Hypothalamic Overexpression of Neurosecretory Protein GL Leads to Obesity in Male C57BL/6J Mice

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Keywords
Neurosecretory protein GL · Hypothalamus · Obesity · Animal model

Abstract
Introduction: The mechanisms underlying obesity are not fully understood, necessitating the creation of novel animal models for the investigation of metabolic disorders. We have previously found that neurosecretory protein GL (NPGL), a newly identified hypothalamic neuropeptide, is involved in feeding behavior and fat accumulation in rats. However, the impact of NPGL on obesity remains unclear in any animal model. The present investigation sought to elucidate whether NPGL causes obesity in the obesity-prone mouse strain C57BL/6J. Methods: We overexpressed the NPGL-precursor gene (Npgl) in the hypothalamus using adeno-associated virus in male C57BL/6J mice fed normal chow (NC) or a high-calorie diet (HCD). After 9 weeks of Npgl overexpression, we measured adipose tissues, muscle, and several organ masses in addition to food intake and body mass. To assess the effects of Npgl overexpression on peripheral tissues, we analyzed mRNA expression of lipid metabolism-related genes by quantitative RT-PCR. Whole body energy consumption was assessed using an O2/CO2 metabolism measurement before an apparent increase in body mass. Results: Npgl overexpression increased food intake, body mass, adipose tissues and liver masses, and food efficiency under both NC and HCD, resulting in obesity observable within 8 weeks. Furthermore, we observed fat accumulation in adipose tissues and liver. Additionally, mRNA expression of lipid metabolism-related factors was increased in white adipose tissue and the liver after Npgl overexpression. Npgl overexpression inhibited energy expenditure during a dark period. Conclusion: Taken together, the present study suggests that NPGL can act as an obesogenic factor that acts within a short period of time in mice. As a result, this Npgl overexpression-induced obesity can be widely applied to study the etiology of obesity from genes to behavior.

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Introduction

The number of obese patients worldwide has recently reached 700 million [1]. Obesity is associated with many serious diseases, such as type 2 diabetes mellitus, hypertension, and hyperlipidemia [2–5]. The excess of energy intake over energy consumption leads to fat accumulation and eventually obesity [6]. Therefore, further research on energy homeostasis is required to prevent and treat metabolic disease. Energy homeostasis is regulated by factors produced by the brain and peripheral tissues. The neuropeptides α-melanocyte-stimulating hormone – derived from proopiomelanocortin – and neuropeptide Y (NPY) are known to be anorexigenic and orexigenic, respectively. Both are produced in the hypothalamic arcuate nucleus (Arc) [7]. Agouti-related peptide is also produced by NPY-expressing neurons and inhibits the effect of α-melanocyte-stimulating hormone by antagonistic binding to melanocortin receptor type 4 [8]. Ghrelin and leptin are other well-known energy-related metabolic factors secreted by peripheral tissues. Ghrelin is an orexigenic factor secreted by the stomach, and it promotes feeding behavior by activating NPY/agouti-related peptide neurons [9]. Leptin is an anorexigenic hormone released from white adipose tissue (WAT) [10, 11]. Moreover, insulin accelerates fat accumulation by inhibiting lipolysis [12]. It is clear that many factors are involved in the regulation of energy intake, but this complex system is still not fully understood. Thus, the identification and functional analysis of novel factors are required for understanding whole body energy homeostasis.

We recently found a novel gene related to energy homeostasis in chickens, rats, mice, and humans [13–15]. The small secretory protein produced by the novel gene was named neurosecretory protein GL (NPGL) because the C-terminus amino acid sequence is Gly-Leu-NH₂ was named neurosecretory protein GL (NPGL) because the C-terminus amino acid sequence is Gly-Leu-NH₂ [13]. Analysis of the genome database revealed that NPGL is evolutionarily conserved throughout vertebrates [13].

To date, we have mainly investigated the functions and features of NPGL in rats and mice. Using morphological analysis, we found that NPGL is produced in the latero-posterior region of the Arc in mice, and NPGL-containing fibers project to several regions within the hypothalamus [15].

We recently showed that NPGL regulates feeding and fat accumulation in rodents. In rats, a 13-day chronic intracerebroventricular (i.c.v.) infusion of NPGL increased the WAT mass without changing body mass and modestly stimulated food intake under a high-calorie diet (HCD) [14]. Analysis of adeno-associated virus (AAV)-driven the NPGL-precursor gene (Npgl) overexpression in rats for 6 weeks had more marked effects on feeding behavior and lipogenesis than the 13-day chronic infusion [14]. Similarly, 13-day chronic i.c.v. infusion of NPGL stimulated feeding behavior and mass gain due to fat accumulation in mice fed a HCD [16]. However, the induction of abnormally high body mass was not a characteristic result of NPGL treatment in these studies, leaving the possibility that NPGL induces obesity as an open question. In general, creation of diet-induced obesity (DIO) in rodents is costly and time-consuming, typically requiring 16–20 weeks [17–19]. Therefore, the discovery of a more efficient way to generate an animal model of obesity would be useful for studies aiming to elucidate the mechanism of energy metabolism regulation.

The aim of the present study was to determine the impact of NPGL on obesity in male obesity-prone C57BL/6J mice. We found that Npgl overexpression in the mouse hypothalamus elicited obesity within 8 weeks under a HCD. The present findings report the effects of Npgl overexpression on food intake, body mass, body composition, blood parameters, the expression of lipid metabolism-related factors, and the whole body energy consumption in mice fed normal chow (NC) or a HCD during the development of obesity.

Table 1. The artificial diets used in this study

| Diet | Protein, kcal% | Carbohydrate (sucrose), kcal% | Fat, kcal% | kcal/g |
|------|---------------|------------------------------|------------|-------|
| NC   | 29            | 56 (0)                       | 15         | 3.45  |
| HCD  | 20            | 48 (20)                      | 32         | 4.4   |

NC, normal chow; HCD, high-calorie diet.

Materials and Methods

Animals

Male C57BL/6J mice (7 weeks old) were purchased from Nihon SLC (Hamamatsu, Japan) and singly housed under standard conditions (25 ± 1°C under a 12-h light/12-h dark cycle) with ad libitum access to water and NC (CE-2; CLEA Japan, Tokyo, Japan) or a HCD (32% of calories from fat/20% of calories from sucrose, D14050401; Research Diets, New Brunswick, NJ, USA). The nutritional compositions are shown in Table 1. Animal surgery was performed under isoflurane anesthesia.

Production of AAV-Based Vectors

We followed a previously reported method to generate the overexpression AAV [14]. The full-length open reading frame of mouse Npgl was amplified from cDNA of the mediobasal hypo-
thalamus and inserted into the pAAV-IRES-GFP expression vector including the sequence of cytomegalovirus promoter (Cell Bioslabs, San Diego, CA, USA). The primers for mouse NPGL were 5′-CGATCGATACCATGGCTGATCCTGGGC-3′ for the sense primer and 5′-CGGAATTCTTATTTTCTCTTTACTTC-3′ for the antisense primer.

AAV-based vectors AAV-DJ/8-NPGL-IRES-GFP for NPGL (AAV-NPGL) and AAV-DJ/8-IRES-GFP for control (AAV-CTL) were produced in 293AAV cells (Cat# AAV-100; Cell Bioslabs) using the AAV-DJ/8 Helper Free Packaging System containing pAAV-DJ/8 and pHelper plasmids (Cell Bioslabs). The triple plasmids (AAV-DJ/8-NPGL-IRES-GFP or AAV-DJ/8-IRES-GFP, pAAV-DJ/8, and pHelper) were mixed with the polyethylenimine MAX transfection reagent (Polysciences, Warrington, PA, USA). The mixture was diluted with Opti-MEM I medium (Life Technologies, Carlsbad, CA, USA) and added to 293AAV cells in 150-mm cell culture dishes. Transfected cells were cultured in DMEM containing 10% fetal bovine serum.

For the purification of AAV-based vectors, 3 days after transfection, the cells and supernatants were harvested and purified using chloroform and were condensed using Amicon Ultra-4 Centrifugal Filter Devices (100K MWCO; Merck Millipore, Billerica, MA, USA). For AAV titration, 1 μL of AAV solution was treated with RQ1 DNase (Promega, Madison, WI, USA) according to the manufacturer’s instructions. Virus titers were determined by quantitative PCR with EGFP primer pairs. The primers for EGFP were 5′-ACCACTACCTGAGCACCAGTC-3′ for the sense primer and 5′-GTCCATGCGAGGATGATCC-3′ for the antisense primer. After titration, the AAV-based vectors were prepared at a concentration of 1 × 10^9 particles/μL and stored at −80°C until use.

Stereotaxic Surgery

For NPGL overexpression, mice were bilaterally injected with 0.5 μL/site (5.0 × 10^8 particles/site) of AAV-based vectors (AAV-NPGL or AAV-CTL) using a Neuros Syringe (7001 KH; Hamilton, Reno, NV, USA) into the mediobasal hypothalamic region with the coordinates 2.2 mm caudal to the bregma, 0.25 mm lateral to the midline, and 5.8 mm ventral to the skull surface. NPGL overexpression was maintained for 9 weeks in mice fed NC or a HCD. NPGL overexpression was confirmed by quantitative RT-PCR at the end point.

Measurement of Body Mass, Food Intake, and Body Composition

Mice were divided into 2 groups according to each diet (NC or HCD). Food intake and body mass were measured weekly. Food efficiency (g/kcal) was calculated as body mass gain (g)/cumulative food intake (kcal) [20]. Nine weeks after stereotaxic surgery, mice were immediately decapitated between 13:00 and 15:00. The mediobasal hypothalamus, adipose tissues, organs, and skeletal muscles were collected, weighed, and frozen in liquid nitrogen. Blood was collected at sacrifice.

Quantitative RT-PCR

Total RNA was extracted using QIAzol lysis reagent (QIAGEN, Venlo, Netherlands) for the inguinal WAT (iWAT) or TRIzol reagent (Life Technologies) for the liver and the mediobasal hypothalamus in accordance with the manufacturer’s instructions. First-strand cDNA was synthesized from total RNA using a ReverTra Ace kit (TOYOBO, Osaka, Japan).

The abbreviations for genes and sequences of primers used in this study are listed in Tables 2 and 3, respectively. PCR amplifications were conducted with THUNDERBIRD SYBR qPCR Mix (TOYOBO) using the following conditions: 95°C for 20 s, followed by 40 cycles each consisting of 95°C for 3 s, and 60°C for 30 s. The PCR products in each cycle were monitored using a Bio-Rad CFX Connect (Bio-Rad Laboratories, Hercules, CA, USA). Relative quantification of each gene was determined by the 2^−ΔΔCt method using ribosomal protein S18 (Rps18) for the iWAT or β-actin (Actb) for the liver and the mediobasal hypothalamus as an inter-
Fatty Acid Analysis
For analysis of endogenous stearoyl-CoA desaturase 1 (SCD1) activity in the iWAT, lipids were extracted according to a previously described method [22]. The iWAT (50 mg) was extracted with 500 μL of chloroform:methanol (2:1) using a bead crusher (μT-12; TAITEC, Saitama, Japan), and 125 μL of distilled water was added and mixed by inversion. After incubation for 30 min, the sample was centrifuged at 3,000 g, and the lower organic phase was collected and evaporated to dryness and kept at −20°C. The residues were solubilized in hexane, and fatty acids were identified by gas chromatography-mass spectroscopy (JMS-T100 GCV; JEOL, Tokyo, Japan). SCD1 activity was estimated as the oleate-to-stearate ratio (18:1/18:0) and palmitoleate-to-palmitate ratio (16:1/16:0) [23]. The de novo lipogenesis index was calculated from the palmitic-to-linoleic acid ratio (16:0/18:2n-6) [24, 25].

Immunohistochemistry
The mediobasal hypothalamus was injected with AAV-CTL or AAV-NPGL, as described above. Four weeks later, the brain tissues were sectioned into 20-μm-thick slices with a cryostat at −20°C following cryoprotection and freezing. Immunohistochemistry on free-floating sections was performed as described previously [14, 15]. The antibody was raised against mouse NPGL (RRID: AB_2636993) which has been used previously [14, 15]. The sections were incubated in blocking buffer (1% bovine serum albumin, 1% normal donkey serum, and 0.3% Triton X-100 in 10 mM phosphate-buffered saline) for 1 h at room temperature before incubation with a rabbit antibody against NPGL (1:100 dilution in blocking buffer), overnight at 4°C. Cy3-conjugated donkey anti-rabbit IgG (RRID: AB_2307443, 1:500 dilution, 711-165-152; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) was used as the secondary antibody. Immunoreactive labeling was observed using a microscope (Eclipse E600; Nikon, Tokyo, Japan).

Oil Red O Staining
To detect fat accumulation in the liver, the liver was fixed in 4% paraformaldehyde at the endpoint of Npgl overexpression, embedded in paraffin, and sectioned to a thickness of 8 μm with a microtome. The sections were then air-dried and deparaffinized in a graded alcohol series. The nucleus and cytoplasm were stained with hematoxylin and eosin (5 min for each stain), and the sections were washed in tap water. After dehydration in a graded alcohol series and clearing with xylene, the sections were mounted on slides and examined under a microscope.

Western Blot Analysis
For the detection of overexpression of mature NPGL in the hypothalamus, Western blot analysis using the mediobasal hypothalamus was performed following a previously reported method [14]. In this study, the hypothalami from 6 mice were used. The blot was probed with the antibody against NPGL (RRID: AB_2636993) and incubated with horseradish peroxidase-labeled donkey anti-rabbit 

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Table 3. Sequences of oligonucleotide primers for quantitative RT-PCR

| Gene     | Sense primer (5’ to 3’) | Antisense primer (5’ to 3’) |
|----------|------------------------|-----------------------------|
| Acc      | TCCGCACGTACGTGAACCACAT | TGTCGCCGACAGATTCTCTCA       |
| Fas      | AAGGGTCGACCTGGTCCCTCA  | GCCATGGCAGGGGTTGTTTGGT     |
| Scd1     | CTGTAACGGGATATCACTGGTTTC | GCCGTGCCTTTGAAAGGTTCTG     |
| Gpat1    | TTCATTTGATGCAGTCCGCAA  | GCCAGAACGCACTCAAGATTTGTT   |
| Chrebp   | CACACTCCACCCACCTCTCTTTC | TGTCACCGGCAGATTTGCTTGCT    |
| Cpt1a    | CTTGCGATGTTCACAAAG     | GGACGCCACACTCCAGATGTT      |
| Atgl     | AACACCAAGCATCCAGTTCAA  | GGTCGATAGGCCATCTCCTCCTC   |
| Hsl      | GTCTGACGCCAAGCTACTG    | GTAACTGACCTGGTTCACAT       |
| Fgf21    | CTCCTAGTTTCTTGCACCATGG | AAGCTCAGGCTCCACGATGTT      |
| Gapdh    | AAGTGCTTCCACCAAGCTGAA  | CTGTCACCCACCTCTGTTTGA      |
| Slc2a2   | GGCAATTTCGACGAGTGTAGG  | TTCTCTGCGGACTGTTCCTCCTC   |
| Slc2a4   | GTAATTTGATGGCAGCAGT    | AAGCTGAACTCGTGCTACAAG      |
| Cd36     | TCCCTGCAATGTCCAGTCTATC | AAAGGCATGTCGAGAGAAAGAA     |
| Ppara    | TCAGATATGGGGGACAAAGG   | GACAGGACACTGGTGAAGAAGA     |
| Ppary    | GCCCTTGTGATCATTTATAGGA | GCACAGGACCTTGTTGAGTTG      |
| Pgc1a    | GCAACATGGCTCAAGCCAAAC  | TGGCTTGCAAGAGTTGCA         |
| Npgl     | TAGTGAAGCTGTGCTCTCAC   | TCTAAGAGCTGAGAATATGCA      |
| Rps18    | CCTGAAGAAGTTCCACGACAT  | TTTCCACGCCCTTTGGTG         |
| Actb     | GCACCCACCACCTCTTCAAT   | AGGTCTCAAAATCATGTTG        |

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IgG (RRID: AB_772206, NA934; GE Healthcare, Little Chalfont, England). The protein bands were detected by Chemi-Lumi One Ultra Western blotting detection reagent (Nacalai Tesque).

Serum Biochemical Analysis

Serum levels of glucose, lipids, and hormones were measured using appropriate equipment, reagents, and kits. The GLUCOMETER G meter was used to measure glucose content (Arkray, Kyoto, Japan). The NEFA C-Test Wako (Wako Pure Chemical Industries, Osaka, Japan) was used to measure free fatty acids levels. Triglyceride E-Test Wako (Wako Pure Chemical Industries) was used to measure triglyceride levels, and the Cholesterol E-Test Wako (Wako Pure Chemical Industries) was used to assess cholesterol content. The LBIS Insulin Mouse T ELISA Kit (Shibayagi, Gunma, Japan) was used to measure insulin levels. The Leptin ELISA Kit (Morinaga Institute of Biological Science, Yokohama, Japan) was used to measure leptin levels.

Indirect Calorimetry and Locomotor Activity

Ten to twelve days after overexpression of Npgl when there were no clear differences in body mass between control and Npgl-overexpressing mice fed NC, indirect calorimetry was conducted to whole body energy consumption using an O₂/CO₂ metabolism measurement system for small animals (MK-5000RQ; Muromachi Industries, Tokyo, Japan). This system monitored VO₂ (mL/min) and VO₂ (mL/kg/h) × [3.815 + (1.232 × RQ)] [26].

Statistical Analysis

Student’s t test was performed for assessment of 2-group differences between AAV-NPGL and AAV-CTL injected animals. To assess the effects of Npgl overexpression in mice fed 2 diets, one-way ANOVA with Tukey’s test for multiple comparisons was conducted. Two-way ANOVA with repeated measures followed by Bonferroni’s test for multiple comparisons was used to evaluate the main effects of Npgl overexpression and time and the effects of interactions between main factors. If significant effects of interactions between main factors were observed, the results of Bonferroni’s test of the main effects of Npgl overexpression were shown in addition to one-way ANOVA with Tukey’s test for multiple comparisons in the same period. p values < 0.05 were considered significant.

Results

Effects of NPGL-Precursor Gene Overexpression on Food Intake, Body Mass, and Food Efficiency

AAV-induced overexpression in the mediobasal hypothalamus was confirmed by quantitative RT-PCR (ANOVA, F, 28 = 30.0, p < 0.005, Tukey, p < 0.005) (online suppl. Fig. 1a; for all online suppl. material, see www.karger.com/doi/10.1159/000518969), Western blot (online suppl. Fig. 1b), and immunohistochemistry (online suppl. Fig. 1c). As colchicine treatment is necessary for the detection of endogenous NPGL in cell bodies of the brain [15], NPGL-immunoreactive cells were not found in AAV control sections from animals without colchicine treatment (online suppl. Fig. 1c).

Effects of chronic (9 weeks) Npgl overexpression in the hypothalamus on cumulative food intake and body mass are presented in Figure 1. Two-way ANOVA by repeated measures revealed the main effects of time (ANOVA, F, 27, 252 = 4,619.7, p < 0.005), group (ANOVA, F, 3, 28 = 9.73, p < 0.005), and a time × group interaction (ANOVA, F, 27, 252 = 11.65, p < 0.005) on food intake (Fig. 1a). Bonferroni’s test revealed that food intake was augmented by Npgl overexpression in mice fed NC (Bonferroni, p < 0.005) and those fed a HCD (Bonferroni, p < 0.005) (Fig. 1a). One-way ANOVA with Tukey’s multiple comparisons showed that food intake was increased by Npgl overexpression in mice fed NC (ANOVA, F, 3, 28 = 11.66, p < 0.005, Tukey, p < 0.005) and was a trend for increase in mice fed a HCD (Tukey, p = 0.05) (Fig. 1b). The main effects of time (ANOVA, F, 9, 252 = 427.0, p < 0.005), group (ANOVA, F, 3, 28 = 62.35, p < 0.005), and a time × group interaction (ANOVA, F, 27, 252 = 38.70, p < 0.005) were significant on body mass by two-way repeated measures ANOVA (Fig. 1c). Multiple comparisons by Bonferroni’s test showed that body mass was augmented by Npgl overexpression in mice fed NC (Bonferroni, p < 0.005) and those fed a HCD (Bonferroni, p < 0.005) (Fig. 1c). One-way ANOVA with multiple comparisons revealed that body mass also was increased by Npgl overexpression in mice fed NC (ANOVA, F, 3, 28 = 58.2, p < 0.005, Tukey, p < 0.005) and those fed a HCD (Tukey, p < 0.005) (Fig. 1d).

To determine whether the increase in body mass was caused by food intake, we next calculated food efficiency, which is a measure of how much mass is gained per unit of food intake [20]. Two-way ANOVA by repeated measures revealed the main effects of time (ANOVA, F, 9, 252 = 50.43, p < 0.005), group (ANOVA, F, 3, 28 = 27.37, p < 0.005), and a time × group interaction (ANOVA, F, 27, 252 = 5.09, p < 0.005) on food efficiency (Fig. 1e). Bonferroni’s test showed that food efficiency was enhanced by Npgl overexpression in mice fed NC (Bonferroni, p < 0.005) and those fed a HCD (Bonferroni, p < 0.005) (Fig. 1e). Food efficiency was markedly increased by Npgl overexpression in mice fed NC (ANOVA, F, 3, 28 = 36.35, p < 0.005, Tukey, p < 0.01) and those fed a HCD (Tukey, p < 0.01) at the end of the treatment using one-way ANOVA with multiple comparisons (Fig. 1e). Npgl-overexpressing mice fed a
Fig. 1. Effects of Npgl overexpression on food intake, body mass, and food efficiency. The panels show the data obtained by injection of the AAV-CTL or the AAV-NPGL in mice fed NC or a HCD for 9 weeks. a Cumulative food intake at all points. b Cumulative food intake at 9 weeks after injection. c Body mass at all points. d Body mass 9 weeks after injection. e Food efficiency is expressed as body weight gain per cumulative food intake per week. f Representative photograph of mice at 8 weeks after injection of the AAV-CTL or the AAV-NPGL under a HCD. Each value represents the mean ± standard error of the mean (n = 8/group). *p < 0.05, **p < 0.01, ***p < 0.005 AAV-CTL (NC) versus AAV-NPGL (NC) in the same period by one-way ANOVA with Tukey’s test for multiple comparisons, †p < 0.05, ††p < 0.005 AAV-CTL (NC) versus AAV-NPGL (NC) by two-way ANOVA by repeated measures with Bonferroni’s test for multiple comparisons, #p < 0.05, ###p < 0.005 AAV-CTL (HCD) versus AAV-NPGL (HCD) in the same period by one-way ANOVA with Tukey’s test for multiple comparisons, †p < 0.05, †††p < 0.005 AAV-CTL (HCD) versus AAV-NPGL (HCD) by two-way ANOVA by repeated measures with Bonferroni’s test for multiple comparisons. NPGL, neurosecretory protein GL; AAV-CTL, AAV-based control vector; AAV-NPGL, AAV-based NPGL-precursor gene vector; NC, normal chow; HCD, high-calorie diet.
HCD were visibly obese, as compared to the AAV-based control vector-injected mice (Fig. 1f).

**Effects of NPGL-Precursor Gene Overexpression on Body Composition and Serum Parameters**

To address the cause of Npgl overexpression-induced body mass increase, we measured the masses of adipose tissues, muscle, and several organs (Fig. 2, 3). In mice fed NC, the masses of WAT and interscapular brown adipose tissue (BAT) were significantly higher in Npgl-overexpressing mice relative to control mice (iWAT; ANOVA, $F_{3, 28} = 71.62, p < 0.005$, Tukey, $p < 0.005$, epididymal WAT [eWAT]; ANOVA, $F_{3, 28} = 46.34, p < 0.005$, Tukey, $p < 0.005$, retroperitoneal WAT [rWAT]; ANOVA, $F_{3, 28} = 48.76, p < 0.005$, Tukey, $p < 0.005$, BAT; ANOVA, $F_{3, 28} = 15.07, p < 0.005$, Tukey, $p < 0.05$) (Fig. 2a, d). Under a HCD, WAT mass and adipocyte size were considerably increased in Npgl-overexpressing mice (iWAT; ANOVA, $F_{3, 28} = 71.62, p < 0.005$, Tukey, $p < 0.005$, eWAT; ANOVA, $F_{3, 28} = 46.34, p < 0.005$, Tukey, $p < 0.01$, rWAT; ANOVA, $F_{3, 28} = 48.76, p < 0.005$, Tukey, $p < 0.005$) (Fig. 2a–c), whereas there was no difference in the BAT mass in Npgl-overexpressing mice (Fig. 2d).

While the mass of WAT increased markedly, the mass of the gastrocnemius muscle was not different in Npgl-overexpressing mice in either diet condition (Fig. 3a). In mice fed NC, the masses of WAT and interscapular brown adipose tissue (BAT) were significantly higher in Npgl-overexpressing mice relative to control mice (iWAT; ANOVA, $F_{3, 28} = 71.62, p < 0.005$, Tukey, $p < 0.005$, epididymal WAT [eWAT]; ANOVA, $F_{3, 28} = 46.34, p < 0.005$, Tukey, $p < 0.005$, retroperitoneal WAT [rWAT]; ANOVA, $F_{3, 28} = 48.76, p < 0.005$, Tukey, $p < 0.005$, BAT; ANOVA, $F_{3, 28} = 15.07, p < 0.005$, Tukey, $p < 0.05$) (Fig. 2a, d). Under a HCD, WAT mass and adipocyte size were considerably increased in Npgl-overexpressing mice (iWAT; ANOVA, $F_{3, 28} = 71.62, p < 0.005$, Tukey, $p < 0.005$, eWAT; ANOVA, $F_{3, 28} = 46.34, p < 0.005$, Tukey, $p < 0.01$, rWAT; ANOVA, $F_{3, 28} = 48.76, p < 0.005$, Tukey, $p < 0.005$) (Fig. 2a–c), whereas there was no difference in the BAT mass in Npgl-overexpressing mice (Fig. 2d).

While the mass of WAT increased markedly, the mass of the gastrocnemius muscle was not different in Npgl-overexpressing mice in either diet condition (Fig. 3a). With regard to peripheral organs, the mass of the liver was increased relative to controls by Npgl overexpression under a HCD feeding conditions (ANOVA, $F_{3, 28} = 18.32, p < 0.005$, Tukey, $p < 0.005$) (Fig. 3b). In addition, Npgl overexpression induced a pronounced whitening of the liver in mice fed a HCD (Fig. 3c). To explore the underlying cause for the whitening of the liver, we performed a histological examination of lipid deposition in the liver. Oil Red O staining revealed that lipid droplets were in-
increased in the liver in mice with Npgl overexpression (Fig. 3c). The masses of the testis and heart were not changed by Npgl overexpression (Fig. 3b). The mass of the kidney increased in Npgl-overexpressing mice fed NC (ANOVA, F3, 28 = 4.38, p < 0.05, Tukey, p < 0.05), whereas there was no effect when these mice were fed a HCD (Fig. 3b).

Fat accumulation can cause hyperglycemia and hyperlipidemia [5]. To reveal the effects of Npgl overexpression on blood parameters, we measured serum levels of glucose, hormones, and lipids. Npgl overexpression increased serum levels of insulin and cholesterol under a HCD feeding conditions (insulin; ANOVA, F3, 27 = 25.71, p < 0.005, Tukey, p < 0.005, cholesterol; ANOVA, F3, 27 = 53.09, p < 0.005, Tukey, p < 0.05), whereas serum levels of glucose, leptin, and free fatty acids were unaffected (Fig. 4a–c, e, f). Moreover, there was a trend for increasing the serum triglyceride level by Npgl overexpression in mice fed NC (ANOVA, F3, 27 = 3.69, p < 0.05, Tukey, p = 0.09) (Fig. 4d).

**Fig. 3.** Effects of Npgl overexpression on the muscle and organs. The panels show the data obtained by injection of the AAV-CTL or the AAV-NPGL in mice fed NC or a HCD for 9 weeks. **a** Mass of the gastrocnemius muscle. **b** Mass of the testis, liver, kidney, and heart. **c** Representative photograph of the liver and liver sections stained by Oil Red O in mice fed a HCD. Scale bars, 1 cm in the top and 100 μm in the bottom. Each value represents the mean ± standard error of the mean (n = 8). Difference between groups was assessed by one-way ANOVA with Tukey’s test for multiple comparisons. *p < 0.05 AAV-CTL (NC) versus AAV-NPGL (NC), †††p < 0.005 AAV-CTL (HCD) versus AAV-NPGL (HCD). NPGL, neurosecretory protein GL; AAV-CTL, AAV-based control vector; AAV-NPGL, AAV-based NPGL-precursor gene vector; NC, normal chow; HCD, high-calorie diet.

**Effects of NPGL-Precursor Gene Overexpression on mRNA Expression and Endogenous Activity of Lipid Metabolism-Related Genes**

We detected fat accumulation in the WAT and liver as a result of Npgl overexpression, so we analyzed the mRNA expression of lipid metabolism-related genes by quantitative RT-PCR in the iWAT and the livers of mice fed NC or a HCD. The following genes were analyzed: acetyl-CoA carboxylase (Acc), fatty acid synthase (Fas), Scd1, and glycerol-3-phosphate acyltransferase 1 (Gpat1) as genes encoding lipogenic enzymes; carbohydrate-responsive element-binding protein α (Chrebpα) as a lipogenic transcription factor; carnitine palmitoyltransferase 1α (Cpt1a), adipose triglyceride lipase (Atgl), hormone-sensitive lipase (Hsl), and fibroblast growth factor 21 (Fgf21) as genes encoding lipolytic enzymes; glyceraldehyde 3-phosphate dehydrogenase (Gapdh) as a carbohydrate metabolism enzyme; solute carrier family 2 member 4 and 2 (Slc2a4 and Slc2a2) as glucose transporters; cluster of differentiation 36 (Cd36) as a fatty acid transporter; peroxisome proliferator-activated receptor α (Ppara) and γ (Pparγ) as genes encoding lipid-activated transcription factors; and peroxisome proliferator-acti-
To confirm the activity of lipogenic factors in the iWAT, we measured the fatty acid ratio using gas chromatography-mass spectroscopy. The fatty acid ratios of palmitoleate-to-palmitate (16:1/16:0) and of oleate-to-palmitate (18:1/16:0) were increased by Npgl overexpression (ANOVA, F3, 28 = 23.16, p < 0.005, Tukey, p < 0.05, Fgf21; ANOVA, F3, 28 = 37.81, p < 0.005, Tukey, p < 0.01, Cd36; ANOVA, F3, 28 = 18.59, p < 0.005, Tukey, p < 0.005). In contrast, mRNA expression levels of Chrebpα and Pparγ were decreased (Chrebpα; ANOVA, F3, 28 = 10.69, p < 0.005, Tukey, p < 0.01, Pparγ; ANOVA, F3, 28 = 7.17, p < 0.005, Tukey, p < 0.01) (Fig. 5a).

Furthermore, there was a trend for increasing mRNA expression level of Gapdh by Npgl overexpression under the same diet (ANOVA, F3, 28 = 3.28, p < 0.05, Tukey, p = 0.08) (Fig. 5a). Under a HCD with Npgl overexpression, mRNA expression level of Cpt1a was upregulated relative to controls, similar to the observations in mice fed NC with Npgl overexpression (ANOVA, F3, 28 = 15.01, p < 0.005, Tukey, p < 0.05) (Fig. 5a).
A Novel Mouse Model of Neurosecretory Protein GL-Induced Obesity

Fig. 5. Effects of Npgl overexpression on the mRNA expression of lipid metabolism-related genes. The panels show the data obtained by injection of the AAV-CTL or the AAV-NPGL in mice fed NC or a HCD for 9 weeks. a mRNA expression levels in the iWAT. b mRNA expression levels in the liver. Data were normalized using internal control (ribosomal protein S18 for the iWAT or β-actin for the liver) and expressed relative to the mRNA expression of the AAV-CTL in mice fed NC. Each value represents the mean ± standard error of the mean (n = 8). Difference between groups was assessed by one-way ANOVA with Tukey’s test for multiple comparisons. *p < 0.05, **p < 0.01, ***p < 0.005 AAV-CTL (NC) versus AAV-NPGL (NC), †p < 0.05, ††p < 0.01, †††p < 0.005 AAV-CTL (HCD) versus AAV-NPGL (HCD). NPGL, neurosecretory protein GL; AAV-CTL, AAV-based control vector; AAV-NPGL, AAV-based NPGL-precursor gene vector; NC, normal chow; HCD, high-calorie diet; WAT, white adipose tissue; iWAT, inguinal WAT.

stearate (18:1/18:0) indicate increased enzymatic activity of SCD1 [23]. The ratio 16:0/18:2n-6 is an index of de novo lipogenesis [24, 25]. The fatty acid ratio of 16:1/16:0 was significantly increased in the iWAT of Npgl-overexpressing mice fed NC (ANOVA, $F_{3, 28} = 5.3$, $p < 0.01$, Tukey, $p < 0.005$), although the 18:1/18:0 and 16:0/18:2n-6 ratios remained unchanged (Fig. 6a–c). This result indicates activation of SCD1 by Npgl overexpression in the iWAT under NC condition. Under the HCD condition, these ratios did not change in the iWAT upon Npgl overexpression (Fig. 6a–c).

Effects of NPGL-Precursor Gene Overexpression on Respiratory Metabolism and Locomotor Activity

To analyze the effects of Npgl overexpression on whole body respiratory metabolism, we measured the VO$_2$ and VCO$_2$ 10–12 days after injection of AAV-based vectors in mice fed NC. VO$_2$ and VCO$_2$ during the dark period were decreased by Npgl overexpression (Student’s $t$ test, VO$_2$: df = 9, $t = 2.28$, $p < 0.05$, VCO$_2$: df = 9, $t = 2.41$, $p < 0.05$) (Fig. 7a, b). As a result of the changes, overall energy expenditure during the dark period was also decreased by Npgl overexpression (df = 9, $t = 2.34$, $p < 0.05$) (Fig. 7c).
In contrast, there was no significant effect on locomotor activity by Npgl overexpression (Fig. 7d).

**Discussion**

We have demonstrated that NPGL is involved in energy homeostasis in vertebrates, including birds and rodents [13–16, 27, 28]. In particular, we recently showed that Npgl overexpression modestly stimulated feeding behavior and fat accumulation without notable body mass gain in male Wistar rats [14]. However, whether NPGL causes obesity across species has not been clearly elucidated. The efficacy of Npgl overexpression in the medio-basal hypothalamus was very similar in mice and rats in the present and previous studies (online suppl. Fig. 1 and [14]). In the present study, we found that Npgl overexpression in male C57BL/6j mice induced a remarkable increase in food intake, body mass, and fat mass under both NC and HCD conditions (Fig. 8). Therefore, these data indicate that NPGL stimulates fat accumulation in WAT very rapidly, resulting in obesity within 8 weeks in male C57BL/6j mice (Fig. 8). Furthermore, this is the first report that Npgl overexpression caused the induction of fatty liver under HCD in addition to fat deposition in the WAT (Fig. 8). We feel that these data are novel and highlight the importance of comparing different rodent strains/mammalian species.

The present study revealed that food efficiency was increased by Npgl overexpression (Fig. 1e). This result suggests that NPGL-induced fat accumulation is caused not only by energy intake but also by other factors. Indeed, this study showed that Npgl overexpression reduced energy expenditure 10–12 days after overexpression when there were no apparent differences in body mass in mice (Fig. 7c). It is known that energy expenditure is under the control of sympathetic nerves. Leptin increases energy expenditure by activation of thermogenesis in the BAT [29]. Our previous study has found that NPGL inhibits the activity of sympathetic nerves in the BAT (unpublished data). Together, our findings may support the hypothesis that NPGL reduces energy consumption through the suppression of sympathetic nerves, resulting in fat accumulation. On the other hand, Npgl overexpression increased food intake, although serum insulin – which has anorexic action – was also elevated in mice under HCD conditions (Fig. 1a, b, 4b). It has been reported that NPY, which is an orexigenic peptide like NPGL, regulates insulin secretion in rodents [30]. Therefore, the present study suggests that NPGL, like NPY, simultaneously elicits food...
intake and secretion of insulin. The present study also showed steady-state levels of blood glucose even in hyperinsulinemia and obesity. Considering our recent report that NPGL mitigates insulin resistance attributed to a high-fat diet [31], NPGL may control the sensitivity of insulin. Further studies are required to reveal the regulation of insulin resistance via NPGL.

Quantitative RT-PCR showed that Npgl overexpression increased mRNA expression of factors involved in lipid metabolism in iWAT, especially under the NC condition (Fig. 5a). This result is not surprising as previous findings establish that lipid metabolism is responsive to nutritional conditions [32]. Docosahexaenoic acid, one of the polyunsaturated fatty acids, upregulates lipid metabolic factors, including Hsl and Pparγ [33]. Specifically, Hsl and Pparγ mRNA expressions were upregulated by Npgl overexpression in the iWAT of mice fed NC, but were unchanged under the HCD condition (Fig. 5a). Therefore, our study suggests that intake of HCD may mitigate the effects of NPGL on lipid metabolism. In addition, Npgl overexpression upregulated mRNA expression for factors involved in both lipogenesis and lipolysis, also activating SCD1 (Fig. 5a, 6). Lipolysis is regulated by several factors, including ATGL and HSL [34]. In particular, HSL is thought to be the rate-limiting enzyme of lipolysis, and the enzyme is activated by phosphorylation [35, 36]. In DIO mice, HSL protein levels were highly similar to those in control lean mice, although the level of HSL phosphorylation was significantly decreased [37]. Therefore, it is possible that HSL might not be activated by phosphorylation despite the abundance of Hsl mRNA. Conversely, the upregulation of mRNA expression related to lipolysis might be a result of feedback control due to fat accumulation. The evaluation of the expression of...
proteins that regulates lipid metabolism is needed to elucidate the effects of NPGL on lipolysis and lipogenesis in these mice.

Npgl overexpression increased liver mass in mice under HCD conditions (Fig. 3b). A previous study reported that elevated insulin stimulates fat accumulation in the liver [38]. In agreement with this finding, we observed increases in serum insulin upon Npgl overexpression, as described above (Fig. 4b). Therefore, the present study suggests that NPGL triggers fat accumulation in the liver by the secretion of insulin. In addition, a previous report revealed that fatty liver leads to hyperlipidemia, including hypercholesteremia and hypertriglyceridemia [39]. It is possible that the fatty liver in mice fed HCD was induced by hyperinsulinemia, resulting in hyperlipidemia (Fig. 4b, f). Fat accumulation in the liver without excessive alcohol consumption is defined as nonalcoholic fatty liver disease [40]. Most patients with nonalcoholic fatty liver disease only exhibit simple steatosis [40]. Based on our data in mice, NPGl may also induce simple steatosis rather than nonalcoholic steatohepatitis. Further investigations are needed to clarify the effects of NPGL on steatosis in the liver.

Although the present study revealed the effects of Npgl overexpression on energy metabolism, this study has several limitations. First, we need to reveal the pathway of lipid metabolism via NPGL in the central and peripheral regions. A previous study reported that NPGL may elicit food intake through neurons in the Arc and the ventromedial hypothalamus [15]. In addition, the expression of Npgl mRNA is downregulated in the hypothalamus of DIO mice [15]. However, the lipid metabolic control of NPGL and its receptor remain unknown. To uncover the mechanism of action of NPGL on lipid metabolism, the analysis of loss of function (i.e., knockdown/knockout), elucidation of the neuronal network of NPGL neurons, and identification of the receptor for NPGL are required. Moreover, recent studies indicate that the sex of mice might interfere with the progress of obesity [41]. In the present study, we reported that Npgl overexpression induces obesity only in male mice. We subsequently plan to conduct analysis using female mice to elucidate whether NPGL induces obesity in both sexes.

In conclusion, Npgl overexpression in mice affects feeding behavior, fat accumulation, energy expenditure, and secretion of insulin, rapidly resulting in obesity observable within 2 months. In our previous study, 13-day chronic i.c.v. infusion of NPGL did not lead to obesity in mice fed a HCD due to the chemical instability of NPGL in the osmotic pumps [16]. Hence, different approaches to research on NPGL will contribute to the understanding of energy metabolism regulation.

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Statement of Ethics

All animal experiments were performed according to the Guide for the Care and Use of Laboratory Animals prepared by Hiroshima University (Higashi-Hiroshima, Japan), and these procedures were approved by the Institutional Animal Care and Use Committee of Hiroshima University (permit numbers: C13-12, C13-17, and C21-1-2).

Conflict of Interest Statement

The authors declare that no competing interests exist.

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Author Contributions

Y.N. and K.U. contributed to conceptualization; Y.N., E.I.-U., K.F., K.S., and M.F. contributed to methodology; Y.N., E.I.-U., K.F., K.S., M.F., M.M., G.E.B., L.J.K., and K.U. contributed to investigation; Y.N. and K.F. contributed to writing and original draft preparation; N., K.F., G.E.B., L.J.K., and K.U. contributed to writing, review, and editing; Y.N. contributed to visualization; K.U. contributed to project administration; and funding was acquired by E.I.-U., K.F., and K.U. All authors have read and agreed to the published version of the manuscript.

Data Availability Statement

The raw data supporting the findings of this study will be made available by the corresponding author, K.U., to any qualified researchers upon reasonable request.

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