically weighed using scales (Department of Biomedical Engineering, UMCU, Utrecht, The Netherlands), which sent data to a computer every 12 s. The data of days 18–20 (last week chow), 27–29 (after 1 week of HFHS), and 48–50 (after 4 weeks of HFHS) were used to analyze meal patterns. A meal was defined as an episode of food intake with a minimal consumption of 0.3 g of chow or 0.1 g of saturated fat and an intermeal interval of 5 min.

Surgical procedures

After 1½ week of acclimatization, surgery was performed under fentanyl/fluanisone (1 ml/kg Hypnorm (0.315 mg/kg fentanyl citrate and 10 mg/kg fluanisone) i.m., Janssen Pharmaceutica, Beerse, Belgium) and midazolam (0.5 ml/kg Dormicum (2.5 mg/kg midazolam hydrochloride) i.p., Roche) anesthesia. Carprofen (0.1 ml/kg Rimadyl (5 mg/kg carprofen) s.c., Pfizer Animal Health, Capelle a/d IJssel, The Netherlands) was administered as pain medication before surgery and 2 days after. During surgery, the rats received an abdominal transmitter (TA10TA-F40; Data Sciences International, St Paul, MN, USA) to measure temperature and activity. This was immediately followed by a bilateral injection (AAV-sh-1, AAV-sh-2, or AAV-sh-c, $n = 16$ per group) at the border of the arcuate nucleus (Arc) and ventromedial hypothalamic nucleus (VMH; coordinates anterior posterior (AP)) -2.6 mm from bregma; mediolateral (ML) ± 1.2 mm from bregma; dorsoventral (DV) -9.9 mm below the skull; with angle of 5 degrees. Per site, 1 µl of virus containing 6×10^9 g.c. of AAV-shRNA was injected at a rate of 0.2 µl/min. Following the injection, needles remained in place for 10 min before removal.

Collection of blood and tissues

On day 51, chow, fat, and sucrose were removed at 0700 h. In the afternoon, the rats were decapitated; the brains were immediately removed, quickly frozen on dry ice, and stored at -80°C . Trunk blood was collected in heparinized tubes containing 83 µmol EDTA and 1 mg aprotinin and immediately placed on ice. After centrifugation, blood plasma was stored at -20°C until further additional analysis. Retroperitoneal, epididymal, mesenteric, and subcutaneous white adipose tissue, thymus, and adrenals were isolated and weighed.

In situ hybridization

To verify injection sites, brains were sectioned on a cryostat at 20 µm thickness in series of 10. One series was used for *in situ* hybridization (ISH) with a 720 bp long digoxigenin (DIG)-labeled eGFP riboprobe (antisense to NCBI gene DQ768212). The ISH was performed as described by Schaeren-Wiemers & Gerfin-Moser (1993) with small modifications in the fixation procedure and hybridization temperature. Briefly, sections were fixed in 4% paraformaldehyde for 20 min and washed in PBS. After acetylation for 10 min (0.25% acetic anhydride in 0.1 M triethanolamine), sections were washed in PBS and prehybridized at RT in hybridization solution, containing 50% deionized formamide, $5 \times$ SSC, $5 \times$ Denhardt's

solution, 250 µg/ml Baker's yeast tRNA, and 500 µg/ml sonicated salmon sperm DNA. After 2 h, 150 µl of hybridization mixture containing 400 ng/ml DIG-labeled riboprobe was applied per slide; slides were covered with Nescofilm and hybridized overnight at 72°C . The next morning, slides were quickly washed in $2 \times$ SSC followed by $0.2 \times$ SSC for 2 h. Both wash steps were performed at 72°C . DIG was detected with an alkaline phosphatase-labeled antibody (1:5000, Roche) using nitroblue tetrazolium and bromochloroindolylphosphate (NBT/BCIP) as a substrate. After overnight incubation at RT with NBT/BCIP mixture, sections were quickly dehydrated in ethanol, cleared in xylene, and mounted using Entellan.

Quantitative ISH

Adjacent cryostat brain sections were used for radioactive ISH with ^{33}P -labeled antisense RNA probes for agouti-related peptide (*Agrp*, 396 bp mouse *Agrp* cDNA (Kas *et al.* 2003)), neuropeptide Y (*Npy*, 287 bp rat *Npy* cDNA), pro-opiomelanocortin (*Pomc*, 350 bp rat *Pomc* cDNA fragment (Kas *et al.* 2003)), and *Socs3* (1200 bp *Socs3* cDNA in PCR-script described above in cell lines and plasmids). The procedure for radioactive ISH and analysis has been described previously (la Fleur *et al.* 2010).

Plasma analysis

Plasma leptin and insulin were measured in duplicate using rat RIA kits (Millipore, Billerica, MA, USA). Plasma glucose was analyzed in triplicate using a glucose/GOD-perid method (Boehringer Mannheim).

Statistical analysis

Data are presented as group means $\pm$ S.E.M. All tissues weights are expressed as percentage of total body weight. Difference in body weight and food intake was assessed using repeated-measures ANOVA with *post hoc* Bonferroni tests to correct for multiple comparisons. Additional statistical analysis was performed using two-tailed *t*-tests. Differences were considered significant at $P < 0.05$.

Results

Knockdown efficiency of *Socs3* shRNAs *in vitro*

To investigate the efficiency of the different shRNA against *Socs3* *in vitro*, we fused genes encoding renilla and SOCS3 and performed renilla luciferase assays. *Socs3*-renilla was co-transfected in a 1:4 molar

ratio with the different pAAV-shRNA constructs against *Socs3* (sh-1 to sh-4), an AAV plasmid with control shRNA (sh-c), and an AAV plasmid without an shRNA insert (empty). In addition, a luciferase construct was co-transfected to correct for the transfection efficiencies. The renilla luciferase assays showed that sh-1 resulted in the lowest levels of

Socs3-renilla, with the highest level of knockdown, namely 81% compared with the empty vector ($t=43.44$, $df=14$, $P<0.0001$; Fig. 2A). Sh-2 was the second best with 74% knockdown, and sh-4 resulted in 68% knockdown compared with empty vector ($t=31.49$, $df=14$, $P<0.0001$ and $t=22.91$, $df=14$, $P<0.0001$ respectively). Sh-3 did not show knockdown of the *Socs3*-renilla construct ($t=1.91$, $df=14$, $P>0.05$). As expected, sh-c also did not reduce *Socs3*-renilla expression.

Figure 2 Knockdown of *Socs3* *in vitro* and *in vivo*. Graphical representation showing the *in vitro* knockdown of *Socs3*-renilla construct by pAAV-sh-1 to sh-4 and sh-c relative to pAAV without an shRNA insert (empty) (average of eight experiments) (A). The photograph in B shows a typical example of a GFP DIG-ISH when AAV-sh-1 was correctly injected bilaterally. The bar graph in C shows the *in vivo* knockdown of *Socs3* in the Arc by AAV-sh-1 relative to AAV-sh-c (C). *** $P<0.0001$ compared with empty vector or sh-c. Full colour version of this figure available via <http://dx.doi.org/10.1677/JME-10-0057>.

Localization of AAV-shRNA injections and knockdown *in vivo*

Based on the renilla luciferase assay, we decided to use AAV-sh-1 and AAV-sh-2 to knock down *Socs3* mRNA in the rat MBH *in vivo*, using AAV-sh-c as a control. Each group consisted of 15 or 16 rats. At the end of the experiment, we performed dig-labeled *Gfp* ISH to determine the injection site in each rat (*Gfp* was a marker in each AAV-shRNA vector). The MBH was considered to be transduced if a *Gfp* signal was observed bilaterally in the Arc and the VMH (AP between -2.1 and -3.6 mm; ML between 0 and 1 mm from bregma; Fig. 2B). Out of 16 rats that received AAV-sh-1, 4 rats were transduced bilaterally in the MBH, and 5 rats were transduced unilaterally. The MBH was missed on both sides in five rats (*Gfp* signal too far lateral), and in two rats, no *Gfp* signal could be detected anywhere. In the AAV-sh-2 group, none of the 15 rats were transduced bilaterally, but 7 rats were transduced unilaterally. In five rats, the MBH was not hit, and in three rats, no *Gfp* signal could be detected at all. In the AAV-sh-c group, correct bilaterally placed injections were observed in 3 out of 16 rats and unilaterally placed injections were observed in 9 rats. In two rats, the *Gfp* signal was observed lateral to the MBH, and in two rats, no *Gfp* signal could be detected. In all rats with *Gfp* expression, we noticed that AAV-shRNA did not transduce all cells at the site of injection.

For analysis, all controls (rats injected with AAV-sh-c vector) were pooled together, excluding one rat, which had hydronephrosis. AAV-sh-1 rats with bilateral *Gfp* expression in the MBH were analyzed thoroughly and compared with control rats before the other groups were analyzed.

To quantify the knockdown of *Socs3* mRNA by AAV-sh-1 *in vivo*, we performed a radioactive ISH with a ^{33}P -labeled *Socs3* RNA probe. For each rat, coronal sections throughout the Arc (200 μm apart) were analyzed. Knockdown of *Socs3* mRNA in the Arc was $59 \pm 4\%$ with AAV-sh-1 compared with *Socs3* mRNA in controls ($t=10.42$, $df=20$, $P<0.0001$; Fig. 2C).

Figure 3 Effect of *Socs3* knockdown on cumulative body weight gain. Body weight gain of bilaterally transduced sh-1 rats was increased compared with sh-c rats (A). In contrast, rats with unilateral or more lateral expression of sh-1 did not differ in body weight compared with rats with sh-c (B). **P* < 0.05 compared with sh-c. *n* = 4 for sh-1 rats and *n* = 15 for sh-c rats.

Effect of *Socs3* knockdown on body weight

After injection of the AAV-shRNA, rats received *ad libitum* access to chow and water for the first 3 weeks (day 0–21 post injection (p.i.)). Subsequently, rats were switched to a HFHS diet for 4 weeks (day 22–50 p.i.) to determine sensitivity to diet-induced obesity. During these 4 weeks, the rats had *ad libitum* access to chow, water, a 30% sucrose solution, and saturated fat (la Fleur *et al.* 2010). Surprisingly, bilateral knockdown of *Socs3* mRNA in the MBH increased body weight gain significantly compared with AAV-sh-c from day 38 onwards (Fig. 3A). Unilateral *Socs3* knockdown in the MBH or other regions (missed injections) by AAV-sh-1 did not affect body weight gain significantly when compared with AAV-sh-c during the experiment (Fig. 3B). At the end of the experiment, the subcutaneous fat mass of bilateral AAV-sh-1 rats was significantly increased compared with AAV-sh-c rats (Table 1). In addition, rats with unilateral knockdown of *Socs3*, using AAV-sh-1 or AAV-sh-2, also showed an increase in fat mass; the values of the unilateral AAV-sh-1 rats were in between those for bilateral AAV-sh-1 and AAV-sh-c (Table 1).

Effects of *Socs3* knockdown on food intake

In the bilateral AAV-sh-1 group, the total caloric intake during the experiment was not significantly increased, except for the first measuring point on HFHS diet (day 24 p.i.) (Fig. 4A). Furthermore, there were no apparent differences in choice of food between AAV-sh-1 and AAV-sh-c rats exposed to the HFHS diet. Chow, fat, sucrose, and water intake were not significantly different between the groups (Fig. 4B–E). Normally, rats do not eat much during the light period and eat most of their food in the dark period. To investigate whether this circadian pattern was affected by MBH *Socs3* mRNA knockdown, we performed an additional analysis for both the light and dark periods separately. On day 18–20, AAV-sh-1 rats ate significantly more calories in the light period, while in the dark period, food intake was similar to the AAV-sh-c rats (Table 2). This increase in caloric intake in the light period was also observed on days 27–29 and 48–50. However, on these days, AAV-sh-1 rats compensated by significantly decreasing their caloric intake in the dark period. AAV-sh-1 rats ate approximately the same amount of calories in the light period as in the dark period, while AAV-sh-c rats showed a normal feeding rhythm, with higher caloric intake in the dark period than in the light period (Table 2).

To investigate whether knockdown of *Socs3* mRNA in the MBH affects feeding behavior independently of total food intake, we analyzed meal patterns at three different time points: after 3 weeks of chow diet (day 18–20 p.i.), after 1 week of HFHS diet (day 27–29 p.i.), and after 4 weeks of HFHS diet (day 48–50 p.i.). On day 18–20 p.i., the AAV-sh-1 rats ate significantly more chow than AAV-sh-c rats in the light phase (Table 3). This was due to a significant increase in average meal size. After 1 and 4 weeks of HFHS diet, the AAV-sh-1 rats did not significantly differ in 24 h chow or lard intake compared with control rats; however, the increased meal size remained after 1 week on HFHS diet, but did not reach significance after 4 weeks on HFHS diet compared with AAV-sh-c rats (Table 3).

The increase in caloric intake of AAV-sh-1 in rats in the light period of day 18–20 was due to a significant increase in both meal size and frequency, while there were no significant effects in the dark period (Fig. 5A and D). On days 27–29 and 48–50, the average frequency of chow meals was significantly

Table 1 Effects of suppressor of cytokine signaling 3 (*Socs3*) knockdown on fat mass at the end of the experiment

	sh-c (<i>n</i> = 15)	sh-1 bilateral (<i>n</i> = 4)	sh-1 unilateral (<i>n</i> = 5)	sh-2 unilateral (<i>n</i> = 7)
SWAT (% BW)	1.34 ± 0.05	2.15 ± 0.17*	1.63 ± 0.08*	1.83 ± 0.20*
AWAT (% BW)	3.81 ± 0.18	5.30 ± 0.14*	3.99 ± 0.11	4.13 ± 0.30

SWAT, subcutaneous white adipose tissue; AWAT, abdominal white adipose tissue. **P* < 0.001 compared with sh-c.

Figure 4 Effects of *Socs3* knockdown on food and water intake. Rats received chow and water for the first 21 days. Subsequently, saturated fat and a sucrose solution were added to the diet. Total caloric intake (A) was calculated by adding calories derived from chow (B), fat (C), and sucrose (E). Water intake (D) did not add any caloric value. * $P < 0.05$ compared with sh-c. $n = 4$ for sh-1 rats and $n = 15$ for sh-c rats.

decreased in the dark period in AAV-sh-1 rats, while in the light period, the average size of chow meals was significantly increased compared with AAV-sh-c rats (Fig. 5B, C, E, and F). In addition, the average size of fat meals was significantly larger in the light phase of the first week of exposure to the HFHS diet (day 27–29); however, this effect did not reach significance when measured at day 48–50 (Fig. 5G, H, I, and J).

Effects of *Socs3* knockdown on locomotor activity and body core temperature

Knockdown of *Socs3* mRNA in the MBH did not significantly alter locomotor activity and body temperature during the third week (day 18–20 p.i.) on chow (Table 4). However, after 1 (day 27–29 p.i.) and 4 weeks (day 48–50 p.i.) on HFHS diet, locomotor activity in the dark period was reduced in AAV-sh-1 rats compared

with control rats. This decrease in locomotor activity was accompanied by a trend towards lower body core temperature in the dark period in week 1 of the HFHS diet and a significant decrease in temperature in week 4 of the HFHS diet (Table 4).

Effects of *Socs3* knockdown on endocrine parameters and body composition

The increase in body weight gain due to knockdown of *Socs3* mRNA in the MBH was accompanied by a significant increase in subcutaneous and abdominal white adipose tissue in AAV-sh-1 rats compared with AAV-sh-c rats (Table 1). AAV-sh-1 rats also showed significantly elevated plasma leptin concentrations and a trend to increased plasma concentrations of insulin and glucose (Table 5). Furthermore, thymus and adrenal weights did not differ between AAV-sh-1 and AAV-sh-c rats.

Table 2 Effects of suppressor of cytokine signaling 3 (*Socs3*) knockdown in the mediobasal hypothalamus (MBH) on caloric intake (kcal) in light and dark phases

	sh-c (n=7)	sh-1 (n=3)
Day 18–20		
Light	13.8 ± 1.49	33.4 ± 0.96 [‡]
Dark	52.3 ± 1.16	52.3 ± 1.72
Day 27–29		
Light	21.1 ± 4.87	47.6 ± 7.60*
Dark	61.1 ± 3.45	39.9 ± 3.99 [‡]
Day 48–50		
Light	12.9 ± 1.23	34.4 ± 2.99 [‡]
Dark	57.2 ± 2.24	31.2 ± 2.96 [‡]

P* < 0.05, [†]*P* < 0.01, [‡]*P* < 0.001 compared with sh-c.Effect of *Socs3* knockdown on *Agrp*, *Npy*, and *Pomc* mRNA in the Arc**

We investigated whether knockdown of *Socs3* mRNA in the MBH had any effects on mRNA expression levels of *Agrp*, *Npy*, and *Pomc*, because leptin signaling is suggested to regulate transcription of these neuropeptides. In addition, the observed phenotype, reduced locomotor activity, and increased feeding in the light phase resemble that of increased NPY signaling (Tiesjema *et al.* 2007). Radioactive ISH showed that AAV-sh-1 rats had significantly increased levels of *Npy* in the Arc compared with AAV-sh-c rats (Fig. 6A). The mRNA levels of *Agrp* and *Pomc* were comparable between AAV-sh-c and AAV-sh-1 rats.

Effects of unilateral *Socs3* knockdown

In unilaterally transduced AAV-sh-1 rats, we compared *Npy* mRNA levels between the side of the Arc that showed knockdown of *Socs3* mRNA to the side that did not.

This revealed an up-regulation of *Npy* levels on the side where *Socs3* mRNA was down-regulated (Fig. 6C and D). The *Socs3* mRNA was down-regulated on the side where *Gfp* was expressed (Fig. 6B). Rats with unilateral expression of AAV-sh-1 or AAV-sh-2 in the MBH showed a significant up-regulation of *Npy* mRNA (39% ± 12.2) compared with control rats (*t* = 3.151, *df* = 9, *P* < 0.05) on the side of transduction. Since bilateral *Socs3* mRNA knockdown clearly affected leptin concentrations and locomotor activity, we also examined these parameters in rats with unilateral *Socs3* mRNA knockdown in the MBH. This revealed an increase in leptin concentrations, while locomotor activity was decreased compared with controls. The values of these parameters in unilaterally transduced rats were in between those of bilaterally transduced AAV-sh-1 and control rats (Tables 4 and 5).

Discussion

In contrast to the current hypothesis that *Socs3* levels are positively correlated with the susceptibility to diet-induced weight gain, we here show that knockdown of *Socs3* mRNA limited to the MBH increased body weight, fat mass, and leptin levels. This was at least partly due to a decrease in locomotor activity during the dark period in MBH *Socs3* knockdown rats compared with control rats. Moreover, MBH *Socs3* knockdown increased meal size in the light phase throughout the experiment, but only transiently increased total daily food intake. Interestingly, *Npy* mRNA levels in the Arc were increased significantly in rats, which received the *Socs3* shRNA in the MBH compared with control rats.

The results obtained in this study are in contrast with previous *Socs3* deletion studies in the mouse brain (Mori *et al.* 2004, Kievit *et al.* 2006, Zhang *et al.* 2008). In these studies, neuronal *Socs3* deletion prevented diet-induced obesity (Mori *et al.* 2004). However, *Socs3* specifically deleted in POMC neurons or in SF-1 neurons had only modest effects on body weight upon exposure to a high-fat diet, although glucose homeostasis did markedly improve. When *Socs3* was deleted in POMC neurons, these effects were due to increased energy expenditure without any change in food intake (Kievit *et al.* 2006). Conversely, when *Socs3* was deleted in SF-1 neurons, a decrease in food intake was observed

Table 3 Effects of suppressor of cytokine signaling 3 (*Socs3*) knockdown in the mediobasal hypothalamus (MBH) on average 24 h food intake (grams), meal size (grams), and frequency

	sh-c (n=7)	sh-1 (n=3)
Day 18–20 (third week chow)		
Chow		
Total intake	20.0 ± 0.50	25.9 ± 0.038 [†]
Average size	1.43 ± 0.11	2.00 ± 0.09*
Average frequency	14.3 ± 0.92	13.0 ± 0.76
Day 27–29 (first week HFHS)		
Chow		
Total intake	14.1 ± 0.80	13.2 ± 2.42
Average size	1.29 ± 0.08	1.67 ± 0.05*
Average frequency	11.2 ± 0.88	8.0 ± 1.76
Lard		
Total intake	3.5 ± 0.78	4.9 ± 1.18
Average size	0.56 ± 0.10	1.37 ± 0.49*
Average frequency	5.9 ± 0.96	4.2 ± 0.88
Day 48–50 (fourth week HFHS)		
Chow		
Total intake	13.4 ± 0.79	10.1 ± 2.01
Average size	1.31 ± 0.06	1.48 ± 0.11
Average frequency	10.4 ± 0.84	7.0 ± 1.53
Lard		
Total intake	3.0 ± 0.46	3.5 ± 0.54
Average size	0.65 ± 0.10	1.24 ± 0.51
Average frequency	4.7 ± 0.17	3.5 ± 0.87

**P* < 0.05, [†]*P* < 0.001 compared with sh-c.

Figure 5 Effect of *Socs3* knockdown on meal patterns. Average size of chow meals in light and dark periods on days 18–20 (A), 27–29 (B), and 48–50 (C). Average size of fat meals in light and dark periods on day 27–29 (D) or 48–50 (E) and average meal frequency for these days (F–J). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with sh-c. $n = 3$ for sh-1 rats and $n = 7$ for sh-c rats.

combined with decreased energy expenditure (Zhang *et al.* 2008). In the present study, knockdown of *Socs3* mRNA in the MBH resulted in an unexpected increase in body weight and fat mass, accompanied by a decrease in locomotor activity. There are several possible explanations for the differences with the previous studies. Firstly, we knocked down *Socs3* in the MBH of adult rats that had normal *Socs3* expression during development. In contrast, neuronal *Socs3* knockout mice lack *Socs3* mRNA in (specific) neurons throughout development, and we cannot, therefore, exclude that developmental compensation may have occurred in these mice. Secondly, our viral vector did not transduce all neurons in the MBH allowing some neurons to continue expressing normal levels of *Socs3* mRNA.

Nevertheless, at least some of the cells transduced in our experiment were likely to be AGRP/NPY neurons that increased their *Npy* expression due to the decrease in *Socs3* mRNA. We would not expect a different transduction pattern by AAV1 vectors of NPY and POMC neurons, since AAV1 vectors predominantly transduce neurons in the hypothalamus and the promoters used to drive the shRNA and *Gfp* are constitutive (de Backer *et al.* 2010). However, we often did not observe *Gfp*-positive cells in the most rostral part of the Arc. Thus, it could well be that due to the localization of the injections, the POMC neurons had a lower change of being transduced. Finally, *Socs3* mRNA levels were only reduced partially, whereas *Socs3* is completely absent in knockout mice.

Table 4 Effects of suppressor of cytokine signaling 3 (*Socs3*) knockdown in the mediobasal hypothalamus (MBH) on locomotor activity and body core temperature

	sh-c (n=15)	sh-1 (n=4)	sh-1 unilateral (n=5)	sh-2 unilateral (n=7)
Activity (a.u.)				
Day 18–20				
Light	89.6±9.2	93.2±8.7	78.8±12.9	80.1±10.7
Dark	325.1±39.2	218.4±57.6	270.7±59.8	218.1±61.8*
Day 27–29				
Light	174.8±34.7	132.9±17.5	142.0±25.3	130.9±17.1
Dark	523.3±40.9	290.1±60.4*	360.4±35.5†	362.9±63.8†
Day 48–50				
Light	150.3±15.5	150.5±21.5	96.2±17.8	131.3±19.3
Dark	463.7±38.2	289.0±66.8*	304.2±63.4†	304.0±46.5†
Body temperature (°C)				
Day 18–20				
Light	37.07±0.05	37.19±0.04		
Dark	37.72±0.06	37.53±0.06		
Day 27–29				
Light	37.17±0.03	37.28±0.03		
Dark	37.73±0.06	37.50±0.02		
Day 48–50				
Light	37.15±0.04	37.12±0.03		
Dark	37.60±0.05	37.34±0.03*		

**P*<0.05 and †*P*<0.01 compared with sh-c.

The marked increase in *Npy* mRNA levels (+93.4%) that we observed in the Arc of MBH *Socs3* mRNA knockdown rats is in line with several *in vitro* studies (Muraoka *et al.* 2003, Higuchi *et al.* 2005). These have shown that, in the absence of SOCS3, leptin activates the *Npy* promoter via STAT3. However, if SOCS3 is present in cells, leptin suppresses *Npy* transcription (Higuchi *et al.* 2005). In addition, SOCS3 also decreases basal *Npy* transcription. Taken together, this suggests that SOCS3 may act as a negative regulator of *Npy* transcription (Higuchi *et al.* 2005). In our experiment, *Socs3* levels in the MBH were decreased by viral vector-mediated knockdown, similar to the *in vitro* experiment by Higuchi *et al.*, allowing leptin to stimulate *Npy* transcription by STAT3 binding to its promoter and simultaneously reducing SOCS3-mediated suppression of basal *Npy* transcription. We, therefore, hypothesize that in cells with reduced levels of *Socs3* mRNA, leptin could no longer inhibit *Npy* expression and leptin even

increased *Npy* expression, which contributed to the development of obesity.

The increased levels of *Npy* mRNA that we observed may explain the unexpected effects on body weight, food intake, fat mass, and locomotor activity after *Socs3* knockdown in the MBH. Viral vector-mediated *Npy* overexpression in several hypothalamic regions results in increased body weight, decreased locomotor activity in the dark period, and increased food intake, in particular, in the light period (Tiesjema *et al.* 2007, Yang *et al.* 2009). In addition, acute i.c.v. injections with NPY increase food intake, and when NPY is infused for longer periods, it also reduces energy expenditure (Clark *et al.* 1984, Morley *et al.* 1987, Zarjevski *et al.* 1993, Baran *et al.* 2002). In rats with MBH *Socs3* knockdown, we observed an increase in chow intake in the light period of day 18–20. This increase in food intake may be due to the increased *Npy* levels in the Arc at this time. Once the rats were switched to the HFHS diet,

Table 5 Effects of suppressor of cytokine signaling 3 (*Socs3*) knockdown on endocrine parameters and body composition

	sh-c (n=13–15)	sh-1 (n=3–4)	sh-1 unilateral (n=4)	sh-2 unilateral (n=5)
Leptin (ng/ml)	15.75±1.69	32.79±5.64*	22.19±3.02†	26.51±4.75*
Insulin (ng/ml)	3.63±0.38	4.73±1.10		
Glucose (mmol/l)	7.71±0.30	8.57±0.09		
Adrenals (% BW)	0.11±0.007	0.09±0.005		
Thymus (% BW)	1.14±0.06	0.97±0.18		

**P*<0.001 and †*P*<0.01 compared with sh-c.

Figure 6 Effect of *Socs3* knockdown on mRNA expression levels in the arcuate nucleus. mRNA expression levels of *Agrp*, *Npy*, and *Pomc* in the ARC of sh-c and sh-1 rats (A). For each mRNA, the sh-c values were set to 100%, and the sh-1 values were correlated to this. *** $P < 0.001$ compared with sh-c. A MCID picture of a section probe of a unilaterally transduced rat which was hybridized with a GFP DIG probe (B). On an adjacent section, *Socs3* RA-ISH was performed. (C) The scan of *Socs3* mRNA film is shown. On the right side of the brain, where *Gfp* and thus the sh against *Socs3* are expressed, *Socs3* mRNA is reduced (C). *Npy* mRNA on another adjacent section of the same rat (D) showed that the *Npy* signal is higher on the right side than on the left side. Full colour version of this figure available via <http://dx.doi.org/10.1677/JME-10-0057>.

hyperphagia was no longer observed, which may be due to counter-regulatory mechanisms. Similarly, overexpression of *Npy* in the PVN (a target area for NPY neurons located in the Arc) was reported to only temporarily increase food intake due to compensatory mechanisms (Tiesjema *et al.* 2007, 2009). The reduced locomotor activity that we observed in the dark period of days 27–29 and 48–50 is not likely due to unspecific illness. Rats did not show abnormal behavior, and weights of thymus and adrenal were not altered (which would occur due to chronic stress and infection). The reduction in locomotor activity is in agreement with both *Npy* overexpression studies and i.c.v. administration studies that show a long-term decrease in locomotor activity (Heilig & Murison 1987, Heilig *et al.* 1989, Tiesjema *et al.* 2007). Since we expected that rats with unilateral knockdown of *Socs3* in the MBH would show similar features as those with bilateral knockdown, we also examined the effects on locomotor activity, fat mass, and leptin concentration in these rats. Despite the fact that rats with unilateral knockdown of *Socs3* in the MBH did not show increased total body weight gain, locomotor activity was reduced, and fat mass and leptin concentrations were increased compared with controls. This underscores the role of MBH SOCS3 in regulating locomotor activity, body composition, and endocrine parameters. However, due to the low number of animals that were transduced bilaterally or unilaterally, the data have to be interpreted with care. Although clear statistically significant effects were found, it could be that non-significant differences may be due to insufficient statistical power.

Although knockdown of *Socs3* mRNA in the MBH did not clearly affect total food intake, it persistently altered meal patterns. Meal size in the light period was increased in rats with MBH *Socs3* knockdown, while there were no effects on the number of meals. On the HFHS diet, rats with MBH *Socs3* knockdown appeared to compensate for the increase in meal size during the light phase by decreasing the number of meals in the

dark period. An increase in meal size and a decrease in frequency were also observed in diet-induced obese rats when they are placed on a HF diet (Farley *et al.* 2003). Similarly, meal size has been reported to increase when rats are placed on a cafeteria-style diet. During the first weeks on cafeteria diet, the meal frequency is also increased, but after several weeks, frequency returns to levels even slightly below the frequency of chow-fed rats (Rogers & Blundell 1984). Taken together, this suggests that in rats that fed on a chow diet, knockdown of *Socs3* in the MBH seems to simulate some of the effects of a high-fat diet on meal patterns. Furthermore, when placed on HFHS diet, susceptibility to the alterations that normally accompany high-fat feeding seems to increase. However, the compensatory decrease in meal frequency that normally accompanies increased meal size in DIO rats only occurs in the dark phase, but not in the light phase.

In conclusion, knockdown of *Socs3* mRNA in the MBH increased NPY levels in the Arc, and this most likely explains the effects observed on energy balance, such as the decreased locomotor activity, the small increase in food intake on chow, and the alterations in meal patterns. These results described here underscore what was observed *in vitro* by Higuchi *et al.* (2005), namely that regulation of gene expression by STAT3 and SOCS3 depends on an intricate balance of signal transduction molecules that together modulate transcriptional activity of genes such as *Npy*.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

MWAdB constructed the (AAV vector) plasmids, produced, and purified AAV vectors with the help of KGM. MWAdB performed and analyzed Socs3 renilla luciferase assays and *in situ* hybridizations; assisted with the animal work; discussed the results; and prepared the manuscript. MADB, EmvDZ, and MCML performed the animal work including stereotaxic injections. OvB provided the original p3xflag-renilla and pcDNA4/TO-luc to MWAdB. MdK cloned the Socs3 cDNA into PCR-script assisted by Moniek Veltman. SELF and RAH participated in an experimental design and supervised the experiments, discussed results, corrected the manuscript, and obtained grant support.

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