Neuronal Expression of Glucosylceramide Synthase in Central Nervous System Regulates Body Weight and Energy Homeostasis

Viola Nordström1*, Monja Willershäuser2†, Silke Herzer1, Jan Rozman2,3*, Oliver von Bohlen und Halbach4, Sascha Meldner1, Ulrike Rothermel1, Sylvia Kaden1, Fabian C. Roth5, Clemens Waldeck5, Norbert Gretz6, Martin Habré de Angelis2**, Andreas Draguhn6, Martin Klingenspor7, Hermann-Josef Gröne1*, Richard Jennemann1*

1 Department of Cellular and Molecular Pathology, German Cancer Research Center, Heidelberg, Germany, 2 German Mouse Clinic, Institute of Experimental Genetics, Helmholtz Zentrum München, Neuherberg, Germany, 3 Molecular Nutritional Medicine, Else-Kröner Fresenius Center, Technische Universität München, Freising-Weißenstephan, Germany, 4 Institute for Anatomy and Cell Biology, University of Greifswald, Greifswald, Germany, 5 Institute for Physiology and Pathophysiology, Heidelberg University, Heidelberg, Germany, 6 Medical Research Center, Heidelberg University, Heidelberg, Germany

Abstract

Hypothalamic neurons are main regulators of energy homeostasis. Neuronal function essentially depends on plasma membrane-located gangliosides. The present work demonstrates that hypothalamic integration of metabolic signals requires neuronal expression of glucosylceramide synthase (GCS; UDP-glucose:ceramide glucosyltransferase). As a major mechanism of central nervous system (CNS) metabolic control, we demonstrate that GCS-derived gangliosides interacting with leptin receptors (ObR) in the neuronal membrane modulate leptin-stimulated formation of signaling metabolites in hypothalamic neurons. Furthermore, ganglioside-depleted hypothalamic neurons fail to adapt their activity (c-Fos) in response to alterations in peripheral energy signals. Consequently, mice with inducible forebrain neuron-specific deletion of the UDP-glucosylceramide glucosyltransferase gene (Ugcg) display obesity, hypothymia, and lower sympathetic activity. Recombinant adeno-associated virus (rAAV)-mediated Ugcg delivery to the arcuate nucleus (Arc) significantly ameliorated obesity, specifying gangliosides as seminal components for hypothalamic regulation of body energy homeostasis.

Citation: Nordström V, Willershäuser M, Herzer S, Rozman J, von Bohlen und Halbach O, et al. (2013) Neuronal Expression of Glucosylceramide Synthase in Central Nervous System Regulates Body Weight and Energy Homeostasis. PLoS Biol 11(3): e1001506. doi:10.1371/journal.pbio.1001506

Academic Editor: Stephen O'Rahilly, University of Cambridge, United Kingdom

Received November 30, 2012; Accepted January 31, 2013; Published March 12, 2013

Copyright: © 2013 Nordström et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported by the European Foundation for the Study of Diabetes (EFSD/Amylin grant to V.N.; http://www.europediabetesfoundation.org) and grants from the Deutsche Forschungsgemeinschaft (DFG; http://www.dfg.de) SFB 938, and GK 888 to H-J.G. Work at the German Mouse Clinic was supported by grants from the European Community (EUMODIC LSHG-2006-037188, Infrafrontier contract No. 211404 to the GMC; http://www.eumodic.org) and grants from the Deutsche Forschungsgemeinschaft (DFG; http://www.dfg.de) SFB 938, and GK 888 to H-J.G. Work at the German Mouse Clinic was supported by grants from the European Community (EUMODIC LSHG-2006-037188, Infrafrontier contract No. 211404 to the GMC; http://www.eumodic.org) to the GMC and from the Bundesministerium für Bildung und Forschung (NGFN-Plus: to M.K. [01GS0822, 01GS0869] and to M.H.A. [01GS0850]; http://www.ngfn.de), Infrafrontier (01KX1012 to the GMC and to the German Center for Diabetes Research [DZD e.V.; http://www.infrafrontier.eu]). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

Abbreviations: 3v, third ventricle; AgRP, agouti-related peptide; alpha-MSH, alpha-melanocyte stimulating hormone; AP, action potential; Arc, arcuate nucleus; ArcH, arcuate hypothalamus; AQP, aquaporin; ATP, adenosine triphosphate; PLA, proximity ligation assay; POMC, proopiomelanocortin; PVN, paraventricular nucleus; rAAV, recombinant adeno-associated virus; RER, rough endoplasmic reticulum; RP, resting membrane potential; RSE, receptor-stimulated endocytosis; SOCS-3, suppressor of cytokine signaling 3; Stat, signal transducer and activator of transcription; TLC, thin layer chromatography; Ugcg, UDP-glucose:ceramide glucosyltransferase; VMH, ventromedial hypothalamus; WAT, white adipose tissue; wk, weeks.

* E-mail: v.nordstroem@dkfz-heidelberg.de
† Member of German Center for Diabetes Research (DZD), Neuherberg, Germany.
‡ Chair of Experimental Genetics, Technische Universität München, Freising-Weißenstephan, Germany.

These authors contributed equally to this work.

Introduction

The investigation of pathogenetic mechanisms underlying obesity has attained significant interest, as obesity has become an endemic metabolic disturbance worldwide. Elevated peripheral energy storage can develop as a consequence of alterations in the neuronal feedback circuits regulating energy homeostasis. The hypothalamus is the main CNS integrator of peripheral energy signals, matching energy intake to energy expenditure for body weight maintenance [1].

Among the most extensively studied peripheral molecules involved in regulating energy homeostasis and feeding behavior...
Obesity is a growing health threat that affects nearly half a billion people worldwide, and its incidence rates in lower income countries are rising dramatically. As obesity is a major risk factor for type II diabetes and cardiovascular disease, significant effort has been put into the exploration of causes, prevention, and potential treatment. Recent research has demonstrated that a region of the brain called the hypothalamus is a major integrator of metabolic and nutrient signals, adapting food intake and energy expenditure to current metabolic needs. Leptin or insulin receptors located in the plasma cell membrane of neurons sense energy signals from the body. They transmit this information inside the cell, which then regulates neuronal function. In this study, we show that leptin receptors interact with gangliosides, a class of plasma membrane lipids. This interaction is a prerequisite for proper receptor activation. Consequently, ganglioside loss in hypothalamic neurons inhibits leptin receptor signal transduction in response to energy metabolites. Furthermore, mice lacking gangliosides in distinct forebrain areas, amongst them the hypothalamus, develop progressive obesity and hypothermia. Our results suggest a previously unknown regulatory mechanism of plasma membrane lipids for hypothalamic control of body weight.

in the CNS are the adipocyte-derived hormone leptin as well as insulin [2,3]. Among numerous leptin- and insulin-sensitive brain areas, the hypothalamic Arc is one of the main regions integrating peripheral energy signals and initiating adaptive metabolic and behavioral responses [4].

Recently, several CNS regions targeted by leptin have emerged that are involved in the regulation of energy metabolism, such as the brain stem nucleus of the solitary tract (NTS) and reward circuits involving the ventral tegmental area [5,6]. Still, leptin is suggested to exert anti-obesity effects by signaling through “long form” leptin receptors (ObR) abundantly present on both orexigenic neuropeptide Y (NPY)/agouti-related peptide (AgRP) neurons and anorexigenic pro-opiomelanocortin (POMC) neurons in the Arc. Excess NPY signaling abates sympathetically mediated thermogenesis, thereby reducing energy expenditure [7]. NPY and AgRP expression is attenuated upon ObR-induced phosphatidylinositol-3-OH-kinase (PI3k) signaling [8]. Conversely, leptin stimulates the expression of the POMC-derived neurotransmitter α-melanocyte-stimulating hormone (α-MSH) through the Janus kinase/signal transducer and activator of transcription (Jak-Stat) pathway [9]. Alpha-MSH, a potent agonist of melanocortin receptors, inhibits food intake and stimulates the expenditure of excess energy in the body, thus preventing obesity development [10].

Insulin exerts its anorexigenic effects in hypothalamic neurons by directly stimulating insulin receptor autophosphorylation and activation of PI3k. Even though both insulin and leptin receptor stimulation leads to activation of PI3k and subsequent formation of phosphatidylinositol (3,4,5)-trisphosphate (PIP3) [11], it has been shown that both hormones exert converging direct actions on POMC neurons, while having opposite effects on AgRP/NPY neurons [12].

GCS is the key enzyme for the biosynthesis of glycosphingolipids (GSLs) and gangliosides, a class of acidic GSLs abundantly expressed by neurons and glial cells [13,14]. Ganglioside-depleted neurons are viable and show apoptosis rates comparable to wild-type neurons [15]. GSLs including gangliosides contribute to the formation of membrane microdomains, which are important mediators of intracellular signal transduction [16]. GCS expression is crucial for initial postnatal brain maturation and Ugg−/−/NesCre mice with constitutive Ugg deletion in brain tissue under the control of the nestin promoter die within 3 wk after birth [15]. In 2003, it was shown that GM3 synthase-deficient mice are more sensitive to insulin, thereby protecting these mice from high-fat-diet-induced insulin resistance [17]. A different ganglioside species, GD1a, has been shown to exert activating effects on tyrosine kinase receptors [18]. To address the functional role of GCS in neuronal regulation of energy homeostasis, we have generated and characterized mice with inducible neuron-specific Ugg deletion in adult mouse CNS (Ugg−/−/CamKCreERT2 mice). Cre activity in this mouse model was restricted to distinct populations of forebrain neurons. Hypothalamic nuclei involved in the regulation of energy homeostasis were targeted by this approach. Explicitly, Cre activity was absent in the brain stem NTS, which also contributes to regulation of energy homeostasis.

The present study highlights GCS-derived gangliosides as mediators for ObR-dependent signal transduction at the hypothalamic neuronal membrane. GCS-depleted mice failed to show ObR activation upon leptin stimulation. Major neuronal gangliosides GM1 and GD1a were recruited to ObR upon ligand stimulation and subsequent signal transduction depended on ganglioside expression in hypothalamic neurons. Ugg−/−/CamKCreERT2 mice deficient in GSLs in hypothalamus developed progressive obesity and decreased sympathetically mediated thermogenesis. rAAV-mediated Ugg delivery to the hypothalamic Arc with ensuing nucleus-specific GSL synthesis significantly ameliorated obesity.

Results

Ganglioside Depletion in Cre-Targeted Neurons in Vivo and in Vitro

Uggfloxflox (Uggf/+) mice were bred with mice expressing the inducible CreERT2 recombinase under the control of the Calcium/Calmodulin-dependent Kinase II-alpha (CamK) promoter, resulting in forebrain neuron-specific Ugg deletion (Uggf/+CamKCreERT2) followed by ganglioside depletion after tamoxifen injection (Figure 1A). Generation of Uggf/− mice and CamKCreERT2 mice has been described earlier [15,19].

Beta-galactosidase (X-Gal) staining of brains from R26R/Uggf/+CamKCreERT2 reporter mice indicated strong Cre activity in distinct hypothalamic nuclei, namely in the Arc (Figures 1B and S1B), in the paraventricular nucleus, and in the median preoptic area (MnPO) (Figure S1A,B). Additional Cre activity was detected in the lateral hypothalamic area (LHA), in hippocampus, and in the cerebral cortex (Figure S1A,B). Notably, Cre activity was absent in the ventromedial hypothalamus and the NTS in the brain stem (Figure S1A,B). Ganglioside depletion was confirmed in Cre-targeted areas by GD1a immunofluorescence, whereas non-targeted areas retained GD1a expression (Figure 1B and Figure S1A).

Consistent with the expected Cre-activity pattern, in situ hybridization showed Ugg mRNA depletion in hippocampus, cerebral cortex, amygdala, as well as hypothalamic nuclei (Figure S1C). Recombination events were absent in peripheral organs and peripheral nervous tissue (Figure S1D).

Neuron-dense total hippocampi showed significant and stable GCS expression in the presence of diet (Figure S1E). Reduced GCS expression in the dissected tissue were measured to result from glial cells as well as from innervating nerve fibers emerging from nontargeted neurons [14]. Ceramide levels in Cre-targeted neuronal
populations were unchanged (Figure 1C), and a slight increase in sphingomyelin could be detected (Figure S1F).

In order to investigate if ganglioside depletion abated general neuronal function and integrity in Ugcg<sup>ff</sup> CamKCreERT2 mice, both electron microscopy and electrophysiological slice recordings were done at late time points p.i. Electron microscopy from Arc neurons displayed normal ultrastructure of the neuronal nucleus, organelles, and an intact, regular plasma membrane of Ugcg<sup>ff//CamKCreERT2</sup> mice both 6 and 12 wk p.i. (Figure 1D). Basic biophysical parameters [spontaneous firing rate, action potential (AP) width, and AP rate of rise] from slice recordings of Arc neurons 12 wk p.i. were unaltered (Figure S2A). The resting

**Figure 1. Normal ultrastructure in ganglioside-depleted neurons.** (A) Major pathway for biosynthesis of GSL including gangliosides in the brain. (B) X-Gal staining in brains of R26R/Ugcg<sup>f/+</sup> CamKCreERT2 reporter mice revealed strong Cre activity in the hypothalamic Arc. GD1a immunofluorescence visualized ganglioside depletion in the Arc of Ugcg<sup>ff//CamKCreERT2</sup> mice 6 wk p.i. Scale bar: 75 μm. (C) Ceramide levels were not significantly altered in hippocampus of Ugcg<sup>ff</sup> CamKCreERT2 mice. Quantification from densitometry analysis of TLC results is depicted (n = 3). (D) Neurons in the Arc of Ugcg<sup>ff//CamKCreERT2</sup> mice showed normal ultrastructural morphology of plasma membrane (pm), nucleus (N), mitochondria (M), endoplasmic reticulum (ER), golgi (G), projections (P), and myelin sheaths (my) 6 and 12 wk p.i. Scale bar: 2 μm. 3<sup>rd</sup>v, third ventricle.

doi:10.1371/journal.pbio.1001506.g001
membrane potential and the AP threshold were marginally increased in Ugcg\textsuperscript{f/f}/CamKCreERT2 mice, however not to an extent that impairs neuronal function (Figure S2B).

In order to confirm these findings in vitro, immortalized mouse hypothalamic cells (N-41 cells) expressing GCS-derived gangliosides (Figure S3A,B) were treated with n-butyldeoxynojirimycin (NB-DNJ) specifically inhibiting GCS [20]. NB-DNJ treatment resulted in approximately 80%-90% ganglioside depletion (Figure S3C). Consistent with the findings in Ugcg\textsuperscript{f/f}/CamKCreERT2 mice, membrane integrity and normal cellular ultrastructure of ganglioside-depleted N-41 cells was confirmed by electron microscopy (Figure S3D). Additionally, passive and active membrane properties of cultured primary GCS-deficient hypothalamic Ugcg\textsuperscript{f/f}/NesCre neurons [15] were examined by whole-cell recordings. There were no differences toward control cells in membrane resistance, capacitance, and resting potential (Figure S3E). Spikes evoked by somatic current injection had unaltered threshold, amplitude, and duration (Figure S3F). These results indicate that basic neuronal integrity and general function are not affected by Ugcg deletion and subsequent lack of plasma membrane gangliosides.

Progressive Body Weight Gain, Hypometabolism, and Hypothermia in Ugcg\textsuperscript{f/f}/CamKCreERT2 Mice

Coinciding with neuronal ganglioside depletion 3 wk p.i. female and male Ugcg\textsuperscript{f/f}/CamKCreERT2 mice displayed progressive body weight increase (Figure 2A,B). This phenotype was not detected in heterozygous mice (Figure S4A), as residual GCS activity accounted for maintenance of neuronal ganglioside biosynthesis [15]. Ugcg\textsuperscript{f/f}/CamKCreERT2 mice were larger than control littermates 16 wk p.i. (Figure 2C).

Hematoxylin and eosin (HE) staining revealed enlarged adipocytes in epigonadal white adipose tissue (WAT) (Figure 2D). In line with this, epigonadal WAT pad weight was significantly elevated (Figure 2E). Whole body nuclear magnetic resonance

![Figure 2. Ugcg\textsuperscript{f/f}/CamKCreERT2 mice develop progressive obesity. Both female (A) and male (B) Ugcg\textsuperscript{f/f}/CamKCreERT2 mice showed a progressive increase in body weight after tamoxifen induction (n=6–9). (C) Ugcg\textsuperscript{f/f}/CamKCreERT2 mice were larger than Ugcg\textsuperscript{f/f} littermates (16 wk p.i.), and body fat mass was prominently elevated. (D) Enlarged adipocytes in Ugcg\textsuperscript{f/f}/CamKCreERT2 mice 9 wk p.i. (E) Increased weight of epigonadal WAT 9 wk p.i. in Ugcg\textsuperscript{f/f}/CamKCreERT2 mice (n=4–5). (F) NMR analysis revealed significant and progressive accumulation of body fat mass in Ugcg\textsuperscript{f/f}/CamKCreERT2 mice (n=9–10). *p<0.05; **p<0.01; ***p<0.001. Means ± SEM. doi:10.1371/journal.pbio.1001506.g002]
(NMR) analysis revealed that body weight increase was due to progressive accumulation of body fat (Figure 2F); lean mass was only marginally elevated 4 wk p.i. (Figure S4B). Adjusted for body weight the initial increase of fat and lean mass was proportional, whereas at later stages fat mass overrode lean mass gain (Figure S4C). Liver steatosis and morphological changes in major peripheral organs of obese Uggg\textsuperscript{f/f}/CamKCreERT2 mice were not detected 9 wk p.i. (Figure S4D). Serum enzyme activities indicative for liver function (glutamate dehydrogenase, glutamic oxaloacetic transaminase, and glutamic pyruvic transaminase) were unaltered (Figure S4E). Likewise, serum cholesterol, urea, glucose, and creatinine did not show any biologically relevant abnormalities (Figure S4F). Coincident with obesity, Uggg\textsuperscript{f/f}/CamKCreERT2 mice were less glucose tolerant than Uggg\textsuperscript{f/f} mice 12 wk p.i. (Figure S4G) and insulin sensitivity was marginally impaired 10 wk p.i. (Figure S4H). These results demonstrate that Uggg\textsuperscript{f/f}/CamKCreERT2 mice develop progressive obesity that is evident in all adipose compartments with constant lean mass and a shift in body composition toward fat accumulation.

As tight regulation of energy homeostasis is crucial for body weight maintenance [1], a metabolic characterization was carried out in order to study the relation of energy intake to energy expenditure. Food intake and metabolizable energy (FAME) adjusted to body weight were slightly elevated in Uggg\textsuperscript{f/f}/CamKCreERT2 mice before the onset of obesity 3 wk p.i. (Figure 3A,B) when gangliosides were already depleted in Cre-targeted brain regions. Hyperphagia was no longer evident 6 and 11 wk p.i., as food intake and FAME were simply elevated due to higher body weight (Figure 3A,B). Fecal excretion of free fatty acids (FFAs) as well as energy content of feces and extraction efficiency from the food (Figure S3A) were unaltered. Thus, abnormalities in food intake do initially contribute to obesity development, but not for obesity maintenance.

Energy expenditure was monitored by indirect calorimetry for 21 h. Before onset of body weight gain, the metabolic rate was indistinguishable from Uggg\textsuperscript{f/f} mice 2 wk p.i. (Figure S3B). When adjusted for body weight, the average metabolic rate tended to be lower in Uggg\textsuperscript{f/f}/CamKCreERT2 mice at 5 and 9 wk p.i. (Figure S3C). Spontaneous locomotor activity is one contributor to daily energy expenditure and has been reported to be decreased in obese rodents [21]. However, both before the onset of weight gain and during progressive adiposity, spontaneous open field activity of Uggg\textsuperscript{f/f}/CamKCreERT2 mice was indistinguishable from control littermates (Figure S3C).

The respiratory exchange ratio (RER) provides information on metabolic fuel preferences [22]. Uggg\textsuperscript{f/f}/CamKCreERT2 mice displayed significantly elevated average daily RER values (Figure 3D). This finding suggests a shift from lipid oxidation toward lipid storage [22]. In line with this, fat mobilization in response to fasting as assessed by measuring plasma nonesterified free fatty acids (NEFAs) was impaired. Significantly decreased plasma NEFAs were detected in Uggg\textsuperscript{f/f}/CamKCreERT2 mice 11 wk p.i. (Figure S3D), suggesting a reduced capability to mobilize lipid stores when challenged by food withdrawal.

After the onset of weight gain, Uggg\textsuperscript{f/f}/CamKCreERT2 mice displayed a prominent drop in core body temperature, as exemplarily depicted 10 wk p.i. (Figure 3E). Adipocytes in intrascapular brown adipose tissue (iBAT) were enlarged (Figure S6A), suggesting reduced triglyceride turnover. Ultrastructural analysis of iBAT furthermore revealed mitochondrial disorganization as well as a lower average mitochondrial size (Figure S6B,C). Thermogenesis in iBAT is regulated by synergistic actions of thyroid hormones and sympathoadrenergic signaling [23]. Free triiodothyronine (fT3) and free thyroxine (fT4) levels were normal in Uggg\textsuperscript{f/f}/CamKCreERT2 mice (Figure S6D,E). Thus, thyroid dysfunction was unlikely to account for inappropriate thermoregulation. Decreased sympathetic outflow to adipose tissue is assumed to be associated with impaired lipid mobilization [24]. In fact, both iBAT sympathetic activity, as assessed by norepinephrine (NE) turnover rate (Figure 3F, Figure S6F), and NE content (Figure S6G) were decreased in Uggg\textsuperscript{f/f}/CamKCreERT2 mice.

These results demonstrate that Uggg\textsuperscript{f/f}/CamKCreERT2 mice develop progressive obesity and a shift in body composition toward fat accumulation initially supported by hyperphagia, but maintained due to hypometabolism and hypothermia.

**Reconstitution of Uggg Gene Expression in the Hypothalamic Arc Ameliorates Obesity**

Several distinct hypothalamic and nonhypothalamic brain regions were targeted by Cre activity in Uggg\textsuperscript{f/f}/CamKCreERT2 mice. Arc neurons in Uggg\textsuperscript{f/f}/CamKCreERT2 mice expressing the long form of the ObR were targeted by Cre activity, as demonstrated by co-immunofluorescence of PStat3 and beta-galactosidase (β-Gal) in R26R/Uggg\textsuperscript{f/f}/CamKCreERT2 reporter mice (Figure 4A). Other leptin-responsive neurons outside the Arc also targeted by Cre activity, such as the MnPO are likely in part contributing to the observed phenotype. However, ObR-expressing neurons in the LHA seem to be recessed by Cre activity (Figure S7A,B).

In order to furtherly clarify the role of the Arc in obesity development, we injected recombinant adeno-associated viruses encoding either Uggg and lacZ (rAAV-Uggg/LacZ) or only lacZ (rAAV-Empty/LacZ) bilaterally into the Arc of Uggg\textsuperscript{f/f}/CamKCreERT2 mice after ganglioside depletion before 4 wk p.i. Injection of rAAV-Uggg/LacZ significantly ameliorated obesity, underlining the importance of Uggg expression in the Arc for body weight maintenance (Figure 4B). Consistently, serum leptin levels tended to be lower in rAAV-Uggg/LacZ-treated mice (Figure 4C). We verified correct targeting of the Arc by X-Gal staining of the brains injected with rAAV-Uggg/LacZ and displayed targeted regions in a schematic drawing as well as a typical staining (Figures 4D-F and S7C). Animals that were not targeted by rAAV-Uggg/LacZ in the Arc (rAAV-Empty/LacZ missed) did not improve their weight gain (Figure S7D). Restored ganglioside biosynthesis in the Arc of rAAV-Uggg-treated animals compared to mice injected with viruses encoding empty plasmid was demonstrated by GD1a immunofluorescence (Figure 4G and Figure S7E).

Taken together, these results indicate that loss of GCS expression in the Arc is significantly involved in part of the metabolic deregulation seen in Uggg\textsuperscript{f/f}/CamKCreERT2 mice.

**GCS-Derived Gangliosides Regulate Leptin Receptor Signaling in Hypothalamic Neurons at the Plasma Membrane**

Since the number of neurons in the Arc did not differ between Uggg\textsuperscript{f/f}/CamKCreERT2 mice and controls (Figure S8A), a functional analysis of the Arc was performed. Leptin signaling in the hypothalamus is crucial for the maintenance of body weight and energy homeostasis. As adipocyte-secreted leptin is a major regulator of body weight in the CNS, we hypothesized that leptin signaling might be disturbed in GCS-deficient neurons of Uggg\textsuperscript{f/f}/CamKCreERT2 mice. In order to test this hypothesis, we investigated hypothalamic Stat3 phosphorylation (PStat3) in the Arc after peripheral leptin stimulation. Decreased PStat3 was detected by immunofluorescence in the Arc (Figure 5A) and by
Figure 3. Obese Ugcg^{f/f}/CamKCreERT2 mice are initially slightly hyperphagic and show hypometabolism and hypothermia. (A) Food intake per day plotted against body weight of Ugcg^{f/f}/CamKCreERT2 mice was slightly but significantly increased 3 wk p.i. and returned to levels not significantly different from control littermates 6 and 11 wk p.i. (n = 9) (LM). Individual means. (B) Metabolizable energy per day plotted against body weight in Ugcg^{f/f}/CamKCreERT2 mice was slightly increased 3 wk p.i. and returned to normal levels 6 and 11 wk p.i. (n = 9) (LM). Individual means. (C) Metabolic rate of Ugcg^{f/f}/CamKCreERT2 mice was decreased 5 and 9 wk p.i., indicating lower energy expenditure (n = 7–10); p = 0.073 week 5; p = 0.77 week 11 (LM). Individual means. (D) Mean respiratory exchange rate (RER), as determined during a 21-h indirect calorimetry measurement, was significantly elevated in Ugcg^{f/f}/CamKCreERT2 mice 5 and 9 wk p.i., indicating reduced lipid oxidation (n = 7–10). (E) Rectal temperature measurements showed a drop in body temperature in Ugcg^{f/f}/CamKCreERT2 mice (10 wk p.i.; n = 9–10). (F) Ugcg^{f/f}/CamKCreERT2 mice showed lower sympathetic activity, indicated by lower norepinephrine turnover (NETO) rate, in total iBAT pad 9 wk p.i. (n = 4). *p≤0.05; **p≤0.01; ***p≤0.001. Means ± SEM unless stated otherwise.

doi:10.1371/journal.pbio.1001506.g003
Western blot in mediobasal hypothalamus (Figure S8B). Interestingly, baseline Stat3 levels were elevated in Ugcgf/f/CamKCreERT2 mice (Figure S8C). The PStat3/Stat3 ratio was decreased both at baseline and upon leptin challenge (Figure S8D).

It has been shown that deficient ObR signaling due to leptin resistance of the Arc in mice with diet-induced obesity (DIO) is a consequence of long-term elevated leptin levels [25–27]. The suppressor of cytokine signaling 3 (SOCS-3) is a major negative regulator of the ObR that is elevated in rodent models of leptin resistance [25,28]. In line with progressive obesity, Ugcgf/f/CamKCreERT2 mice show indeed elevated leptin levels 7 wk p.i. (Figure 5B). However, expression of hypothalamic Stat3-3 did not rise with increasing obesity and leptin levels, as measured 2, 6, and 9 wk p.i. (Figure 5C). Moreover, hypothalamic ObR expression, usually elevated in leptin-resistant rodents [29,30], was normal in Ugcgf/f/CamKCreERT2 mice 6 wk p.i. (Figure 5D).

To further investigate if GCS-derived gangliosides regulate proper leptin receptor signaling at the level of the plasma membrane in hypothalamic neurons, we first assured that loss of gangliosides would not interfere with ObR transport to the membrane, which would have impaired ObR signaling per se. ObR was labeled by an in situ proximity ligation assay (PLA) on non-detergent-perturbed cells by two ObR antibodies. The number of detected surface ObR PLA spots on cells treated with NB-DNJ was similar to control cells (Figure 5E), indicating that ObR at the plasma membrane of ganglioside-depleted hypothalamic cells is not significantly changed compared to control cells.

As GCS-derived gangliosides have previously been shown to modulate the activity of plasma-membrane-located receptors through close interactions in both adipocytes [17] and neurons [31], we investigated ObR interactions with major neuronal gangliosides. The PLA indicating close proximity events [32] indeed revealed proximity between ObR and gangliosides GM1 and GD1a. In demonstration of activity-dependent interaction between GSL and ObR, the number of GD1a/ObR and GM1/ObR PLA spots increased upon stimulation with leptin (Figures 5F,G and S8E). Complex formation between GD1a/GM1 with ObR was further corroborated by co-immunoprecipitation (Co-IP) of ObR and GD1a/GM1 in saline- and leptin-stimulated N-41 cells (Figures 5H and S8F). As N-41 cells do not express the complex neuronal gangliosides GD1b and GT1b, potential interactions with ObR had to be analyzed in hypothalamic tissue of Ugcgf/f mice. GD1b and GT1b could not be co-precipitated with ObR (Figure 5H).

Ganglioside-depleted cells were then assessed for leptin-dependant signal transduction. Ganglioside-depleted cells did not show the leptin-stimulated increased complex formation between ObR and Jak (Figures 5I and S8G). Time- and dose-dependent Jak phosphorylation could be induced by leptin treatment in N-41 cells and was decreased in NB-DNJ-treated GSL-depleted cells (Figures 5J and S8H). It has to be noted that NB-DNJ evokes ganglioside depletion by only approximately 80–90% (Figure S3C). Thus, residual gangliosides in the plasma cell membrane may explain the appearance of a P-Jak signal at a late time point after stimulation of NB-DNJ-treated cells. Ganglioside-depleted N-41 cells showed decreased Jak phosphorylation 30 min after stimulation with 0.5 μg/ml leptin (Figure 5J,K).

These results have now shown that two major neuronal GCS-derived gangliosides, GD1a and GM1, form dynamically leptin-stimulated complexes with ObR on the plasma membrane and that loss of gangliosides decreases signal transduction in hypothalamic neurons.

**Distinct Hypothalamic Neurons of Ugcgf/f/CamKCreERT2 Mice Are Less Responsive to Peripheral Leptin**

It is known that mice with deficient leptin receptor (db/db mice) function develop obesity and lack hypothalamic responsiveness to leptin stimulation [33]. Regarding the finding that neuronal gangliosides enhance ObR signaling, we hypothesized that hypothalamic neuronal function may be altered in Ugcgf/f/CamKCreERT2 mice. In order to investigate this question, neuronal activity after intraperitoneal (i.p.) leptin injection was evaluated by c-Fos staining [34]. Leptin-induced c-Fos formation was normal in non-obese Ugcgf/f/CamKCreERT2 mice 1–2 wk p.i. (Figure 6A). Since ganglioside depletion coincides with the start of the obesity development, Ugcgf/f/CamKCreERT2 mice that were weight-matched to control littermates were analyzed 3–4 wk p.i. Decreased leptin responsiveness could already be observed in the Arc of these mice (Figure 6B) as well as in the Arc of obese mice 6 wk p.i. (Figure 6C).

Neurons in the nontargeted and non-ganglioside-depleted VMH retained responsiveness to leptin at all time points (Figure 6D–F). As expected, the nontargeted brain stem NTS of Ugcgf/f/CamKCreERT2 mice showed regular leptin-induced c-Fos staining 6 wk p.i. (Figure S9).

All together, these results indicate a primary deficiency of ganglioside-depleted hypothalamic neurons to respond adequately to peripheral leptin signals.

**Ganglioside-Depleted NPY/AgRP and POMC Neurons in the Arc Are Less Responsive to Leptin**

Antagonistic orexigenic NPY and anorexigenic POMC neurons in the hypothalamic Arc are first-order responsive neurons initiating metabolic adaptations to altered peripheral leptin levels [4]. In order to determine leptin-dependent NPY and POMC neuronal function, neuronal activity and ObR activation were assessed by semiquantitative analysis of c-Fos, PStat3, and PIP3 formation in response to peripheral leptin injections. Leptin engaged POMC neurons (α-MSH positive) in control mice, as indicated by increased c-Fos (Figure 7A). Significantly elevated PStat3 (Figure 7B) and PIP3 formation (Figure S10A) confirmed activation of their ObR. Before ganglioside depletion (1–2 wk p.i.), POMC neurons of Ugcgf/f/CamKCreERT2 mice responded normally to leptin. However, c-Fos, PStat3, and PIP3 formation were not elevated in response to leptin in obese GSL-deficient mice 6 wk
GCS Regulates ObR in Hypothalamic Neurons

**Figure A**

- Upper panel: Immunohistochemistry images of hypothalamic area showing the percentage of cells positive for PhStat3 in Ugcg^fl/fl^ and Ugcg^fl/fl^/CamKCreERT2 mice treated with saline or leptin.
- Lower panel: Bar graph showing the percentage of PhStat3-positive cells in Ugcg^fl/fl^ and Ugcg^fl/fl^/CamKCreERT2 mice treated with saline or leptin.

**Figure B**

- Serum leptin levels in Ugcg^fl/fl^ and Ugcg^fl/fl^/CamKCreERT2 mice at weeks 3 and 7 after leptin treatment.

**Figure C**

- mRNA expression levels of ObR in arc-enriched hypothalamic tissue from Ugcg^fl/fl^ and Ugcg^fl/fl^/CamKCreERT2 mice at 2, 6, and 9 weeks after leptin treatment.

**Figure D**

- mRNA expression levels of leptin in MBH from Ugcg^fl/fl^/CamKCreERT2 mice at 6 weeks.

**Figure E**

- Surface ObR expression levels in saline and NB-DNJ (100 mM, 7 days) treated mice.

**Figure F**

- Images showing ObR and ObR in combination with CD11b and GM1 in saline and leptin-treated mice.

**Figure G**

- Images showing ObR and ObR in combination with CD11b and GM1 in saline and leptin-treated mice.

**Figure H**

- Detection of ObR and ObR in combination with CD11b and GM1 in saline and leptin-treated Ugcg^fl/fl^ mice.

**Figure I**

- Western blot analysis showing phosphorylation of Jak in control and NB-DNJ (100 mM, 7 days) treated mice.

**Figure J**

- Western blot analysis showing phosphorylation of Jak in control and NB-DNJ (100 mM, 7 days) treated mice.

**Figure K**

- Western blot analysis showing phosphorylation of Jak and ObR in control and NB-DNJ (100 mM, 7 days) treated mice.
Ugcg effect in plasma cell membrane, thereby facilitating ObR-dependent signal revealed that GCS-derived gangliosides interact with ObR on the most prominent regulators of CNS metabolic control [35,36].

Analysis of the leptin receptor signaling pathway, being one of the most critical animal groups were analyzed. (B) Serum leptin levels were unchanged 3 wk p.i. in Ugcg−/−/CamKCreERT2 mice, reflecting increased body fat mass (n = 12–14). (C) mRNA expression analysis for suppressor of cytokine signaling 3 (SOCS-3) expression in Arc-enriched hypothalamic tissue was carried out 2, 6, and 9 wk p.i. Sox-3 expression normalized to the housekeeping gene tubulin was unaltered (n = 3–5). (D) mRNA expression analysis for the long form of the leptin receptor, Lepr, in mediobasal hypothalamus was carried out 6 wk p.i. Leprb expression normalized to the housekeeping gene tubulin was unaltered at that time point (n = 4–5). (E) Immortalized mouse hypothalamic cells (N-41 cells) were analyzed for cell surface expression of ObR. Non-detergent-treated cells were fixed and simultaneously stained with two ObR antibodies. A proximity ligation assay (PLA) indicated quantifiable and unchanged ObR expression on the surface of controls and cells treated with the specific GCS inhibitor NB-DNJ (n = 41–47 cells). PLA principle is depicted on the right side. (F–G) N-41 cells were incubated with either saline or 100 ng/ml leptin (10 min). Close interactions between GCS-derived neuronal gangliosides GD1a/ObRb and GM1/ObR (G) were detected by PLA. Leptin treatment dynamically increased the GD1a/ObRb and GM1/ObR PLA spots per cell (n = 48–67 cells). (H) Extracts from saline- and leptin-treated N-41 cells were immunoprecipitated with an ObR antibody, lipids were extracted, and GD1a and GM1 were visualized by immune overlay TLC. GD1a and GM1 co-immunoprecipitated (Co-IP) with ObR, which tended to be stronger in leptin-treated cells. Addition of a blocking peptide almost totally abolished ganglioside signals. Gangliosides GD1b and GT1b, expressed in mouse brain tissue, were not co-precipitated with ObR from hypothalamic tissue of Ugcgf/f//CamKCreERT2 mice 6 wk p.i. (Figure 7D) and did not have any direct effect on PIP3 formation (Figure S10C). Remarkably, basal mRNA expression of Agrp and Npy was markedly elevated in the MBH of Ugcg−/−/CamKCreERT2 mice 6 and 9 wk p.i., with Agrp already increasing 2 wk p.i. (Figure S10D).

In summary, this study has indicated that GCS expression and sufficient gangliosides in neurons of the adult CNS play a seminal role in the regulation of body weight and energy homeostasis. Analysis of the leptin receptor signaling pathway, being one of the most prominent regulators of CNS metabolic control [35,36], revealed that GCS-derived gangliosides interact with ObR on the plasma cell membrane, thereby facilitating ObR-dependent signal transduction (Figure 8A). In Ugcg−/−/CamKCreERT2 mice, leptinresponsiveness and neuronal function are impaired in hypothalamic neurons involved in the regulation of energy metabolism (Figure 8B). Consequently, defective ObR signaling contributes to the observed metabolic imbalance and obesity development of mice with ganglioside deficiency in the CNS.

Discussion

Although the seminal role of CNS feedback responses to peripheral energy signals for the regulation of energy homeostasis has been extensively studied, the role of the lipid microenvironment for energy signal receptor function has not yet been addressed. The present study demonstrates that GCS-derived GSLs are critically involved in a to-date unknown mechanism of hypothalamic control of body weight. In line with the finding that neurons of the constitutive Ugcg−/−/NesCre mouse do not show increased apoptosis [15], ganglioside-deficient hypothalamic neurons are viable and they show normal membrane and organelle appearance both in vivo and in vitro. Electrophysiological recordings from Arc neurons in slices of Ugcg−/−/CamKCreERT2 mice at 12 wk p.i. did not show a major disruption of membrane functions. However, resting membrane potential and action potential threshold were both shifted to slightly more depolarized values. The molecular mechanism underlying the altered membrane potential remains presently elusive. However, it is well feasible that the shift of threshold is secondary to the slight depolarization, which might inactivate a fraction of Na+ channels.

In line with the largely normal properties of neurons from brain slices, biophysical parameters of primary hypothalamic neurons devoid of gangliosides were unaltered. Thus, failure of basic electrophysiological membrane functions is unlikely to cause the observed phenotype of mice with ganglioside deficiency.

Therefore, the present work focuses on interactions of leptin receptors with the ganglioside-containing lipid microenvironment in which receptors are embedded. We show with independent methods that two major neuronal GCS-derived gangliosides, GD1a and GM1, closely interact with leptin receptors on the neuronal membrane. This interaction is dynamically enhanced by stimulation with leptin. Both Ugcg−/−/CamKCreERT2 mice and ganglioside-depleted hypothalamic cells display deficient ObR signal transduction upon leptin stimulation, as assessed by decreased leptin-induced Jak phosphorylation, Stat3 phosphorylation, and PIP3 formation. Corroborated in situ by deficient leptin responsiveness in Arc neurons of Ugcg−/−/CamKCreERT2 mice, these results indicate that GCS-derived GSLs, primarily gangliosides, are seminal regulators for neuronal leptin signal transduction. Consequently, Ugcg−/−/CamKCreERT2 mice with deficient leptin-induced hypothalamic neuronal responsiveness develop progressive obesity.

Numerous hypothalamic feedback systems involved in body weight maintenance are known [1,4]. Admittedly, the robust phenotype of Ugcg−/−/CamKCreERT2 mice may be caused by several peripheral hormones and defective ensuing signaling events occurring in various Cre-targeted CNS regions of this mouse model. The brain stem NTS, though an important mediator of metabolic control [37], is not targeted by activity under the CamK II alpha-dependent Cre recombinase used in this study. Consequently, the NTS shows normal responsiveness to leptin in obese Ugcg−/−/CamKCreERT2 mice and can be excluded to contribute to the observed phenotype. Recent reviews also highlight the LHA as an important regulator of energy balance [38,39]. In fact, compensating neurocircuits involving nontargeted CNS regions may be considered for the return of food intake from initial hyperphagia to normal levels in obese mice despite the striking increase in orexigenic neuropeptides. Even though X-Gal staining could be seen in parts of the LHA, we could not verify Cre targeting of a major part of ObR-expressing LHA neurons in Ugcg−/−/CamKCreERT2 mice (Figure S7B). In strong support to this line of reasoning, we demonstrate that partial Ugcg replenishment in the Arc mediated by stereotactic injection of rAAV significantly
Figure 6. Hypothalamic neurons of \textit{Ugcg}^{f/f}//CamKCreERT2 mice are less responsive to peripheral leptin. (A–C) Brains of leptin-stimulated mice were analyzed for neuronal activity indicated by c-Fos immunofluorescence. Detailed pictures in the upper lane indicate regions of the Arc that are outlined in overview pictures (frames). Arrowheads mark c-Fos-positive neurons located in the VMH. Axis indicators were included indicating the medial (m) and ventral (v) axes. (A) \textit{Ugcg}^{f/f}//CamKCreERT2 mice showed leptin-induced neuronal activation comparable to \textit{Ugcg}^{f/f} mice in the Arc 1–2 wk p.i. (B) Leptin response in the Arc was decreased in nonobese \textit{Ugcg}^{f/f}//CamKCreERT2 mice weight-matched to controls 3–4 weeks p.i. (C) Decreased c-Fos staining in the Arc was also observed in obese leptin-induced \textit{Ugcg}^{f/f}//CamKCreERT2 mice 6 wk p.i. The percentage of c-Fos-positive neurons per Arc section was depicted as values normalized to saline-injected \textit{Ugcg}^{f/f} mice (\(n = 14–22\) sections). Depicted sections are located between bregma levels 2.21.5 to 2.1.8. Quantification contains data from bregma levels 2.1.4 to 2.2.0. Datasets for each time point were acquired individually. Two (1–2 and 3–4 wk) or three (6 wk) independent animal groups were analyzed. Immunofluorescence and image acquisition for each dataset (treated and untreated controls and knockouts) were performed simultaneously.

Scale bar: 75 \(\mu m\); 3rdv, 3rd ventricle; *p < 0.05; **p < 0.01; ***p < 0.001. Means ± SEM.

doi:10.1371/journal.pbio.1001506.g006
ameliorates obesity and hyperleptinemia in Ugcg<sup>f/f</sup>/CamKCreERT<sup>2</sup> mice. Even though limited infection of closely attached tissue by rAAV injection could not be definitely excluded, mainly Arc neurons were targeted by this approach, as assessed by X-Gal stainings of brains co-injected with LacZ-expressing viruses. The present investigation has thus been restricted to GCS effects focused on the MBH harboring Arc neurons.

Deficient leptin signaling as a consequence of leptin resistance occurs predominantly in the Arc of DIO mice with severe long-term hyperleptinemia [25–27,40]. Socs-3 is a major negative feedback pathway of ObR signaling [41]. Thus, elevated Socs-3 expression levels are found in the hypothalamus of leptin-resistant rodent models [25,42]. In line with observations in obese db/db mice with nonfunctioning ObR [25,42], Socs-3 expression in the Arc remains indistinguishable from control littermates in nonobese and obese Ugcg<sup>f/f</sup>/CamKCreERT<sup>2</sup> mice 2, 6, and 12 wk p.i. Elevated hypothalamic ObR expression, as it occurs in DIO mice [29,30], has also been proposed as a potential mechanism playing a role in the development of leptin resistance [28]. However, normal ObR expression in Ugcg<sup>f/f</sup>/CamKCreERT<sup>2</sup> mice supports the hypothesis

---

**Figure 7. POMC and NPY neurons of Ugcg<sup>f/f</sup>/CamKCreERT<sup>2</sup> mice are less responsive to leptin.** (A) Leptin engages POMC neurons in the Arc of control (Ugcg<sup>f/f</sup>) mice and Ugcg<sup>f/f</sup>/CamKCreERT<sup>2</sup> mice 1–2 wk p.i., as indicated by elevated c-Fos. This response was decreased in Ugcg<sup>f/f</sup>/CamKCreERT<sup>2</sup> mice 6 wk p.i. (B) Elevated leptin-induced PStat3 levels in POMC neurons of Ugcg<sup>f/f</sup> mice and Ugcg<sup>f/f</sup>/CamKCreERT<sup>2</sup> mice 1–2 wk p.i. This response was blunted in Ugcg<sup>f/f</sup>/CamKCreERT<sup>2</sup> mice 6 wk p.i. (C) Leptin slightly decreased the activity of NPY neurons in Ugcg<sup>f/f</sup> mice and Ugcg<sup>f/f</sup>/CamKCreERT<sup>2</sup> mice 1–2 wk p.i. This was not detected in Ugcg<sup>f/f</sup>/CamKCreERT<sup>2</sup> mice 6 wk p.i. (D) Unlike 1–2 wk p.i., leptin did not elevate PStat3 in NPY neurons of Ugcg<sup>f/f</sup>/CamKCreERT<sup>2</sup> mice 6 wk p.i. Datasets for each time point were acquired individually, and quantification contains normalized data from two (1–2 wk p.i.; n = 4–11) or three (6 wk p.i.; n = 18–27) independent animal groups. Immunofluorescence and image acquisition for each dataset (treated and untreated controls and knockouts) were performed simultaneously. Scale bar: 20 μm; *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001. Means ± SEM. doi:10.1371/journal.pbio.1001506.g007

---

**Figure 8. Proposed model for GCS-derived ganglioside GD1a and GM1 regulation of hypothalamic leptin signaling and energy homeostasis.** (A) GCS-derived gangliosides form complexes with ObR, thereby facilitating leptin-dependent Jak and Stat3 phosphorylation, and formation of PIP3. These pathways are crucial contributors to regulation of energy homeostasis. (B) In obese Ugcg<sup>f/f</sup>/CamKCreERT<sup>2</sup> mice, ObR signal transduction is abolished in GCS-depleted neurons. doi:10.1371/journal.pbio.1001506.g008
that the ObR signaling in their neurons must be deficient due to ganglioside loss and not merely due to secondary leptin resistance. Furthermore, the non-targeted hypothalamic VMH and brain stem NTS retain leptin responsiveness even in obese mice 6 wk p.i. These results in combination with the decreased ObR signal transduction in ganglioside-depleted and non-leptin-resistant N-41 cells strongly suggest that loss of GCS-derived GSLs including gangliosides GD1a and GM1 is the reason for failing ObR activation and subsequently inhibited intracellular signaling.

GCS-depleted Arc neurons display normal leptin sensitivity 1–2 wk p.i., a time point when gangliosides are still present. Furthermore, onset of body weight gain, deficient neuronal activity in the Arc, and abolished ObR signaling coincide with ganglioside depletion 3 wk p.i. This strongly suggests that the mentioned defects are due to ganglioside depletion in these cells rather than due to lack of the enzyme GCS itself.

Further evidence for the postulate that ganglioside deficiency-dependent inhibition of ObR signaling in hypothalamic neurons leads to impaired neuronal function is based on our in situ results in ganglioside-depleted Arc of both obese and nonobese Ugg\textsuperscript{f/f//CamKCreERT2} mice. Whereas leptin injection increases c-Fos immunoreactivity and thus neuronal activity in the Arc neurons of fasted lean mice, this response did not occur in GCS-deficient neurons. Leptin specifically engages POMC neurons. Even though the effects of PI3k- and Stat3-dependent signaling in POMC neurons do not overlap [12,43] and contribute to maintenance of energy homeostasis [46]. In ganglioside-depleted POMC neurons, neither PStat3 nor PIP3 formation is increased by peripheral leptin injections, strongly suggesting that defects in both pathways may contribute to partial failure of obesity prevention. As peripheral leptin stimulates both pathways through ObR activation [11,47], defective ObR function is very likely to be assumed.

In NPY neurons, it has been demonstrated that Jak-Stat3 signaling plays an important role in maintaining NPY/AgRP-mediated energy homeostasis [48]. Additional ObR-mediated PI3k activation seems to be required for inhibiting Npy and Agrp gene expression [8]. Npy and Agrp expression is markedly increased in the MBH of Ugg\textsuperscript{f/f//CamKCreERT2} mice, which may be a consequence of absent leptin-induced PStat3 formation in NPY neurons. On the other hand, leptin-induced PI3k formation does not differ in neither of the groups, which goes in line with the hypothesis that leptin-dependent PI3k formation in AgRP/NPY neurons is stimulated by an indirect mechanism involving synaptic transmission [12]. Overactive NPY neurons in obese ObR-deficient \textit{Lepr}\textsuperscript{ob/ob} rats were shown to inhibit sympathetic nervous outflow to BAT and cause hypothermia [7] as observed in Ugg\textsuperscript{f/f//CamKCreERT2} mice. With regard to the fact that Npy and Agrp expression differ in Ugg\textsuperscript{f/f//CamKCreERT2} mice, the role of GCS expression in regulating neuropeptide expression and secretion has to be elucidated. Especially the role of hypothalamic insulin receptor signaling, which also regulates the expression of Pomp and Npy/Agrp in part similar to ObR signaling [11] and is antagonized by GM3 in the periphery [17], constitutes a promising target for further clarifying the differential neuropeptide expression. Moreover, a potential contribution of Cre-targeted ObR-expressing neurons in the median preoptic area of Ugg\textsuperscript{f/f//CamKCreERT2} mice to hypothermia may also be considered.

Dynamic membrane microdomains are widely accepted as critical components involved in membrane receptor functions [16,49]. Since GCS-derived gangliosides are important constituents of these microdomains, they potentially interact with and regulate a variety of membrane components including receptors such as Trk receptors [31] and insulin receptors [17]. In contrast to mice with neuron-specific insulin receptor deletion, which only display a gender- and diet-dependent subtle increase in body weight [11,30], the obesity and glucose intolerance observed in db/db mice can be rescued by neuron-specific re-expression of ObR [51]. Furthermore, deficient ObR signaling in POMC neurons of the Arc itself leads to the development of mild obesity [52]. In consideration of these findings—despite the existence of potential alternative pathways that might be impaired in neurons of Ugg\textsuperscript{f/f//CamKCreERT2} mice—we ascribe ObR and its regulation of activity to a major function in our model pointing to a novel mechanism for CNS metabolic regulation.

We demonstrate that GCS-derived gangliosides GD1a and GM1 closely interact with ObR. The leptin-induced increase in GD1a/ObR and GM1/ObR interaction assumes recruitment of these gangliosides to the ObR upon leptin stimulation. These results in combination with the demonstrated deficient ObR signaling in ganglioside-depleted hypothalamic neurons both in vivo and in vitro leads us to surmise that the lipid microenvironment surrounding the ObR can significantly modulate leptin-dependent intracellular signal transduction in hypothalamic neurons. Altogether, these results provide evidence that GM1 and GD1a are actively involved in enhancing the effects of leptin in hypothalamic neurons.

As insulin receptors contain a lysine residue predicted for interaction with GM3 [53], loss of GM3 synthase showed already a prominent effect on peripheral insulin receptor signaling [17]. It is a widely accepted concept that in the state of insulin resistance in peripheral adipocytes, the IR segregates from caveolae into GM3-enriched microdomains [53], an endogenous inhibitory mechanism [17]. Indeed, elevated GM3 synthase expression could be detected in adipose tissue of obese Zucker \textit{fa/fa} rats and \textit{ob/ob} mice [54]. Pharmacologic GCS inhibition in the periphery has been shown to exert beneficial effects on peripheral insulin sensitivity and liver steatosis [55,56]. With regard to the fact that different ganglioside species can exert either stimulatory [18,57] or inhibitory [17] effects on membrane receptors, the mentioned studies including the present work support the concept that any perturbation, either loss or excess, of membrane GSLs can alter receptor function. Contributions of GCS-derived lipid raft components apart from gangliosides, namely neutral GSLs in the CNS, to leptin receptor function have yet to be elucidated and constitute a challenging target for future investigations. Besides gangliosides, lactosylceramide has been shown to contribute to formation of lipid microdomains [58]. We, however, propose in the present study that in line with the findings for the insulin receptor, hypothalamic leptin receptor signaling is to a significant extent regulated through interactions with the dominant gangliosides GD1a and GM1.

Recent studies have highlighted the central role of systemic ceramide biosynthesis and GCS in the regulation of energy homeostasis [59,60]. In accordance with earlier findings [15,61], we show that neuronal ceramide levels in Ugg\textsuperscript{f/f//CamKCreERT2} mice are indistinguishable from control mice, virtually excluding any effects of ceramides.
influence on hormone signaling. Ugcg deletion in adult mouse CNS leads to development of progressive obesity, hyperleptinemia, and glucose intolerance. The obesity can be partially ameliorated by restoration of GCS activity and ganglioside expression in the hypothalamic Arc of Ugcg<sup>fl/fl</sup>/CamKCreERT2<sup>+</sup> mice. Neuronal GCS expression therefore constitutes a novel mechanism for hypothalamic regulation of body weight maintenance.

**Materials and Methods**

**Ugcg<sup>fl/fl</sup>/CamKCreERT2<sup>+</sup> Mice**

Animals were kept in specific-pathogen-free barrier facilities. Ugcg<sup>fl/fl</sup> mice [15] and inducible CamKCreERT2 mice were bred to generate Ugcg<sup>fl/fl</sup>/CamKCreERT2<sup>+</sup> mice and control littermates. Mice were induced with tamoxifen 6 wk after birth for 1 wk as described [19]. We performed experiments in female mice, unless stated otherwise.

**Glucose Tolerance and Insulin Sensitivity**

Mice were fasted overnight (o/n). Blood glucose levels were analyzed prior to i.p. injection of glucose (2 g/kg body weight). Glucose levels were determined from tail vein blood (Glucometer Accu Check, Aviva, Roche). Food was withdrawn 4 h prior to the insulin sensitivity assay. Mice were injected i.p. with 0.75 U/kg human insulin (Eli Lilly), and glucose levels were determined as described above (see also Text S1).

**GD1a Immunofluorescence**

Staining was carried out as described earlier [62]. Cryosections (male mice) were incubated with mouse-α-GD1a (1:100, Millipore) followed by secondary donkey-α-goat-Alexa-Fluor 546, donkey-α-sheep-Peprotech) or saline between 8.00 a.m. and 10.00 a.m. Animals were kept in specific-pathogen-free barrier facilities. Mice were fasted overnight (o/n). Blood glucose levels were analyzed prior to i.p. injection of glucose (2 g/kg body weight). Glucose levels were determined from tail vein blood (Glucometer Accu Check, Aviva, Roche). Food was withdrawn 4 h prior to the insulin sensitivity assay. Mice were injected i.p. with 0.75 U/kg human insulin (Eli Lilly), and glucose levels were determined as described above (see also Text S1).

**Leptin Injections and Double-Immunofluorescence**

Mice were fasted o/n and injected with leptin (5 mg/kg, Peprotech) or saline between 8.00 a.m. and 10.00 a.m. Animals were sacrificed at indicated time points and transcardially perfused with 4% paraformaldehyde (PFA). We prepared 40 μm cryosections covering the Arc. Alternating sections were collected in series for subsequent free-floating section immunostainings. First antibodies used for immunostaining were rabbit-α-PStat3 (1:100, Cell Signaling Technology), rabbit-α-c-Fos (1:100, Santa Cruz), and FITC-conjugated α-PIP3 (1:100, Echelon). Secondary antibody was donkey-α-rabbit-Alexa-Fluor 488 (1:200, Invitrogen). Analysis was performed by confocal microscopy (TCS-SL, Leica).

**Proximity Ligation Assay (PLA)**

Eight thousand N-41 cells were seeded onto coverslips and incubated at 37° o/n. The 3 h serum-starved cells were stimulated with leptin (100 ng/ml, Peprotech) for 10 min, washed with PBS and fixed in 4% PFA for 15 min. Cells were blocked with 5% skim milk/PBS. PLA was performed with primary antibodies against ObR (1:50, Santa Cruz), GD1a (1:100, Millipore), and GM1 (1:10, Matreya). PLA was performed according to the manufacturer's guidelines (Duolink Orange Detection System, Olink Biosciences). Formation of PLA spots was analyzed by fluorescence microscopy (Zeiss Cell Observer).

**Western Blot**

Mice were injected with leptin or saline as described above and sacrificed 30 min later. The MBH was dissected homogenized on ice in lysis buffer (20 mM HEPES, 25 mM KCl, 250 mM sucrose, 2 mM MgCl<sub>2</sub>, 0.5 mM DTT, 1% digitonin) containing protease inhibitor (Roche) and phosphatase inhibitor cocktail (Sigma). Immortalized hypothalamic cells were treated with either saline or 100 μM NB-DNJ for 7 d, serum starved for 4 h, and subsequently treated with either saline or leptin (1,000 ng/ml, Peprotech, 1 h). Cells were lysed on ice in lysis buffer. Protein concentrations were determined by Bradford assay (Sigma). Western blots were performed as described earlier [25]. Primary antibodies: rabbit-α-PI3K, rabbit-α-PStat3, rabbit-α-PI3K, rabbit-α-PJak, rabbit-α-Jak (1:1,000, Cell Signaling Technology), mouse-α-tubulin (1:5,000, Zymed Labs), and rabbit-α-Actin (1:1,000, Santa Cruz). Secondary antibodies: HRP-conjugated rabbit-α-IgG (1:1,000, Dako) and HRP-conjugated mouse-α-IgG (1:5,000, Santa Cruz). Bands were visualized by chemiluminescence (Amersham) and quantified (ImageJ, NIH).

**Co-Immunoprecipitation (Co-IP)**

Four hours serum-starved N-41 cells were treated with leptin (1,000 ng/ml, 25 min). Cells were lysed in IP buffer [50 mM HEPES, pH 7.0, 150 mM NaCl, 10% glycerol, 1% Triton-X, 1.5 mM MgCl<sub>2</sub>, 1 mM EDTA, proteinase inhibitor cocktail (Roche)]. Co-IP for ObR/Jak was performed as described earlier [36]. ObR/GD1a- and ObR/GM1-Co-IP and subsequent lipid extraction and analysis was performed as described earlier [31,53]. Anti-ObR were incubated at 4°C o/n. Immunoprecipitated lipids were desalted on an RP-18 column, spotted on a TLC, and run in solvent (chloroform/methanol/0.2% CaCl<sub>2</sub>; 60:35:8, by vol.). GD1a was visualized with mouse-α-GD1a (1:1,000, 4°C overnight, Millipore) on the TLC by immune overlay staining as described earlier [66].
Measurement of Serum Leptin and Nonesterified Free Fatty Acids (NEFAs)

Serum leptin and NEFAs were determined by commercially available kits according to the manufacturer’s guideline [Leptin-ELISA (Linco); NEFA-HR2 kit (WAKO Chemicals)]. NEFAs were measured in male mice.

Determination of NETO and NE Content in iBAT

NETO rate in iBAT was determined as described earlier [67]. Tissue NE was measured by reversed-phase HPLC with electrochemical detection (Chrome Systems, Germany) (see also Text S1).

Metabolic Characterization, Core Body Temperature, and Locomotor Activity

Body weight was measured once a week. Metabolic measurements were carried out in an open circuit respiratory system (SM-MARS, Sable Systems, USA). VO₂ and VCO₂ per mouse were analyzed for 21 h to determine the RER = VCO₂/VO₂ and HP (mW). Whole body composition was determined by noninvasive NMR analysis (Mini-Spec, Bruker Optics). Core body temperature was measured with a rectal probe (ALMEN 2390-1, Alibhorn) (see also Text S1).

Quantitative mRNA Analysis

Total RNA of the MBH was extracted from nonfasted mice as described earlier [68]. RNA was reversely transcribed by Superscript II Reverse Transcriptase (Invitrogen) and cDNA was quantified using the LC FastStart DNA Master SYBR Green I kit (Roche) according to the manufacturer’s guidelines and the Light Cycler (Roche) (see also Text S1).

X-Gal Stainings

R26R/UGGf/f/CamKCreERT2 mice and R26R/UGGf/+ mice were induced with tamoxifen i.p. 6 wk after birth as described. At 3 d p.i., animals were sacrificed, and brains were removed and frozen on dry ice. X-Gal staining was performed as described previously [69]. Similarly, β-galactosidase activity in brains of rAAV-UGGf/LacZ-, rAAV-Empty/LacZ-, and rAAV-LacZ-injected mice was visualized 7 d after virus injection.

Extraction and Analysis of GSLs and Ceramide

GSLs were extracted and separated into neutral and acidic fractions containing gangliosides as described earlier [15]. The amount of GSLs spotted onto a plate by a TLC applicator (Camag, USA) was normalized to tissue protein content determined by the Lowry method [70]. TLC running solvent for acidic GSLs was chloroform/methanol/0.2% CaCl₂ (45:45:10 by vol). GSLs were visualized with 0.2% orcinol in 10% sulphuric acid at 120°C for 10 min. Ceramide was extracted as described earlier [15] and spotted onto a TLC plate. Running solvent for ceramide was chloroform/methanol/acetie acid (190:9:1 by vol), and ceramide was visualized with 10% CuSO₄ in 8% H₃PO₄ at 180°C for 10 min. Lipid content was quantified by densitometry (Shimadzu, Japan).

Statistical Analysis

Unless stated elsewhere, results were analyzed by a two-tailed, unpaired Student’s t test (Graph Pad Prism, Graph Pad Software, Inc.). To analyze main effects of genotype on metabolizable energy or energy expenditure, body weight was employed as a co-factor in a linear regression model to account for the confounding effect of body size on energy metabolism parameters [71]. p≤0.05 was considered statistically significant and marked *, p≤0.01 was marked **, and p≤0.001 was marked ***.

Supporting Information

Figure S1 Generation and characterization of inducible UGGf/f/CamKCreERT2 mice. (A) R26R/UGGf/f/+ CamKCreERT2 reporter mice and R26R/UGGf/+ mice were induced with tamoxifen and brains were removed 3 d after the last injection. X-Gal staining revealed Cre activity in hypothalamic MnPO, paraventricular nucleus (PVN), and lateral hypothalamus (LH). Cre activity was absent in ventromedial hypothalamus (VMH) and in the brain stem nucleus of the solitary tract (NTS; cc, central canal). Cre activity could be detected in hippocampus and cerebral cortex. GD1a immunofluorescence visualized ganglioside depletion in Cre-targeted regions of male UGGf/f/CamKCreERT2 mice (6 wk p.i.). Scale bar: 100 μm. (B) Overview of brain slices from R26R/UGGf/f/+ CamKCreERT2 reporter mice indicating Cre activity. 3v, 3rd ventricle; CP, caudoputamen; LSN, lateral septal nucleus; SFO, subformical organ; CTX, cortex; HC, hippocampus; DMH, dorsomedial hypothalamus. (C) In situ hybridization showed depletion of UGG mRNA in hypothalamic PVN (a), SCN (b), as well as in the amygdala (c), hippocampus (d), and cerebral cortex (e) of male UGGf/f/CamKCreERT2 mice (4 wk p.i.). Respective areas were visualized by thionine staining. (D) Southern blot did not show recombination events in peripheral tissues of female UGGf/f/CamKCreERT2 mice 2 wk p.i. The UGG null allele could solely be detected in cerebral cortex, hippocampus, and hypothalamus. (E) Stable ganglioside depletion 3 wk p.i. in targeted neuronal populations of totally dissected hippocampus as shown by TLC. Residual gangliosides from nontargeted cells and innervating fibers were still visible. (+ UGGf/f, − UGGf/f/CamKCreERT2). Laidom amounts equaling 50 μg of tissue protein were loaded. Quantification from densitometry analysis of thin layer chromatography results is depicted (n = 3). (F) Sphingomyelin was slightly elevated in hippocampus of UGGf/f/CamKCreERT2 mice. *p≤0.05; **p≤0.01. Means ± SEM. (TIF)

Figure S2 Intrinsic electrical properties of Arc neurons from brain slices of female UGGf/f/CamKCreERT2 mice at 12 wk p.i. compared to controls. (A) Spontaneous firing frequency (left), maximal rate of rise of action potentials (AP; middle), and AP width at half-maximal amplitude (right) were not different between both strains. (B) Resting membrane potential (RMP, left) and logical (N-41) and biophysical membrane properties (primary AP; right) were both shifted to more depolarized values in cells from UGGf/f/CamKCreERT2 mice. Cell numbers are stated in brackets above each column. *p≤0.05. Mean values ± SEM. (TIF)

Figure S3 Immortalized hypothalamic cells (N-41 cells) express gangliosides and ganglioside-depleted cells show normal morphological (N-41) and biophysical membrane properties (primary neurons). (A) Ganglioside expression pattern as determined by TLC after lipid extraction from immortalized hypothalamic cell lines and subsequent separation of neutral and acidic glycosphingolipids including gangliosides (St, standard). (B) Immediate overlay TLC confirmed the identity of GD1a and GM3 bands (St, standard). (C) The 7-d treatment of immortalized hypothalamic cells with the GCS inhibitor NB-DBJ (100 μM) led to inhibition of ganglioside biosynthesis, as shown by TLC (St, standard). Quantification of the TLC bands is depicted. (D) Normal membrane appearance and assessed by electronmicroscopy (n, nucleus; nl, nucleoli; m, mitochondria; er, endoplasmic

PLOS Biology | www.plosbiology.org 16 March 2013 | Volume 11 | Issue 3 | e1001506
PLOS Biology | www.plosbiology.org 17 March 2013 | Volume 11 | Issue 3 | e1001506

Figure S4  Fat mass in Ugg<sup>f/f</sup>//CamKCreERT2 mice was elevated, while organ morphology was unaltered. (A) Unaltered body weight in heterozygous Ugg<sup>f/f</sup>//CamKCreERT2 mice (n = 4). (B) NMR analysis revealed that lean mass was only slightly increased 4 wk p.i. (n = 9–10). (C) Significantly increased body fat mass in Ugg<sup>f/f</sup>//CamKCreERT2 mice 7 and 10 wk p.i. when adjusted for body weight, as determined by nuclear magnetic resonance imaging (n = 9–10 per group); **p≤0.01 (LM). Individual values. Relative lean mass, when plotted against body weight, decreased during progressive weight gain 7 and 10 wk p.i. in Ugg<sup>f/f</sup>//CamKCreERT2 mice (n = 9–10 per group); *p≤0.05; ***p≤0.001 (LM). Individual values. (D) Unaltered morphology of major peripheral organs in female mice was shown by hematoxylin and eosin stainings 9 wk p.i. No liver steatosis could be detected. (Scale bars: Liver, 50 μm; Kidney, Lungs, Spine, 200 μm). (E) Parameters indicative for liver function did not show any significant changes 19 wk p.i., indicating normal liver function in Ugg<sup>f/f</sup>//CamKCreERT2 mice (n = 12; GOT, n = 4). (F) Serum levels for cholesterol, fasting glucose, urea, and creatinine 19 wk p.i. did not show any biologically relevant differences in mice (n = 12). (G) Ugg<sup>f/f</sup>//CamKCreERT2 mice displayed impaired glucose tolerance 12 wk p.i. (n = 9; Mann-Whitney Rank Sum Test). (H) Slight but significant insulin insensitivity was detected in Ugg<sup>f/f</sup>//CamKCreERT2 mice 10 wk p.i. (n = 8–9; Mann-Whitney Rank Sum Test). *p≤0.05; **p≤0.01; ***p≤0.001. Means ± SEM unless stated otherwise. (TIF)

Figure S5  Normal excretion and unaltered spontaneous locomotor activity in Ugg<sup>f/f</sup>//CamKCreERT2 mice, but plasma NEFAs are altered. (A) Fecal free fatty acids (8 wk p.i.), fecal energy density and size were detected in Ugg<sup>f/f</sup>//CamKCreERT2 mice 3, 6, and 11 wk p.i. Means ± SEM. (B) Metabolic rate of Ugg<sup>f/f</sup>//CamKCreERT2 mice was unaltered 2 wk p.i. (n = 7–10). Individual means. (C) Spontaneous locomotor activity measured at different time points after tamoxifen induction did not reveal any statistically significant difference between Ugg<sup>f/f</sup>//CamKCreERT2 mice and control littersmates (n = 9) (Mann-Whitney Rank Sum Test). Medians and individual values. (D) Decreased plasma NEFA values in fasted Ugg<sup>f/f</sup>//CamKCreERT2 mice 11 wk p.i., indicating decreased fat mobilization (n = 4–6). **p≤0.01. Means ± SEM. (TIF)

Figure S6  Hypothemia in Ugg<sup>f/f</sup>//CamKCreERT2 mice is not due to defective thyroid function, but decreased sympathetic activity in iBAT. (A) Enlarged lipid droplets were found in iBAT of Ugg<sup>f/f</sup>//CamKCreERT2 mice 9 wk p.i. (B) Altered mitochondrial density and size were detected in Ugg<sup>f/f</sup>//CamKCreERT2 mice by ultrastructural analysis of iBAT 9 wk p.i. (C) Mitochondrial area was reduced in Ugg<sup>f/f</sup>//CamKCreERT2 mice (n = 3 mice, 195 mitochondria). (D) Serum-free thyroxine (TT4) was determined by ELISA and did not show any alterations in Ugg<sup>f/f</sup>//CamKCreERT2 mice (n = 5–6 per group). (E) Serum-free triiodothyronine (rT3) was determined by ELISA and did not show any alterations in Ugg<sup>f/f</sup>//CamKCreERT2 mice (n = 3–6 per group). (F) High performance liquid chromatography (HPLC) revealed lower sympathetic activity (NETO rate) per mg iBAT 9 wk p.i. (n = 4). (G) NE content was also decreased in Ugg<sup>f/f</sup>//CamKCreERT2 iBAT 9 wk p.i. (n = 4). **p≤0.01; ***p≤0.001. Means ± SEM. (TIF)

Figure S7  rAAV-mediated Ugg<sup>f/f</sup> gene delivery to the hypothalamic Arc ameliorates obesity in Ugg<sup>f/f</sup>//CamKCreERT2 mice. (A) and (B) Double immunofluorescence showed that Cre activity, indicated by beta galactosidase staining (b-gal), was targeted to MnPO neurons (A) expressing the long form of the ObR (see arrowheads), but not to the majority of ObR neurons in the LHA (B), as indicated by PStat3 staining in leptin-injected R26R/ΔCre<sup>f/f</sup>/Ugg<sup>f/f</sup>//CamKCreERT2 mice (5 mg/kg leptin, 120 min). (C) Stereotactic rAAV-LacZ delivery to the Arc was exemplarily demonstrated by X-Gal-staining. Morphology was depicted in the HE section. (D) Ugg<sup>f/f</sup>//CamKCreERT2 mice that were not targeted in the Arc by stereotactic delivery of rAAV viruses encoding Ugg<sup>f/f</sup> and lacZ did not show improvement in body weight increase compared to rAAV-Empty/lacZ-injected Ugg<sup>f/f</sup>//CamKCreERT2 mice (n = 4; rAAV-Ugg/lacZ missed, n = 8; rAAV-Empty/lacZ). The graph depicting rAAV-Empty/lacZ-targeted mice is taken from Figure 4B for comparison. (E) Restored ganglioside biosynthesis in the Arc of rAAV-Ugg<sup>f/f</sup>-injected Ugg<sup>f/f</sup>//CamKCreERT2 mice, as shown by GD1a immunofluorescence 8 wk p.i. Shown are overview pictures for Figure 4G (also taken for Figure S7E). Scale bar: 18 μm. Means ± SEM. (TIF)

Figure S8  GCS in hypothalamic neurons regulates neuronal leptin signaling. (A) Neuron count in the Arc was normal in Ugg<sup>f/f</sup>//CamKCreERT2 mice (n = 115–122 sections). Quantification contains normalized data from 12 mice each. (B) Stat3 phosphorylation in MBH of male mice was investigated by Western blot 6 wk p.i. We loaded 100 μg of protein in each lane. Elevated PStat3 levels were not seen in Ugg<sup>f/f</sup>//CamKCreERT2 mice upon leptin stimulation. Quantification for PStat3/Tubulin is depicted (n = 3). (C) Baseline and leptin-stimulated Stat3 levels were elevated in Ugg<sup>f/f</sup>//CamKCreERT2 mice. Stat3 was normalized for tubulin expression (n = 3). (D) The PStat3/Stat3 ratio is decreased in Ugg<sup>f/f</sup>//CamKCreERT2 mice 6 wk p.i. both at baseline and after leptin stimulation. PStat3 and Stat3 levels were normalized for tubulin (n = 3). (E) Immortalized hypothalamic cells (N-41 cells) were incubated with either saline or 100 ng/ml leptin (10 min). A proximity ligation assay (PLA; principle depicted in Figure 5E) for GD1a/ObR similar to the experiment depicted in Figure 5F was performed using a different ObR-specific antibody. GD1a/ObR interactions were detected and leptin treatment dynamically increased the GD1a/ObR PLA spots per cell similar to the result depicted in Figure 5F. Pre-adsorption of the antibody by a blocking peptide abolished PLA signals (n = 74–150 cells). (F) Similar to the experiments depicted in Figure 5H, extracts from saline- and leptin-treated N-41 cells were immunoprecipitated with a second ObR antibody, lipids were extracted, and GD1a and GM1 were visualized by immunoblotting (TLC). Similar to the results obtained in Figure 5H, GD1a and GM1 co-immunoprecipitated (Co-IP) with ObR. (G) Similar to the result in Figure 5I, Jak was co-precipitated with the ObR, which increased upon leptin stimulation of N-41 cells, using a second ObR antibody. Addition of the blocking peptide abolished the signal. (H) Weak Jak phosphorylation was induced in N-41 cells after 30 min of leptin treatment (0.1 μg/ml). *p≤0.05; **p≤0.01; ***p≤0.001. Means ± SEM. (TIF)
Datasets for each time point were acquired individually. Immunofluorescence and image acquisition for each dataset (treated and untreated controls and knockouts) were performed simultaneously. *p<0.05; **p<0.01; ***p<0.001. Means ± SEM. (TIF)

Text S1  Supplemental experimental procedures and supplemental references. (DOC)

Acknowledgments
We would like to thank Gunther Schütz and Stefan Berger for providing us with CamKCreERT2 mice. We are grateful to G. Schütz and Rohini Kuner for critical comments on this manuscript. We thank the DKFZ Light Microscopy Facility for imaging systems, Richard Hertel for HPLC measurements, Hannah Monyer and Ulla Amtmann for help with in situ hybridization, Jurgen Kleinschmidt for advice with rAAV purification, Lena Hoffmann for advice with stereotactic injections, and Maria Meister, Gabi Schmidt, Martina Volz, and Ann-Elisabeth Schwarz for expert technical assistance.

Author Contributions
The author(s) have made the following declarations about their contributions: Conceived and designed the experiments: VN MW HJG JR MK JR AD MHA. Performed the experiments: VN MW SH SM UR SK FCR CW. Analyzed the data: VN MW JR FCR CW AD MK. Contributed reagents/materials/analysis tools: OBH NG. Wrote the paper: VN MW HJG JR MK.

Figure S8
The brain stem NTS of Ugcg f/f//CamKCreERT2 mice retained leptin responsiveness 6 wk p.i. c-Fos expression was unaltered in the non-targeted NTS (n = 2–4 sections). Datasets for each time point were acquired individually. Immunofluorescence and image acquisition for each dataset (treated and untreated controls and knockouts) were performed simultaneously. *p<0.05; **p<0.01; ***p<0.001. Means ± SEM. (TIF)

Figure S10
Reduced PIP3 formation in POMC neurons of Ugcg f/f//CamKCreERT2 mice upon leptin stimulation. (A) Fasted mice were injected with either saline or leptin (5 mg/kg body weight) and sacrificed 45 min later. Either POMC or NPY staining identified individual neuronal populations. Leptin did not directly lead to increased PIP3 formation in NPY neurons in neither of the groups (n = 3–7). (B) Unaltered mRNA expression of the anorexigenic peptides POMC and CART in the MBH of Ugcg f/f//CamKCreERT2 mice 6 and 9 wk p.i. (n = 3–6). (C) Fasted mice were injected with either saline or leptin (5 mg/kg body weight) and sacrificed 45 min later. NPY staining identified individual neuronal populations. Leptin did not directly lead to increased PIP3 formation in NPY neurons in neither of the groups (n = 3–7). (D) Increased Npy and Ugcg mRNA expression in the MBH of Ugcg f/f//CamKCreERT2 mice 6 and 9 wk p.i. (n = 3–6). Datasets for each time point were acquired individually.

References
1. Morton GJ, Cummings DE, Baskin DG, Barsh GS, Schwartz MW (2006) Central nervous system control of food intake and body weight. Nature 443: 289–295.
2. Lee GH, Proenza R, Monteiz JM, Carroll KM, Darvishzadeh JG, et al. (1996) Abnormal splicing of the leptin receptor in diabetic mice. Nature 379: 632–635.
3. Konner AC, Klockener T, Bruning JC (2009) Control of energy homeostasis by insulin and leptin: targeting the arcuate nucleus and beyond. Physiol Behav 97: 632–638.
4. Schwartz MW, Woods SC, Jr DP, Seeley RJ, Baskin DG (2000) Central nervous system control of food intake. Nature 404: 661–671.
5. Opland DL, Louis GW, Leinninger GM, Patterson CM, et al. (2010) Ventral terminal area leptin receptor neurons specifically project to and regulate cocaine- and amphetamine-regulated transcript neurons of the extended central amygdala. J Neurosci 30: 5713–5723.
6. Myers MG, Jr, Munzberg H, Flier JS, Bjorbaek C (2004) Region-specific leptin resistance within the hypothalamus of diet-induced obese mice. Endocrinology 145: 4880–4889.
7. Halaas JL, Boozer C, Blair-West J, Fidahusein N, Denton DA, et al. (1997) Reduced PIP3 formation in POMC neurons of Ugcg f/f//CamKCreERT2 mice before ganglioside depletion was completed (1–2 wk p.i.). This response was blunted in ganglioside-depleted POMC neurons 6 wk p.i. The percentage of POMC/PIP3-double-positive neurons per Arc section normalized to Ugcg f/f saline is depicted (n = 3–7). (B) Unaltered mRNA expression of the anorexigenic neuropeptides POMC and CART in the MBH of Ugcg f/f//CamKCreERT2 mice 6 and 9 wk p.i. (n = 3–6).
8. Schwartz MW, Woods SC, Jr DP, Seeley RJ, Baskin DG (2000) Central nervous system control of food intake. Nature 404: 661–671.
9. Myers MG, Jr, Munzberg H, Flier JS, Bjorbaek C (2004) Region-specific leptin resistance within the hypothalamus of diet-induced obese mice. Endocrinology 145: 4880–4889.
52. Balthasar N, Coppari R, McMinn J, Liu SM, Lee CE, et al. (2004) Leptin activation of Stat3 in the hypothalamus of wild-type and ob/db mice but not db/db mice. Nat Genet 14: 95–97.

53. Kabayama K, Sato T, Saito K, Lobero N, Prinetti A, et al. (2007) Dissociation of the insulin receptor and caveolin-1 complex by ganglioside GM3 in the state of insulin resistance. Proc Natl Acad Sci U S A 104: 13678–13683.

54. Inokuchi J (2007) Insulin resistance as a membrane microdomain disorder. Trends Endocrinol Metab 18: 269–272.

55. van Eijk M, Arens J, Bijl N, Ottenhoff R, van Roomen CP, et al. (2009) Reducing glycopospholipid content in adipose tissue of obese mice restores insulin sensitivity, adipogenesis and reduces inflammation. PlOS One 4: e1723. doi:10.1371/journal.pone.0002637

56. Zhao H, Przybyska M, Wu IH, Zhang J, Maniatis P, et al. (2009) Inhibiting glycoprophospholipid synthesis ameliorates hepatic steatosis in obese mice. Hepatology 50: 85–93.

57. Lang Z, Guerrero M, Li R, Ladisch S (2001) Ganglioside GD1a enhances VEGF-induced endothelial cell proliferation and migration. Biochim Biophys Acta 1522: 1031–1037.

58. Nakayama H, Yoshizaki F, Prinetti A, Sommio S, Mauri L, et al. (2008) Lipid-coupled LacCer-enriched lipid rafts are required for CD11b/CD18-mediated neurphageal phagocytosis of nonopsonized microorganisms. J Leukoc Biol 83: 729–741.

59. Yang G, Badeanlu L, Belavsky J, Roberts AJ, Hamun Y, et al. (2009) Central role of ceramide biosynthesis in body weight regulation, energy metabolism, and the metabolic syndrome. Am J Physiol Endocrinol Metab 297: E211–E224.

60. Kohyama-Koganea Y, Nabetani T, Miura M, Hiraibayashi Y (2011) Glucocerebroside synthase in the fat body controls energy metabolism in Drosophila. J Lipid Res 52: 1392–1399.

61. Aerts JM, Ottenhoff R, Woodson AS, Greffhorst A, van Eijk M, et al. (2007) Pharmacological inhibition of glucocerebrosidase synthase enhances insulin sensitivity. Diabetes 56: 1341–1349.

62. Lunn JP, Johnson LA, Fromholt SE, Itouni S, Huang J, et al. (2000) High-affinity anti-ganglioside IgG antibodies raised in complex ganglioside knockout mice: reexamination of GD1a immunolocalization. J Neurochem 75: 404–412.

63. Latvanlehto A, Fox MA, Sorunen R, Tu H, Oikarainen T, et al. (2010) Muscle-derived collagen XIId regulates maturation of the skeletal neuromuscular junction. J Neurosci 30: 12304–12314.

64. Zolotukhin S, Byrne BJ, Mason E, Zolotukhin I, Potter M, et al. (1999) Recombinant adeno-associated virus purification using novel methods improves infectious titer and yield. Gene Ther 6: 973–985.

65. Cetin A, Kosma S, Elavaza M, Seeburg PH, Osten P (2006) Stereotaxic gene delivery in the rodent brain. Nat Protoc 1: 3166–3173.

66. Jeenemann R, Sandhoff R, Grone HJ, Hienhui H (2001) Human heterophile receptor LRP1 regulates leptin signaling and energy homeostasis in the adult hypothalamic mechanisms controlling food intake. Physiol Behav 104: 40–46.

67. Myers MG, Cowley MA, Munzberg H (2008) Mechanisms of leptin action and leptin resistance. Ann Rev Physiol 70: 537–556.

68. Munzberg H, Myers MG, Jr. (2005) Molecular and anatomical determinants of central leptin resistance. Nat Rev Endocrinol 1: 566–570.

69. Björbaek C, Elmquist JK, Frantz JD, Shoelson SE, Flier JS (1998) Identification of SOCS-3 as a potential mediator of central leptin resistance. Mol Cell 1: 619–625.

70. Plum L, Ma X, Hampel B, Balthasar N, Coppari R, et al. (2006) Enhanced PIP3 signaling in POMC neurons causes decreased pomc expression, mild obesity, and defects in central thermoregulation. Diabetes 55: 567–573.

71. Lunn MP, Johnson LA, Fromholt SE, Itouni S, Huang J, et al. (2000) High-affinity anti-ganglioside IgG antibodies raised in complex ganglioside knockout mice: reexamination of GD1a immunolocaiization. J Neurochem 75: 404–412.

72. Latvanlehto A, Fox MA, Sorunen R, Tu H, Oikarainen T, et al. (2010) Muscle-derived collagen XIId regulates maturation of the skeletal neuromuscular junction. J Neurosci 30: 12304–12314.

73. Zolotukhin S, Byrne BJ, Mason E, Zolotukhin I, Potter M, et al. (1999) Recombinant adeno-associated virus purification using novel methods improves infectious titer and yield. Gene Ther 6: 973–985.

74. Cetin A, Kosma S, Elavaza M, Seeburg PH, Osten P (2006) Stereotaxic gene delivery in the rodent brain. Nat Protoc 1: 3166–3173.

75. Jeenemann R, Sandhoff R, Grone HJ, Hienhui H (2001) Human heterophile receptor LRP1 regulates leptin signaling and energy homeostasis in the adult hypothalamic mechanisms controlling food intake. Physiol Behav 104: 40–46.