

NOVEL PHYSIOLOGICAL SUBSTANCE NESFATIN, SUBSTANCE
RELEVANT THERETO, AND USE OF THE SUBSTANCES

5 FIELD OF THE INVENTION

The present invention relates to a novel method of obtaining a factor that is involved in food intake control and/or body weight control. The present invention also relates to a polypeptide obtained by said method, said polypeptide being involved in food intake control and/or body weight control, a nucleic acid molecule encoding said polypeptide, as well as a means for treating, preventing and diagnosing diseases associated with the suppression of food intake and/or the suppression of body weight gain using them. The present invention also relates to a means for treating, preventing and diagnosing diseases associated with enhanced appetite and/or enhanced body weight gain using a substance that inhibits the effect of said polypeptide or said gene. Furthermore, the present invention relates to an animal model of diseases associated with food intake control and/or body weight control obtained by said polypeptide, said gene, and a substance that inhibits activities thereof, and a method of screening compounds that controls the effect or expression of said polypeptide using the model. The present invention also relates to a compound that is selected by said screening method, and a diagnostic means and therapeutic agents for diseases using said compound.

30 BACKGROUND OF THE INVENTION

Obesity is a condition in which body weight, especially white adipose tissue, occurs in excess, and is generally classified as having a body mass index (BMI) of $\geq 25 \text{ kg/m}^2$ and as having a body fat percentage of 25% or greater for male adults and 30% or greater for female adults. Due to habits of eating high-fat diets and a lack of exercises in modern society, the percentage of

people classified as obese is on the rise. According to the result of the national nutrition survey by the Ministry of Health, Labor and Welfare in 2000, the number of men who are diagnosed as obese have certainly
5 increased during the last 10 and 20 years and about 30% of men from 40-69 years old are diagnosed as obese, and for women as well, about 30% of those 60-69 years old are diagnosed as obese.

10 In the past, obesity was viewed as an aesthetic problem, but today, rather than obesity per se, health impairments accompanied (or potentially accompanied) by obesity pose major clinical challenges, providing a medical grounds for the prevention and treatment of obesity. Under such circumstances, the Japan Society for
15 the Study of Obesity (JASSO) has defined obesity as "a pathological condition that is accompanied or suspected of being accompanied by health impairments resulting from or associated with obesity and that requires medical reduction in body weight", and proposes to deal with it
20 as a disease entity. The health impairments as used herein include type 2 diabetes and impaired glucose tolerance as well as hypertension, hyperlipidemia, hyperuricemia, fatty liver, cardiac and cerebral vascular diseases, sleep apnea syndrome, orthopedic diseases such
25 as osteoarthritis, menstrual disorders and the like (The Japanese Journal of Clinical Medicine (Nippon Rinsho), Supplement "Adiposis", issued by Nippon Rinshosha Co., Ltd. on July 28, 2003). As diseases resulting from obesity, malignant tumors are mentioned, and
30 specifically, obesity has been reported to be a risk factor for the onset of breast cancer, uterine cancer, colon cancer, kidney cancer, esophageal cancer, pancreatic cancer, liver cancer, and gallbladder cancer (The Japanese Journal of Clinical Medicine (Nippon
35 Rinsho), Supplement "Adiposis", issued by Nippon Rinshosha Co., Ltd. on July 28, 2003; Non-patent document 1, Abu-Abid et al., Journal of Medicine (USA), January 1,

2002, Vol. 33, Nos. 1-4, pp. 73-86; and Nair et al.,
Hepatology (USA), July 1, 2002, Vol. 36, No. 1, pp. 150-
155). In recent years, furthermore, there has been
5 of arteriosclerotic diseases (myocardial infarction,
cerebral infarction etc.) called "metabolic syndrome",
which is attracting attention since cerebral vascular
diseases and cardiovascular diseases account for 30% of
all deaths in Japan. Therefore, the Japan Society for
10 the Study of Obesity, the Japan Atherosclerosis Society,
the Japan Diabetes Society, the Japanese Society of
Hypertension, the Japanese Circulation Society, the
Japanese Society of Nephrology, the Japanese Society on
Thrombosis and Hemostasis, the Japanese Society of
15 Internal Medicine collaborated to draw up its diagnostic
criteria and announced the criteria at the press
conference of the Meeting of the Japanese Society of
Internal Medicine on April 8, 2005. According to the
criteria, with visceral fat (fat accumulation in internal
20 organs) set at the center of the criteria, men with a
waist circumference of 85 cm or greater and women with a
waist circumference of 90 cm or greater who have two or
more risks of serum lipid abnormality (either one or both
of a triglyceride value of 150 mg/dL or more and a HDL
25 cholesterol value of 40 mg/dL or less), high blood
pressure value (either one or both of a systolic pressure
of 130 mmHg or more and a diastolic pressure of 85 mmHg)
and high blood glucose (a fasting blood glucose level of
110 mg/dL or more) are diagnosed as having the metabolic
30 syndrome (Journal of the Japanese Society of Internal
Medicine, A research committee on the diagnostic criteria
for metabolic syndrome, April, 2005 issue, Vol. 94, pp.
188-203). When this criteria was used, it is reported,
among 290 male adults who were undergoing health
35 screening, 61 people (21%) were diagnosed as obese,
whereas 27 people (9%) were diagnosed as having the
metabolic syndrome, and 9 people (3%) were not included

in obesity but were diagnosed as having the metabolic syndrome (Igaku no Ayumi, Kazuo Takahashi and Yasushi Saito, 2005, Vol. 213, No. 6, pp. 549-554).

5 Since the possible cause of obesity is essentially
the persistent excess of energy (calorie) taken in over
energy (calorie) consumed, it is recommended that obese
people or people with obesity undergo the diet therapy
and/or the exercise therapy in order to lower body
weight, especially body fat percentage. However, since
10 the continuance of these therapies poses considerable
stress on an enhanced appetite, adaptation to changes in
life styles, and exercise tolerance, various difficulties
must be overcome to continue the therapies. It is likely
that when the calorie intake was decreased in the diet
15 therapy, the so-called rebound phenomenon, i.e., that the
intestinal absorption of nutrients increases and energy
metabolism lowers, may occur, and therefore the
continuance of the diet therapy may be abandoned. Though
medical treatments of obesity include central anorectic
20 drugs, agents promoting thermal metabolism, absorption-
inhibiting agents, steatogenesis-inhibiting agents, etc.,
the only agent that can be used under the health
insurance system in Japan at present is mazindol which is
classified as a central anorexigenic drug. However,
25 mazindol is a stimulant-like compound, and has side
effects of excitation, irritation, cardiovascular load,
dysuria etc., and the period of use has been limited to
within 3 months, and thus it is not considered a drug
that can be easily used (Novartis Pharma KK, "Sanorex
30 0.5mg tablet", package insert).

Excessive reduction in body weight (so-called
"emaciation") or food intake (so-called "anorexia")
concerning obesity is problematic since it can cause
infection due to a reduced defense reaction (immunity),
35 hematopoietic disorders, amenorrhea or irregular
menstruation, infertility, mental disorders, peripheral
nerve paralysis, hypotension, osteoporosis etc.

Generally, when BMI is $<18.5 \text{ Kg/m}^2$, or men with a body fat percentage of 10% or less and women with a body fat percentage of 15% or less are classified as emaciated. According to the result of a national nutrition survey by the Ministry of Health, Labor and Welfare in 2000, the percentage of women with BMI of $<18.5 \text{ Kg/m}^2$ in the 20-39 year-old bracket has risen steadily in the past 10 and 20 years, and in the 20-29 year-old bracket about 24% are classified as "emaciated". This is possibly caused by intentional reduction in food intake by young women due to excessive concern over weight. However, in anorexia nervosa (food refusal), one of the central food intake disorders prevalent among this age group, appetite per se extremely decreases and hence the nutritional condition aggravates, sometimes leading to death due to general prostration. Also, as appetite-lowering diseases that include concepts formerly called gastroparesis, gastroparesis, or neurogenic gastritis, there is a disease termed functional dyspepsia, which is said to exhibit symptoms of early satiety after meals and reduced appetite etc. (Talley et al., Gut 1999, 45, Suppl. 2:1137-42). Furthermore, as causes of anorexia, there can be mentioned cancer, inflammatory diseases, reduced function of the pituitary, the thyroid, or the adrenal etc., post-surgery, excessive stress and the like, and persistent anorexia for a long time under these conditions may cause body weakening.

Under these circumstances, in recent years, vigorous research has been undertaken on biological factors that control food intake and also on the relationships of factors such as leptin, adiponectin and ghrelin on the control of food intake. At present, however, no conclusions have been made on factors that play a leading role in food intake control and/or body weight control, and no factors such as those described above have yet been used in therapies. Thus, there is a strong need for identifying factors that play a leading role in the

control of food intake and/or the control of body weight and for applying them into the treatment of obesity and adipogenesis. However, few factors have been reported to be involved in food intake control and/or body weight control and for the PPAR γ agonist widely used as a therapeutic agent for diabetes mellitus, no direct involvement in food intake control and/or body weight control has been reported.

On the other hand, nuclear EF-hand acidic (NEFA) is also called nucleobindin II (NUCB 2), and a polypeptide encoded by the NEFA gene has a calcium-binding domain (EF domain) and a DNA-binding domain (Biol Chem Hoppe Seyler 1994, Aug; 375(8):497-512). NEFA has a high homology with nucleobindin and is considered to be a member of the DNA-binding factor called the EF-hand superfamily having a reactivity with calcium (Karabinos et al., Mol Biol Evol 1996 Sep; 13(7):990-8). Though NEFA is being investigated regarding its calcium-binding ability, its binding with necdin, a cellular growth control factor, etc. (Kroll et al., Biochem. Biophys. Res. Commun. 1999, 24, pp. 1-8 and Tanimura et al., J. Biol. Chem. 2000, Oct 13:275(41):31674-81), there are no reports on its detailed functions. NEFA has been studied regarding the possibility of being a causative gene of Usher's syndrome, an ophthalmological disease, and gastric cancer (Doucet et al., Biochim. Biophys. Acta. 1998 July 1; 1407(1):84-91 and Line et al., Br. J. Cancer 2002, June 5:86(11):1824-30). Furthermore, though the possibility of the NEFA polypeptide being extracellularly secreted has been demonstrated because it has a signal sequence at the amino terminal end (Non-patent document 5), there are no reports on the physiological or pharmacological role as a result of extracellular secretion thereof. Also, there are no reports that suggest a relationship between NEFA and food intake control and/or body weight control.

OBJECTS AND SUMMARY OF THE INVENTION

Problems to be solved by the present invention are

to provide a novel method of obtaining a factor that is involved in food intake control and/or body weight control, and to provide a gene obtained by said method, a polypeptide encoded by said gene and a novel polypeptide obtained using information on the polypeptide encoded by said gene as a means for treating, controlling and diagnosing food intake disorders and/or diseases associated with the control of body weight. It is also to provide a substance that inhibits the effect of said gene or polypeptide as a means for treating, controlling and diagnosing diseases associated with food intake control and/or body weight control. Furthermore, it is to provide an animal model of diseases associated with the control of food intake and/or the control of body weight obtained by said gene or polypeptide, or a substance that inhibits them. It is also to provide a method of screening compounds that control the effect or expression of said polypeptide using the model, compounds selected by said screening method, and a diagnostic means and therapeutic agents using said compounds.

After intensive and extensive study to find a novel method of obtaining a factor involved in food intake control and/or body weight control, the present inventors have found that, by using thiazolidine diones having a PPAR γ agonist activity, genes and polypeptides involved in the suppression of food intake and/or the reduction in body weight can be obtained. Furthermore, it was found that the factor obtained by said method was NEFA whose function has not reported, and thus said polypeptide factor was designated as nesfatin. After investigating on nesfatin, the present inventors have found that the partial sequence for which no functional domains were indicated in the previous reports has an activity on the suppression of food intake and/or the reduction in body weight, and therefore have disclosed novel polypeptides nesfatin-1, nesfatin-1M30, nesfatin-1M16, nesfatin-1M14 and nesfatin-1M10. In Nucleobindin I (NUCB1) having a

high homology in the amino acid sequence and the base sequence of the gene with NEFA/nesfatin and belonging to the same family as the NEFA/nesfatin, it was also found, NUCB1-M30 which is a site corresponding to the nesfatin-1M30 of NUCB1 exhibits a similar activity.

Furthermore, it was found that an antibody that binds to nesfatin, nesfatin-1 or nesfatin-1M30 has an activity of enhancing food intake and increasing body weight, and it was confirmed that the inhibition of activity of nesfatin, nesfatin-1 or nesfatin-1M30 is effective for enhancing food intake and increasing body weight.

Thus, the present invention provides the following:

(1) A method of obtaining a factor related to food intake control and/or body weight control, said method comprising the steps of:

acting a thiazolidine dione compound having a PPAR γ agonist activity to a mammalian cell, and

identifying a gene of which expression is induced by said compound;

(2) The method according to (1) wherein said thiazolidine dione compound is troglitazone;

(3) The method according to (1) or (2) wherein said mammalian cell is a non-small cell lung cancer cell line, an adipose cell or a cerebral nerve-derived cell;

(4) The method according to (1), (2) or (3) wherein food intake control and/or body weight control is the suppression of food intake and/or the suppression of body weight gain;

(5) A polypeptide comprising an amino acid sequence set forth in any of SEQ ID NOs: 65-73 or SEQ ID NOs: 107-115;

(6) A polypeptide comprising an amino acid sequence set forth in any of SEQ ID NOs: 39-41 or SEQ ID NOs: 101-103;

(7) A polypeptide comprising an amino acid sequence set forth in SEQ ID NOs: 13-15;

(8) A polypeptide comprising an amino acid sequence set forth in SEQ ID NOs: 3, 6 and 9, said polypeptide having an activity of suppressing food intake and/or suppressing body weight gain;

5 (9) A polypeptide having an activity of suppressing food intake and/or suppressing body weight gain, said polypeptide comprising an amino acid sequence having a homology of at least 60% with any of the amino acid sequence set forth in SEQ ID NOs: 13-15, 39-41, 65-73,
10 101-103 and 107-115; or an amino acid sequence in which some of the amino acids have been deleted, inserted or substituted in an amino acid sequence set forth in any of SEQ ID NOs: 13-15, 39-41, 65-73, 101-103 or 107-115;

 (10) A polypeptide having an activity of suppressing
15 food intake and/or suppressing body weight gain, said polypeptide comprising an amino acid sequence having a homology of at least 60% with any of the amino acid sequence set forth in SEQ ID NOs: 3, 6 and 9; or an amino acid sequence in which some of the amino acids have been
20 deleted, inserted or substituted in an amino acid sequence set forth in SEQ ID NO: 3, 6 or 9; or an amino acid sequence in which some of the amino acids have been deleted, inserted or substituted in any of the amino acid sequence set forth in SEQ ID NOs: 3, 6 and 9, said
25 polypeptide comprising at least one recognition site for a cleaving enzyme contained in a living body in an amino acid sequence corresponding to amino acid numbers 82-162 in SEQ ID NO: 3, 6 or 9;

 (11) The polypeptide according to any of (5) to (10)
30 wherein at least one amino acid has been added to the N terminal or the C terminal;

 (12) The polypeptide according to any of (5) to (10) wherein at least one amino acid residue has been modified by a compound or a peptide;

35 (13) The polypeptide according to any of (5) to (12) wherein said activity of suppressing body weight gain is an activity of suppressing body fat gain;

(14) A nucleic acid molecule encoding a polypeptide set forth in any of (5) to (13);

5 (15) A nucleic acid molecule comprising a base sequence set forth in any of SEQ ID NOs: 74-82 and 116-124;

(16) A nucleic acid molecule comprising a base sequence set forth in any of SEQ ID NOs: 44-46 and 104-106;

10 (17) A nucleic acid molecule comprising a base sequence set forth in SEQ ID NOs: 18-20;

(18) A nucleic acid molecule that comprises a base sequence set forth in SEQ ID NO: 10, 11 or 12 and that encodes a polypeptide having an activity of suppressing food intake and/or suppressing body weight gain;

15 (19) A nucleic acid molecule that hybridizes to a base sequence set forth in SEQ ID NOs: 10-12, 18-20, 44-46, 74-82, 104-106, or 116-124, or a partial sequence thereof under a stringent condition, and that encodes a polypeptide having an activity of suppressing food intake and/or suppressing body weight gain;

20 (20) The nucleic acid molecule according to any of (14) to (19) wherein said activity of suppressing body weight gain is an activity of suppressing body fat gain;

(21) A vector comprising the nucleic acid molecule according to any of (14) to (20);

25 (22) The vector according to (21) to which a nucleic acid molecule is operably linked under the control of a regulatory nucleic acid molecule that controls the expression of said nucleic acid molecule;

30 (23) A transformant comprising the nucleic acid molecule according to any of (14) to (20);

(24) The transformant according to (23) that expresses the transcription product of said nucleic acid molecule;

35 (25) The transformant according to (23) or (24) that expresses the polypeptide encoded by said nucleic acid molecule;

(26) The transformant according to (23), (24) or (25) wherein the transformant is a microorganism;

(27) The transformant according to (26) wherein said microorganism is *Escherichia coli*;

5 (28) The transformant according to (23), (24) or (25) wherein the transformant is a mammalian cell;

(29) The transformant according to (23), (24) or (25) wherein the transformant is a plant cell;

10 (30) A pharmaceutical composition for suppressing food intake and/or suppressing body weight gain, said composition comprising, as an active ingredient, the polypeptide according to any of (5) to (13) or a peptide that contains some of the amino acid sequence of said polypeptide, the vector according to (21) or (22), or the
15 transformant according to any of (23) to (29);

(31) The pharmaceutical composition according to (30) wherein said activity of suppressing body weight gain is an activity of suppressing body fat gain;

20 (32) The pharmaceutical composition according to (30) or (31) for patients with a disease selected from obesity, diabetes mellitus, hypertension, hyperlipidemia, hyperuricemia, fatty liver, cardiac diseases, cerebral vascular diseases, sleep apnea syndrome, orthopedic diseases, menstrual disorders and malignant tumors;

25 (33) The pharmaceutical composition according to (30) or (31) wherein the malignant tumor is any of breast cancer, uterine cancer, colon cancer, kidney cancer, esophageal cancer, pancreatic cancer, liver cancer and gallbladder cancer;

30 (34) The pharmaceutical composition according to any of (30) to (33) comprising a pharmaceutically acceptable additive;

(35) An antibody that binds to any polypeptide according to (5) to (13);

35 (36) The antibody according to (35) that binds to a peptide comprising an amino acid sequence set forth in SEQ ID NO: 24, 32;

(37) A substance that suppresses the activity or production of the polypeptide according to any of (5) to (13);

5 (38) The substance according to (37) that suppresses the activity of said polypeptide by binding to said polypeptide;

(39) The substance according to (37) wherein said substance that suppresses the activity of said polypeptides is the antibody according to (35) or (36);

10 (40) A substance that suppresses the expression of a gene encoding the polypeptide according to (5) to (13);

(41) The gene expression-suppressing substance according to (40) wherein said gene expression-suppressing substance is an antisense oligonucleotide molecule;

15

(42) The gene expression-suppressing substance according to (41) wherein the antisense oligonucleotide molecule comprises a base sequence set forth in SEQ ID NO: 31;

20 (43) The gene expression-suppressing substance according to (40) wherein said gene expression-suppressing substance is a RNAi molecule;

(44) A vector for producing an antisense oligonucleotide molecule or a RNAi molecule, said vector comprising a nucleic acid molecule comprising a base sequence that is complementary to the nucleic acid sequence of the antisense oligonucleotide molecule according to (41) or (42) or of the RNAi molecule according to (43);

25

30 (45) A pharmaceutical composition for enhancing appetite or enhancing body weight gain, said composition comprising the substance according to any of (37) to (43) or the vector according to (44);

(46) The pharmaceutical composition according to (45) containing a pharmaceutically acceptable additive;

35

(47) A transgenic non-human organism comprising the nucleic acid molecule according to any of (14) to (20) or

the vector according to any of (21) to (22);

(48) The transgenic non-human organism according to (47) wherein the nucleic acid molecule according to any of (14) to (20) is expressed;

5 (49) The transgenic non-human organism according to (47) or (48) wherein the transformant according to any of (23) to (28) has been introduced;

(50) The transgenic non-human organism according to (47), (48) or (49) wherein said transgenic non-human
10 organism is a transgenic non-human animal that exhibits the state of suppressed food intake or the state of suppressed body weight gain;

(51) The transgenic non-human organism according to (47), (48) or (49) wherein said transgenic non-human
15 organism is a transgenic plant;

(52) A transgenic non-human animal that has introduced therein the antibody according to (35) or (36), the suppressing substance according to any of (37) to (39), the gene expression-suppressing substance
20 according to any of (40) to (43) or the vector according to (44), and that exhibits the state of enhanced food intake or the state of enhanced body weight gain;

(53) A knock-out non-human animal wherein the entire region or part thereof of a gene comprising a nucleotide
25 sequence set forth in any of SEQ ID NOs: 10-12, 18-20, 44-46, 74-82, 104-106 and 116-124 has been deleted;

(54) The knock-out non-human animal according to (53) that exhibits enhanced appetite or enhanced body weight gain;

30 (55) The non-human animal according to (52), (53) or (54) that can be used as an animal model for a disease selected from obesity, diabetes mellitus, hypertension, hyperlipidemia, hyperuricemia, fatty liver, cardiac diseases, cerebral vascular diseases, sleep apnea
35 syndrome, orthopedic diseases, menstrual disorders and malignant tumors;

(56) A method of producing the peptide according to

any of (5) to (13) by a cell-free protein synthetic method or a chemical synthetic method;

5 (57) The method of producing the peptide according to any of (5) to (13) using the transformant according to any of (23) to (29), the transgenic non-human organism according to any of (47) to (51), or the non-human animal according to any of (52) to (55);

10 (58) The production method according to (56) or (57) comprising a purification process by desorption of said peptide from the antibody according to (35) or (36);

(59) The production method according to (56) or (57) comprising a process wherein said peptide is expressed as a GST-fused protein and then is purified using a glutathione-bound carrier;

15 (60) The production method according to (56) or (57) comprising a process wherein said peptide is expressed as a His tag-fused protein and then is purified using a metal ion chelate carrier;

20 (61) The production method according to (56) or (57) comprising a process wherein said peptide is expressed as a FLAG tag-fused protein and then is purified using an anti-FLAG tag antibody-bound carrier;

25 (62) An assay method of predicting or diagnosing the state of enhanced food intake or enhanced body weight gain comprising the step of detecting the amount contained of a nucleic acid molecule comprising a nucleotide sequence set forth in any of SEQ ID NOs: 10-12, 18-20, 44-46, 74-82, 104-106 and 116-124, or of a polypeptide comprising an amino acid sequence set forth
30 in any of SEQ ID NOs: 3, 6, 9, 13-15, 39-41, 65-73, 101-103 and 107-115 in a biological sample from a mammal;

(63) The assay method according to (62) comprising a step of comparing the amount contained of said nucleic acid molecule or said polypeptide in a biological sample
35 from a mammal with that in a biological sample from a normal individual;

(64) The assay method according to (62) or (63)

comprising a step of judging the state of decreased amount contained of said nucleic acid molecule or said polypeptide in a biological sample from a mammal as a state of enhanced food intake or enhanced body weight gain;

5 (65) The assay method according to any of (62) to (64) comprising the step of judging the state of decreased amount contained of said nucleic acid molecule or said polypeptide in a biological sample from a mammal as a state or a risk of developing a disease selected from obesity, diabetes mellitus, hypertension, hyperlipidemia, hyperuricemia, fatty liver, cardiac diseases, cerebral vascular diseases, sleep apnea syndrome, orthopedic diseases, menstrual disorders and malignant tumors;

15 (66) The assay method according to (62) or (63) comprising the step of judging the state of increased amount contained of said nucleic acid molecule or said polypeptide in a biological sample from a mammal as the state of suppressed food intake or suppressed body weight gain;

(67) The assay method according to any of (62) to (66) wherein the amount contained of said polypeptide is detected using the antibody according to (35) or (36);

25 (68) The assay method according to any of (62) to (66) wherein the amount contained of said nucleic acid molecule is detected using at least one of a PCR primer, a probe or a DNA chip for detecting a nucleic acid molecule comprising a nucleotide sequence set forth in any of SEQ ID NOs: 10-12, 18-20, 44-46, 74-82, 104-106 and 116-124;

30 (69) An assay kit for use in the assay method according to any of (62) to (68), said kit comprising at least one of a PCR primer, a probe or a DNA chip for detecting a nucleic acid molecule comprising a nucleotide sequence set forth in any of SEQ ID NOs: 10-12, 18-20, 44-46, 74-82, 104-106 and 116-124; or an antibody

recognizing a polypeptide comprising an amino acid sequence set forth in SEQ ID NOs: 3, 6, 9, 13-15, 39-41, 65-73, 101-103 or 107-115, a standard peptide, or a modified peptide for the binding competitive reaction;

5 (70) A method of screening a therapeutic or preventive agent having an effect of suppressing food intake and/or suppressing body weight gain, said method comprising the steps of:

10 contacting a test substance with a mammalian cell, and

 detecting the increased expression of a gene in said cell comprising a nucleotide sequence set forth in any of SEQ ID NOs: 10-12, 18-20, 44-46, 74-82, 104-106 and 116-124, or the increased amount of a polypeptide
15 intracellularly contained in said cell or extracellularly secreted comprising an amino acid sequence set forth in any of in SEQ ID NOs: 3, 6, 9, 13-15, 39-41, 65-73, 101-103 and 107-115;

 (71) The screening method according to (70) wherein
20 the mammalian cell is one in which a regulatory nucleic acid molecule that controls the expression of a gene comprising a nucleotide sequence set forth in any of SEQ ID NOs: 10-12, 18-20, 44-46, 74-82, 104-106 and 116-124 and the nucleic acid molecule of a reporter gene have
25 been introduced, and the induced expression of a gene comprising a nucleotide sequence set forth in any of SEQ ID NOs: 10-12, 18-20, 44-46, 74-82, 104-106 and 116-124 is detected by the induced expression of the reporter gene;

30 (72) A method of screening a therapeutic or preventive agent having an effect of suppressing food intake and/or suppressing body weight gain, said method comprising the steps of:

 administering a test substance to a mammal, and

35 detecting the enhanced expression of a gene comprising a nucleotide sequence set forth in any of SEQ ID NOs: 10-12, 18-20, 44-46, 74-82, 104-106 and 116-124

or the enhanced production of a polypeptide comprising an amino acid sequence set forth in any of SEQ ID NOs: 3, 6, 9, 13-15, 39-41, 65-73, 101-103 and 107-115 in a biological sample from said test animal;

5 (73) A method of screening a therapeutic or preventive agent having an effect of suppressing food intake and/or suppressing body weight gain, said method comprising the steps of:

10 administering a test substance to the transgenic non-human organism according to any of (47) to (50) or the non-human animal according to any of (52) to (55), and

15 detecting the suppression of food intake or the suppression of body weight gain in said transgenic non-human organism or said non-human animal;

20 (74) The screening method according to any of (70) to (73) wherein the therapeutic or preventive agent having an effect of suppressing food intake and/or suppressing body weight gain is a therapeutic or preventive agent for a disease selected from obesity, diabetes mellitus, hypertension, hyperlipidemia, hyperuricemia, fatty liver, cardiac diseases, cerebral vascular diseases, sleep apnea syndrome, orthopedic diseases, menstrual disorders and malignant tumors;

25 (75) The therapeutic or preventive agent having an effect of suppressing food intake and/or suppressing body weight gain, said agent being obtained by the method according to any of (70) to (74);

30 (76) The therapeutic or preventive agent according to (75) wherein the therapeutic or preventive agent having an effect of suppressing food intake and/or suppressing body weight gain is a therapeutic or preventive agent for a disease selected from obesity, diabetes mellitus, hypertension, hyperlipidemia, hyperuricemia, fatty liver, cardiac diseases, cerebral vascular diseases, sleep apnea syndrome, orthopedic diseases, menstrual disorders and malignant tumors;

35

(77) A method of screening a therapeutic or preventive agent having an effect of enhancing food intake and/or enhancing body weight gain, said method comprising the steps of:

5 contacting a test substance with a mammalian cell, and

 detecting the decreased expression of a gene comprising a nucleotide sequence set forth in any of SEQ ID NOs: 10-12, 18-20, 44-46, 74-82, 104-106 and 116-124
10 in said cell, or the decreased amount of a polypeptide comprising an amino acid sequence set forth in any of SEQ ID NOs: 3, 6, 9, 13-15, 39-41, 65-73, 101-103 and 107-115 intracellularly contained in said cell or extracellularly secreted;

15 (78) The screening method according to (77) wherein the mammalian cell is one in which a regulatory nucleic acid molecule that controls the expression of a gene comprising a nucleotide sequence set forth in any of SEQ ID NOs: 10-12, 18-20, 44-46, 74-82, 104-106 and 116-124
20 and the nucleic acid molecule of a reporter gene have been introduced, and the suppressed expression of a gene comprising a nucleotide sequence set forth in any of SEQ ID NOs: 10-12, 18-20, 44-46, 74-82, 104-106 and 116-124 is detected by the suppressed expression of the reporter
25 gene;

(79) A method of screening a therapeutic or preventive agent having an effect of enhancing food intake and/or enhancing body weight gain, said method comprising the steps of:

30 administering a test substance to a mammal, and
 detecting the suppressed expression of a gene comprising a nucleotide sequence set forth in any of SEQ ID NOs: 10-12, 18-20, 44-46, 74-82, 104-106 and 116-124 or the suppressed production of a polypeptide comprising
35 an amino acid sequence set forth in any of SEQ ID NOs: 3, 6, 9, 13-15, 39-41, 65-73, 101-103 and 107-115 in a biological sample from said test animal;

(80) A method of screening a therapeutic or preventive agent having an effect of enhancing food intake and/or enhancing body weight gain, said method comprising the steps of:

5 administering a test substance to the transgenic non-human organism according to any of (47) to (50) or the non-human animal according to any of (52) to (55), and

10 detecting the enhancement of food intake or the enhancement of body weight gain in said transgenic non-human organism or said non-human animal; and

(81) The therapeutic or preventive agent having an effect of enhancing food intake and/or enhancing body weight gain, said agent being obtained by the method
15 according to any of (77) to (80).

BRIEF EXPLANATION OF THE DRAWINGS

Fig. 1 is an image of Northern blotting using a NEFA probe, which shows that the expression of the NEFA gene is induced by troglitazone, and that the NEFA gene is
20 constantly expressed in 3T3-L1 cells that were differentiated to precursor adipose cells, in human cerebrospinal blastocytoma cell line HBT185 cells and SQ-5 cells.

A of Fig. 2 is a drawing that shows a schematic diagram of the domain structure of a polypeptide encoded by the NEFA gene and a figure of the sequence of the NAP peptide used for preparing anti-nesfatin antibody. B of
25 Fig. 2 is an image of Western blotting using a polyclonal antibody prepared with the NAP peptide, showing that a polypeptide encoded by the NEFA gene is present in the
30 extract of the rat brain.

Fig. 3 is an image of immunohistochemical staining using a polyclonal antibody against the NAP peptide, showing that the NEFA gene is expressed in sites
35 associated with food intake control such as the arcuate nucleus (Arc), paraventricular nucleus (PVN), the supraoptic nucleus (SON) and the lateral hypothalamic

area (LH) of the hypothalamus of the rat brain.

5 A of Fig. 4 is an image of Western blotting using a polyclonal antibody against the NAP peptide showing the expression and the purification of GST-NEFA which is a bound form of GST and mouse matured nesfatin. In A of Fig. 4, lanes 1-4 represent the rainbow marker, the preinduced bacteria, the post-sonicated pellet and the purified GSTNAP, respectively. Also, B of Fig. 4 is an image of Western blotting using anti-nesfatin antibody
10 showing the process of GST-NEFA being cleaved with thrombin and purified. In B of Fig. 4, lane 1 represents the marker, lane 2 represents the sample before purification, lanes 3-6 represent washed samples, and lane 7 the purified sample.

15 Fig. 5 is a graph showing that the food intake behavior by rats is suppressed by the administration of recombinant nesfatin into the third ventricle of the rat brain. In Fig. 5, * and ** represent a significant difference $P < 0.05$ and $P < 0.01$, respectively, relative to
20 the control group.

Fig. 6 is a graph showing that the food intake behavior by rats is enhanced by the administration of anti-nesfatin antibody into the third ventricle of the rat brain. In Fig. 6, * represents a significant
25 difference $P < 0.001$ relative to the control IgG.

Fig. 7 shows an image of in situ hybridization in the brain tissue, showing that fasting can lower the expression of the nesfatin gene in the rat hypothalamus and re-feeding can restore the expression. In Fig. 7, A
30 represents the control group, B represents the fasting group and C represents the re-feeding group, and the upper figures represent 100-magnified images and the bottom figures represent 400-magnified images.

Fig. 8 shows an image of immunohistochemical stain
35 using anti-nesfatin antibody, showing that fasting can lower the expression of nesfatin in the rat hypothalamus. In Fig. 8, A represents the control group and B

represents the fasting group. It is also an image of immunohistochemical stain (C) using anti-C-Fos antibody, showing that the reduced expression of nesfatin during fasting is due to enhanced appetite. In Fig. 8 the upper figures represent paraventricular nucleus (PVN) and the bottom figures represent arcuate nucleus (Arc).

Fig. 9A is a drawing that shows the amino acid sequences of human and rat nesfatin and the estimated cleavage site for prohormone convertase. ▼ represents the estimated cleavage site for prohormone convertase.

Fig. 9B is a schematic diagram showing the position of peptides in nesfatin for preparing antibody against nesfatin-1, nesfatin-2, nesfatin-3, nesfatin-2/3, as well as nesfatin-1, nesfatin-2/3 and nesfatin-3 thought to be formed by prohormone convertase.

Fig. 9C is a drawing of Western blotting showing that the antibody per se against nesfatin-1 and nesfatin-3 bind to the antigen of interest. In Fig. 9C, the drawing on the left shows the result of an experiment in which the nesfatin-1 peptide was migrated and subjected to Western blotting with nesfatin-1 IgG, and the drawing on the right shows the result of an experiment in which the nesfatin-3 peptide was migrated and subjected to Western blotting with nesfatin C2 IgG.

Fig. 10 is an image of double immunohistochemical stain using anti-nesfatin-1 antibody and anti-PC-1/3 antibody or anti-PC-2 antibody showing the presence of a cell that is simultaneously expressing nesfatin-1 and prohormone convertase (PC-1/3 or PC-2) in the rat brain. The upper panel and the lower panel of A of Fig. 10 represent the stained images with nesfatin-1 IgG in the immunohistochemical image of the rat hypothalamus tissue, and the upper panel in B of Fig. 10 represents a fluorescent image with PC-1/3 and the lower panel in B of Fig. 10 represents a fluorescent image with PC-2.

Fig. 11 is a graph showing that the administration of nesfatin-1 into the third ventricle of a rat

suppresses the food intake behavior of the rat, but the administration of nesfatin-2 or nesfatin-3 does not cause any changes in food intake behavior.

5 Fig. 12 is a graph showing that the continuous administration of nesfatin-1 into the third ventricle of a rat results in persistent suppression in food intake behavior (A) and persistent suppression in body weight gain (B).

10 Fig. 13A is a graph showing that the administration of an antibody against nesfatin-1 into the ventricle of a rat enhances appetite. In Fig. 13A, * and ** represent a significant difference $P < 0.05$ and $P < 0.001$, respectively, relative to the control IgG-administration group.

15 Fig. 13B is a graph showing that the administration of a mutant in which nesfatin-1 cannot be excised from nesfatin into the ventricle of a rat does not cause enhanced appetite relative to nesfatin.

20 Fig. 14 is a graph showing that the continuous administration of antisense RNA against nesfatin into the ventricle of a rat results in suppression in food intake behavior (A) and suppression in body weight gain (B). In Fig. 14, * and ** represent a significant difference $P < 0.05$ and $P < 0.01$, respectively, relative to the missense.

25 Fig. 15 is a graph showing the result of measuring the amount of food intake after the administration of nesfatin-1 into the ventricle of a Lean rat and a Zucker rat which is an animal model of leptin-resistant obesity. In the Zucker rat, as in the Lean rat (normal animal),
30 the suppression of food intake is noted by the intraventricular administration of nesfatin-1. In Fig. 15, * and ** represent a significant difference $P < 0.05$ and $P < 0.001$, respectively, relative to the physiological saline-administration group. White boxes and hatched
35 boxes represent the physiological saline-administration group and the nesfatin-1 administration group, respectively.

Fig. 16 is a graph showing the effect of intraperitoneal nesfatin-1 administration into a mouse on the amount of food intake. It shows that the activity by nesfatin-1 of suppressing food intake can be seen in the intraperitoneal administration into the mouse as well (A). It also shows the result when nesfatin-1 was intraperitoneally administered into an Agouti-yellow mouse which is a mouse model of obesity and the control mouse. It shows that the intraperitoneal administration of nesfatin-1 yields a similar effect in the control mouse (B) and the Agouti-yellow mouse (C) as well.

Fig. 17 is a graph showing the effect of subcutaneous nesfatin-1 administration into a mouse on the amount of food intake. Though nesfatin-1 exhibited an effect of suppressing food intake both by the intraperitoneal administration (ip) and the subcutaneous administration (sc), the development of the effect tended to lag in the subcutaneous administration. In Fig. 17, * and ** represent a significant difference $P < 0.05$ and $P < 0.005$, respectively, relative to the physiological saline-administration group.

Fig. 18A is a graph showing the effect on the amount of food intake by the intraperitoneal administration of nesfatin-1N23, nesfatin-1M30, and nesfatin-1C29 into a mouse. Among the partial peptides of nesfatin-1, nesfatin-1M30 was only shown to exhibit the effect of suppressing food intake. In Fig. 18A, * represents a significant difference $P < 0.02$ relative to the physiological saline-administration group.

Fig. 18B is a drawing that shows the result of amino acid alignment of human, mouse and rat nesfatin-1 and the sites of nesfatin-1N23, nesfatin-1M30 and nesfatin-1C29. It was shown that amino acid sequences are highly conserved between species in the site of nesfatin-1M30.

Fig. 19A-1 is a graph (A-1) showing a standard curve in a competitive EIA system that determines the concentration of nesfatin or nesfatin-1 in a sample, and

is a table (A-2) showing the result of measurement in the cerebrospinal fluid. The equation of the standard curve: $Y=D+(A-D)/1+(\text{Log}(X)/C^B)$. Plot #1 (standard value: concentration value vs measured value). $A=8.4672E-001$;
5 $B=4.3850E+000$; $C=3.5938E+000$; $D=-2.8957E-001$; $R^2=0.9998$.

Fig. 19A-2 is a graph (A-1) showing a standard curve in a competitive EIA system that determines the concentration of nesfatin or nesfatin-1 in a sample and
10 is a table (A-2) showing the result of measurement in the cerebrospinal fluid.

Fig. 19B is a graph (b-1 and b-2) showing the assay result in which peptide samples extracted from the hypothalamus tissue and the cerebrospinal fluid were
15 fractionated by HPLC and nesfatin-1 in the fractions were determined by a competitive EIA system.

Fig. 20 is a graph showing the effect on the amount of food intake by the intraperitoneal administration of partial peptides of nesfatin-1M30 into a mouse. In all cases of nesfatin-1M16M (M16M), nesfatin-1M10M (M10M) or
20 nesfatin-M14 (M14) administered, the effect of suppressing food intake was noted. In Fig. 20, * and ** represent a significant difference $P<0.02$ and $P<0.002$, respectively, relative to the physiological saline-administration group.

Fig. 21A is a graph showing the effect on the amount of food intake by the intraperitoneal administration of human nesfatin-1M30 and mouse NUCB1-M30 into a mouse. It shows that the effect of suppressing food intake can be
25 noted for human nesfatin-1M30 (human/nesfatin-1M30) and mouse NUCB1-M30 (mouse NUCB1) as well as mouse nesfatin-1M30 (mouse/nesfatin-1M30). In Fig. 20, * represents a significant difference $P<0.02$ relative to the
30 physiological saline-administration group.

Fig. 21B is a drawing that shows the result of amino acid alignment of human, rat and mouse nesfatin and
35 human, rat and mouse NUCB1 and the site corresponding to nesfatin-1 and the site corresponding to nesfatin-1M30.

It is shown that amino acid sequences are highly conserved at a site corresponding to nesfatin and nesfatin-1 of NUCB2, specifically nesfatin-1M30.

5 Fig. 21C is a drawing that shows the result of amino acid alignment of human, rat and mouse nesfatin and human, rat and mouse NUCB1 and the site corresponding to nesfatin-1 and the site corresponding to nesfatin-1M30, and represents the continuation of Fig. 21B.

10 Fig. 22 shows an image of in situ hybridization using a NEFA probe in various tissues. A of Fig. 22 represents a tissue section containing paraventricular nucleus (PVN) and supraoptic nucleus (SON), B of Fig. 22 represents a tissue section containing zona incerta (Zi) and arcuate nucleus (Arc), and C of Fig. 22 represents a
15 tissue section containing lateral hypothalamic area (LHA) of the hypothalamus.

A of Fig. 23 is a graph showing that the administration of recombinant nesfatin into the third ventricle of the rat brain can suppress the amount of
20 food intake by the rat. B of Fig. 23 is a drawing that shows the amount of food intake during 0-1 hour, 1-3 hours, 3-6 hours and 6-12 hours for the nesfatin administration group (hatched box) that received 5 pmol of nesfatin into the third ventricle of the rat brain and the control group (0 pmol of nesfatin, white box). In A
25 of Fig. 23, * represents a significant difference $P < 0.01$ relative to 0 pmol, and in B of Fig. 23, * represents a significant difference $P < 0.05$ and ** represents a significant difference $P < 0.01$.

30 A of Fig. 24 shows the result of image analysis by in situ hybridization of the nesfatin mRNA expression at various sites of arcuate nucleus (Arc), paraventricular nucleus (PVN), lateral hypothalamic area (LHA) and supraoptic nucleus (SON) in the hypothalamus region of
35 the rat brain in the feeding ad libitum group (control group) and those in the fasting group. B of Fig. 24 shows the result of image analysis by a competitive EIA

method of the expression of nesfatin-1 peptides at paraventricular nucleus (PVN) among the hypothalamic area of the rats in the feeding ad libitum group (control group: white box) and those in the fasting group (hatched box).

Fig. 25 is a drawing that shows the amount of food intake during 0-1 hour, 1-3 hours, 3-6 hours and 6-12 hours for the nesfatin administration group (hatched box) that received 5 pmol of nesfatin into the third ventricle of the rat brain and the control group (0 pmol of nesfatin, white box). * represents a significant difference $P < 0.01$ relative to 0 pmol.

Fig. 26 is a graph showing the amount of food intake for 1 hour after administration for the control group that received physiological saline alone (nesfatin-1 IgG/nesfatin-1/leptin: -/-/-), the group that received nesfatin-1 alone (nesfatin-1 IgG/nesfatin-1/leptin: -/+/-), the group that received nesfatin-1 and anti-nesfatin-1 antibody (nesfatin-1 IgG/nesfatin-1/leptin: +/+/-), the group that received leptin alone (nesfatin-1 IgG/nesfatin-1/leptin: -/-/+), and the group that received leptin and anti-nesfatin-1 antibody (nesfatin-1 IgG/nesfatin-1/leptin: +/-/+). * represents a significant difference $P < 0.01$ relative to the control group.

A of Fig. 27 shows an image of Western blotting carried out with anti-nesfatin-1 antibody using protein extracts from the rat brain. B of Fig. 27 shows an image of Western blotting at about 47.5 kd carried out after anti-nesfatin-1 antibody and various peptides were previously reacted. In the top of B of Fig. 27, the types of peptides reacted to anti-nesfatin-1 antibody were no peptides, NAP1-Ab peptide (cognate peptide), leptin, α MSH and CART from the left, and in the bottom of B of Fig. 27, no peptides, NAP1-Ab peptide (cognate peptide) NPY, MCH and Orexin-A from the left.

A of Fig. 28 shows an image of immunohistochemical

stain using a NAP peptide antibody in the brain tissue containing the medulla oblongata. B of Fig. 28 shows an image of immunohistochemical stain carried out after an antibody against the NAP peptide and the NAP peptide (cognate peptide) were previously reacted.

A of Fig. 29 is a graph showing the body weight of the group (TGZ: +) in which the troglitazone-containing food was given to the normal rats (Lean) and the Zucker fa/fa rats (Zucker), and the group (TGZ: -) in which the troglitazone-free food was given thereto. B of Fig. 29 is a graph showing the concentration of leptin in the blood of the group (TGZ: +) in which the troglitazone-containing food was given to the normal rats (Lean) and the Zucker fa/fa rats and the group (TGZ: -) in which the troglitazone-free food was given thereto. C of Fig. 29 is a graph showing the concentration of nesfatin in the brain of the group (TGZ: +) in which the troglitazone-containing food was given to the normal rats (Lean) and the Zucker fa/fa rats and the group (TGZ: -) in which the troglitazone-free food was given thereto. In Fig. 29, * and ** represent a significant difference $P < 0.05$ and $P < 0.01$, respectively, relative to the group (TGZ: -) in which the troglitazone-free food was given to the normal rats (Lean), and in Fig. 29 # and ## represent a significant difference $P < 0.05$ and $P < 0.01$, respectively, relative to the group (TGZ: -) in which the troglitazone-free food was given to the Zucker fa/fa rats.

A of Fig. 30 shows an image of Western blotting of the fraction No. 45 obtained by fractionating a peptide extract from the rat hypothalamus by HPLC. B of Fig. 30 shows an image of the part at a molecular weight of about 9.7 kd in the Western blotting image of the fraction Nos. 43-47 obtained by fractionating a peptide extract from the rat hypothalamus by HPLC.

A of Fig. 31 shows the result of immunohistochemical stain using an anti-nesfatin-1 antibody in the rat brain tissue containing the arcuate nucleus, and the result of

immunohistochemical stain carried out after the anti-nesfatin-1 antibody and various peptides were previously reacted. In A of Fig. 31, a-1 represents an image of immunological stain with anti-nesfatin-1 antibody, a-2 to a-6 represent an image of immunological stain when the anti-nesfatin-1 antibody was previously reacted with the nesfatin-1 peptide (a-2), leptin (a-3), α MSH (a-4), CART (a-5) and NPY (a-6), respectively.

B of Fig. 31 shows the result of immunohistochemical stain using an anti-nesfatin-1 antibody in the rat brain tissue containing the paraventricular nucleus, and the result of immunohistochemical stain carried out after the anti-nesfatin-1 antibody and various peptides were previously reacted. In B, b-1 represents an image of immunological stain with the anti-nesfatin-1 antibody, b-2 to b-6 represent an image of immunological stain when the anti-nesfatin-1 antibody was previously reacted with nesfatin-1 peptide (b-2), leptin (b-3), α MSH (b-4), CART (b-5) and NPY (b-6), respectively.

Fig. 32 is a graph of the result showing the ratio (tissue weight/body weight, mg/g) of tissue weight of the abdominal subcutaneous adipose tissue (A), the epididymal adipose tissue (B), the mesenteric adipose tissue (C), the retroperitoneal adipose tissue (D), the brown adipose tissue (E) and the gastrocnemial muscle (F) obtained from the rats that were given nesfatin-1 or physiological saline alone for 10 days relative to the body weight of each individual. In Fig. 32, * and ** represent a significant difference $P < 0.05$ and $P < 0.005$, respectively, relative to the physiological saline-administration group.

Fig. 33 is a graph of the assay result of the amount of food intake, blood glucose, total cholesterol and triglyceride when nesfatin-1 or physiological saline alone was intraperitoneally given to the mouse. In Fig. 33, the white box and the hatched box represent the physiological saline administration group and the

nesfatin-1 administration group, respectively.

In accordance with the present invention, a factor involved in food intake control and body weight control can be obtained by using a PPAR γ agonist. Also, by using
5 nesfatin, nesfatin-1, nesfatin-1M30, nesfatin-1M16, nesfatin-1M14, nesfatin-1M10M, NUCB1-M30, NUCB1-M16, NUCB1-M14 and NUCB1-M10M, diseases associated with metabolic and food intake disorders such as obesity or adiposis and nervous hyperphagia, and type 2 diabetes
10 mellitus, impaired glucose tolerance, hypertension, hyperlipidemia, hyperuricemia, fatty liver, cardiac diseases, cerebral vascular diseases, sleep apnea syndrome, orthopedic diseases such as osteoarthritis, menstrual disorders and diseases associated with adiposis
15 such as malignant tumors can be prevented or treated. Furthermore, by using a substance such as antibody that suppresses the activity of nesfatin, nesfatin-1 or nesfatin-1M30, diseases associated with nutritional and food intake disorders such as anorexia and cibophobia in
20 post-surgery and/or cancer patients can be prevented or treated.

BEST MODE FOR CARRYING OUT THE INVENTION

<Method of obtaining a factor involved in food intake control and/or body weight control>

25 The present invention relates to a method of obtaining a factor that is involved in food intake control and/or body weight control, comprising the steps of reacting a thiazolidine dione compound having a PPAR γ agonistic activity to a mammalian cell, and identifying a
30 gene of which expression is induced by said compound.

As used herein "food intake control" refers to the control of the amount of food in animals or meals in humans (both are collectively referred to hereinafter as feeding etc.) during a certain period, or the control of
35 the total calories taken from feeding etc. during a certain period. The control of food intake also includes the control of events such as appetite or the satiety

that motivates feeding.

As used herein "the suppression of food intake" refers to the state in which the amount of feeding etc. or the total amount of calories taken in by feeding etc. is decreased compared to when no control of food intake is being done, or the state in which the growing trend of the amount of feeding etc. or the total amount of calories taken in by feeding etc. is being suppressed compared to when no control of food intake is being done. Also, the suppression of food intake includes states such as reduced appetite and enhanced satiety feeling.

On the other hand, "the enhancement of food intake" as used herein refers to the state in which the amount of feeding etc. or the total amount of calories taken in by feeding etc. is increased compared to when no control of food intake is being done, or the state in which the declining trend of the amount of feeding etc. or the total amount of calories taken in by feeding etc. is being suppressed compared to when no control of food intake is being done. Also, enhanced appetite includes states such as an increased appetite and a suppressed feeling of satiety.

"The control of body weight" as used herein refers to controlling the absolute body weight value, the body mass index (an index that employs body weight and body length) or the body fat percentage. As used herein "the suppression of body weight gain" refers to the state in which the absolute body weight value, the body mass index or the body fat percentage are decreased or maintained compared to when no control of body weight is being made, or the state in which the growing trend of the absolute body weight value, the body mass index or the body fat percentage is being suppressed compared to when no control of body weight is being made.

"The suppression of the body fat gain" as used herein refers to the state in which the body fat percentage is decreased or maintained compared to when no

control of body weight is being done, or the state in which the growing trend of the body fat percentage is suppressed compared to when no control of body weight is being done. "The enhancement of body weight gain" refers to the state in which the absolute body weight value, the body mass index or the body fat percentage are increased or maintained compared to when no control of body weight is being done, or the state in which the growing trend of the absolute body weight value, the body mass index or the body fat percentage is suppressed compared to when no control of body weight is being done, and it is also called herein "the suppression of body weight reduction". In the case of humans, as the representative body mass index, height BMI (body mass index) is used, which is calculated from body weight (kg) $\div$ height (m) $\div$ height (m), and is expressed in units of Kg/m^2 . Thus, the effect of such a suppression of body weight gain or an enhancement of body weight gain can also be expressed using BMI as an index. Also, the body fat percentage is indicated by a percentage of the weight of body fat in body weight, it can be determined by the body density method, the body moisture method, the body potassium-determining method, the impedance method, the dual X-ray absorption method, the neutron activation method, the near infrared spectroscopic method, the skinfold thickness-determining method, the imaging method and the like (The Japanese Journal of Clinical Medicine (Nippon Rinsho) Vol. 61, Supplement 6, pp. 357-396, 2003, issued by Nippon Rinsho K.K.).

As used herein "thiazolidine dione compounds having a PPAR γ agonist activity" include, for example, troglitazone, pioglitazone, rosiglitazone, and roboglitazone etc., and troglitazone was first put into clinical use.

Cells as used in the present invention include a non-small cell lung cancer cell line, an adipose cell and a cerebral nerve-derived cell, but they are not limiting

and any cells that express PPAR γ can be used.

As the method of reacting said compound to a mammalian cell, as described below, there is a method in which said cell is cultured under stimulation by said compound (Sato et al., Oncogene, England, 2002, Vol. 21, pp. 2171-2180).

The gene, of which expression is induced by the above compound, can be identified using, for example, a method in which the gene of which expression was specifically induced is subjected to the subtraction method or the DNA array analysis and the like (Sato et al., Oncogene, England, 2002, Vol. 21, pp. 2171-2180). In order to select a gene encoding a factor that is extracellularly secreted from among the genes that are specifically induced by the activation of PPAR γ , the nucleotide sequence of the gene is analyzed and can be selected depending on whether the secretory signal peptide is being encoded or not. Furthermore, as the method of selecting a gene involved in food intake control and/or body weight control among those genes, there can be mentioned immunological detection (illustrated in Working Example 3) or a histochemical method (illustrated in Working Examples 4 and 9) with a tissue extract using a brain sample containing the hypothalamus of a human or an animal, and an antibody that binds to a polypeptide encoded by said gene, and a method of confirming the expression in the hypothalamus using such as the in situ hybridization method (illustrated in Working Example 8) and the RT-PCR method.

For the gene thus obtained that is involved in food intake control and/or body weight control, the nucleotide sequence can be analyzed to identify the amino acid sequence of the encoded polypeptide. A peptide or a polypeptide comprising the amino acid sequence of the polypeptide obtained or a partial amino acid sequence thereof can be prepared using a genetic engineering method or a chemical synthetic method.

By introducing the polypeptide thus obtained or a nucleic acid molecule encoding said peptide introduced in a form that allows in vivo expression locally or systemically into a test animal, and then by examining
5 changes in the amount of food intake by said animal and/or body weight, the polypeptide or the gene involved in food intake control and/or body weight control can be selected from among the polypeptide obtained or the gene encoding said polypeptide. In another method, by
10 introducing an antibody that binds to the polypeptide thus obtained or an antisense oligonucleotide molecule or a RNAi molecule capable of suppressing the expression of the gene encoding said polypeptide locally or systemically into a test animal, and then by examining
15 changes in the amount of food intake by said animal and/or body weight, the polypeptide or the gene involved in food intake control and/or body weight control can be selected from among the polypeptide obtained or the gene encoding said polypeptide.

20 <A polypeptide having an activity of suppressing food intake and/or suppressing body weight gain>

The present invention relates to a polypeptide obtained by the above method, said polypeptide having an activity of suppressing food intake and/or suppressing
25 body weight gain. As such a polypeptide, there can be mentioned a polypeptide encoded by the nesfatin gene of which the function had not been identified, and it was found for the first time by the present inventors that said polypeptide has the above function. In accordance
30 with the present invention, it was found that the nesfatin gene is expressed in the hypothalamus of the brain that is said to control appetite (illustrated in Working Examples 4, 8, 9, 24 and 26), and that the nesfatin polypeptide administered into an animal brain
35 causes reduction in the amount of food intake and the body weight of the animal (illustrated in Working Examples 6 and 25). It was further demonstrated that, by

5 suppressing the function of the nesfatin polypeptide or
by inhibiting the expression of the nesfatin gene, the
enhancement in food intake and body weight gain can be
induced in animals (illustrated in Working Example 7 and
Working Example 15).

10 As examples of the nesfatin polypeptide, there can
be mentioned those that include amino acid sequences set
forth in SEQ ID NOs: 3, 6 and 9. A precursor nesfatin
polypeptide containing a human signal peptide is shown in
SEQ ID NO: 2. When the precursor nesfatin polypeptide is
extracellularly secreted the signal peptide is cleaved,
and thus a human matured nesfatin polypeptide, that
substantially has an activity, produces a form set forth
in SEQ ID NO: 3. As used herein, the nesfatin
15 polypeptide is simply called nesfatin.

Furthermore, the continued intensive and extensive
study on the nesfatin polypeptide having said activity of
suppressing food intake and/or suppressing body weight
gain led to the invention of a polypeptide with a novel
20 structure having an activity of suppressing food intake
and/or suppressing body weight gain. The discovery of
this polypeptide with a novel structure was based on the
investigation on various peptides derived from the
nesfatin polypeptide considering a possibility that the
nesfatin polypeptide may undergo cleavage by proteolytic
25 enzymes when they are extracellularly secreted. As a
result, it was found that a polypeptide comprising 82
amino acids having a sequence corresponding to the amino
acids No. 25 to 106 of the nesfatin polypeptide set forth
30 in SEQ ID NO: 5 has an activity of suppressing food
intake and/or suppressing body weight gain, and reducing
body fat percentage (illustrated in Working Example 12,
Working Example 13 and Working Example 34), and that by
inhibiting the suppression of function of the nesfatin-1
35 polypeptide, enhancement in food intake can be induced in
animals. Based on the above, said polypeptide was named
nesfatin-1 (SEQ ID NO: 14). Though the nesfatin

polypeptide has a calcium-binding domain, a DNA-binding domain etc. in its structure, the sequence of nesfatin-1 polypeptide has no such existing domain structures, and thus to obtain this nesfatin-1 polypeptide could not be absolutely expected from the conventional technology. Also, peptide hormones in vivo are known to be expressed in the form of a precursor protein, which then is cleaved by a proteolytic enzyme etc., and there are many reports that a prohormone convertase (or proprotein convertase: PC) is involved in the activity. In the human full-length nesfatin polypeptide set forth in SEQ ID NO: 2, the mouse precursor nesfatin polypeptide set forth in SEQ ID NO: 5 and the rat precursor nesfatin polypeptide set forth in 8, there is a common site (see Working Example 10) that is likely to be cleaved by a subtype of the prohormone convertase, PC1/3 (EC 3.4.21.93, Seidahi et al., DNA and Cell Biology, USA, Vol. 9, 1990, pp. 415-424) or PC2 (EC 3.4.21.94, Seidah et al., DNA and Cell Biology, USA, Vol. 9, 1990, pp. 415-424), and a possibility was demonstrated that nesfatin-1 polypeptides set forth in SEQ ID NO: 13 to SEQ ID NO: 15, respectively, are excised.

Also, when a nesfatin (Mut) having a mutation at the site cleaved with prohormone convertase was given to the ventricle of the rat brain, an unexpected result was obtained that the effect of suppressing food intake could not be noted (Working Example 14). Therefore, this suggested a possibility that the nesfatin-1 polypeptide is a functional molecule involved in food intake control and/or body weight the control in a living body, and that in order for the nesfatin polypeptide to be functional, the process of being processed by a protease such as prohormone convertase is important.

From the foregoing, the fact was not known at all that nesfatin/NEFA functions as a hormone precursor like proinsulin, and thus it was found for the first time in the present invention after the expression site for

nesfatin/NEFA and the expression of nesfatin/NEFA in cells that express PC1/3 and PC2 were analyzed and the activity and structure were intensively studied. Also, since there are secretory proteins that are not processed by PC1/3 and PC2 despite the presence of sequences Arg-Arg or Lys-Arg that are recognition sites of the prohormone convertase, it cannot be easily inferred that nesfatin-1 is excised from nesfatin/NEFA and exhibits the activity of suppressing food intake and/or suppressing body weight gain.

From the foregoing, the present invention also relates to nesfatin-1 polypeptides set forth in SEQ ID NOs: 13-15. As described above, said nesfatin-1 polypeptides have an activity of suppressing food intake and/or suppressing body weight gain. The amino acid sequence of mouse nesfatin-1 polypeptide is shown in SEQ ID NO: 14. A nesfatin polypeptide having such a sequence can be obtained by cleaving the nesfatin polypeptide set forth in SEQ ID NO: 14 with the prohormone convertase followed by purification with a technique such as reverse phase chromatography or by performing the step of binding to and releasing from an antibody against the nesfatin-1 polypeptide described below.

Furthermore, after continued intensive investigation on the structure of the nesfatin-1 polypeptide and the activity of suppressing food intake and/or suppressing body weight gain, it was found that a novel polypeptide comprising 30 amino acids having a sequence corresponding to amino acids 24 to 53 of the nesfatin polypeptide set forth in SEQ ID NO: 14 exhibits an activity of suppressing food intake and/or suppressing body weight gain (illustrated in Working Example 20), and said polypeptide was named nesfatin-1M30 (SEQ ID NO: 41). The discovery of the nesfatin-1M30 polypeptide indicates that when a polypeptide that contains the part corresponding to nesfatin-1M30 is present even after the nesfatin polypeptide or the nesfatin-1 polypeptide was

physiologically or artificially cleaved or digested, said polypeptide retains an activity of suppressing food intake and/or suppressing body weight gain.

Furthermore, for the structure of nesfatin-1M30 comprising 30 amino acids, the activity of the site having an activity of suppressing food intake was investigated. The result indicated that nesfatin-1M16 comprising 16 amino acids which is a partial peptide of its peptide, nesfatin-1M14 comprising 14 amino acids, and nesfatin-1M10M comprising 10 amino acids have an activity of suppressing food intake and/or suppressing body weight gain (Working Example 22).

When the sequences of nesfatin-1 comprising 82 amino acids set forth in SEQ ID NO: 13, SEQ ID NO: 14 or SEQ ID NO: 15 was analyzed on homology with the amino acid sequence of a factor having an activity of controlling food intake, no sequences having a high homology were found, and thus it was impossible to estimate that these nesfatin-1M30, nesfatin-1M16, nesfatin-1M14 and nesfatin-1M10M have an activity of suppressing food intake and/or suppressing body weight gain based on the conventional technology. It was also demonstrated that these nesfatin-1M30, nesfatin-1M16, nesfatin-1M14 and nesfatin-1M10M are active even if they are derived from inactive human-derived nesfatin or nesfatin-1.

Thus, the present invention relates to the nesfatin-1M30 polypeptide set forth in SEQ ID NOs: 39-41, the nesfatin-1M16 polypeptide set forth in SEQ ID NO: 65, 68 or 71, the nesfatin-1M14 polypeptide set forth in SEQ ID NO: 66, 69 or 72, or the nesfatin-1M10M polypeptide set forth in SEQ ID NO: 68, 70 or 73. As described above, said nesfatin-1M30 polypeptide, nesfatin-1M16 polypeptide, nesfatin-1M14 polypeptide and nesfatin-1M10M polypeptide have an activity of suppressing food intake and/or suppressing body weight gain. Also, the amino acid sequence of human nesfatin-1M30 polypeptide is shown in SEQ ID NO: 39. Also, the polypeptides comprising an

amino acid sequence set forth in SEQ ID NO: 39, SEQ ID NO: 40 or SEQ ID NO: 41 excluding the polypeptides comprising an amino acid sequence set forth in SEQ ID NO: 3, SEQ ID NO: 6 or SEQ ID NO: 9, SEQ ID NO: 13, SEQ ID NO: 14 or SEQ ID NO: 15 are included in the nesfatin-1M30 polypeptide. As examples of such polypeptides, there can be mentioned those polypeptides that were physiologically or artificially cleaved or digested from the nesfatin polypeptide or the nesfatin-1 polypeptide and that contain a sequence corresponding to nesfatin-1M30.

With regard to Nucleobindin I (NUCB1) that has a high homology with NEFA/nesfatin in terms of the amino acid sequence and the nucleotide sequence of the gene and that belongs to the same family, it was investigated whether NUCB1-M30 which is a site corresponding to nesfatin-1M30 of NUCB1 exhibits a similar activity. The result revealed that NUCB1-M30 also has an activity of suppressing food intake and/or suppressing body weight gain (Working Example 23). When human, rat and mouse nesfatins were compared, it was found, the amino acid sequence has been highly conserved at the site corresponding to nesfatin-1 of nesfatin and NUCB1 of each species, specifically at the site corresponding to nesfatin-1M30. From the foregoing, it is inferred that NUCB1-M16 comprising 16 amino acids of NUCB1, NUCB1-M14 comprising 14 amino acids and NUCB1-M10M comprising 10 amino acids of NUCB1 also have an activity of suppressing food intake and/or suppressing body weight gain similarly to the nesfatin-1M16 polypeptide, the nesfatin-1M14 polypeptide and the nesfatin-1M10M polypeptide.

Thus, the present invention relates to the NUCB1-M30 polypeptide set forth in SEQ ID NOs: 101-103, the NUCB1-M16 polypeptide set forth in SEQ ID NO: 107, 110 or 113, the NUCB1-M14 polypeptide set forth in SEQ ID NO: 108, 111 or 114, or the NUCB1-M10M polypeptide set forth in SEQ ID NO: 109, 112 or 115. Said NUCB1-M30 polypeptide, NUCB1-M16 polypeptide, NUCB1-M14 polypeptide and NUCB1-

M10M polypeptide also have an activity of suppressing food intake and/or suppressing body weight gain as described above.

5 The present invention also relates to a polypeptide that has a homology of at least 60% with any of the amino acid sequences set forth in SEQ ID NOs: 13-15, 39-41, 65-73, 101-103 and 107-115 and that has an activity of suppressing food intake and/or suppressing body weight gain. The homology with the amino acid sequence set
10 forth in SEQ ID NOs: 13-15, 39-41, 65-73, 101-103 and 107-115 is preferably 70% or greater and more preferably 80% or greater. A representative example thereof includes the nesfatin-1M30 polypeptide of a non-human animal. For example, as a polypeptide that has a
15 homology of 60% or greater with the amino acid sequence of the human nesfatin-1M30 polypeptide (SEQ ID NO: 39) and that has an activity of suppressing food intake and/or suppressing body weight gain, there can be mentioned, but not limited to, a mouse nesfatin-1M30
20 polypeptide (SEQ ID NO: 41) and a rat nesfatin-1M30 polypeptide (SEQ ID NO: 40).

Among the polypeptides comprising an amino acid sequence that has a homology of at least 60% with any of the amino acid sequences set forth in SEQ ID NOs: 13-15,
25 39-41, 65-73, 101-103 and 107-115, the selection of a polypeptide having an activity of suppressing food intake and/or suppressing body weight gain may be performed by introducing said polypeptide or a nucleic acid molecule encoding said polypeptide locally or systemically into a
30 test animal, and then selecting a polypeptide that suppresses the amount of food intake and/or the body weight of said animal. In an alternative method, the selection may be performed by introducing an antibody against said polypeptide or an antisense oligonucleotide
35 molecule or a RNAi molecule that can suppress the expression of a gene encoding said polypeptide locally or systemically into a test animal, and then selecting a

polypeptide that suppresses the amount of food intake and/or the body weight of said animal. Such a polypeptide is referred to hereinafter as altered nesfatin-1M30, altered nesfatin-1M16, altered nesfatin-1M14, altered nesfatin-1M10M, altered NUCB1-M30, altered NUCB1-M16, altered NUCB1-M14 and altered NUCB1-M10M.

Furthermore, the present invention also relates to a polypeptide that comprises an amino acid sequence in which some of the amino acids have been deleted, inserted or substituted in any of the amino acid sequences set forth in SEQ ID NOs: 13-15, 39-41, 65-73, 101-103 and 107-115 and that has an activity of suppressing food intake and/or suppressing body weight gain. Such a polypeptide can be obtained by replacing one or more amino acid residues with amino acids that are chemically or structurally similar to said amino acids in an amino acid sequence set forth in, for example, SEQ ID NOs: 13-15, 39-41, 65-73, 101-103 or 107-115. Specific embodiments of the substitution of amino acids that are chemically or structurally similar, i.e. the substitution of highly conserved amino acids, are well known to a person skilled in the art. For example, chemically or structurally, glycine (Gly) is similar to proline (Pro), alanine (Ala) and valine (Val), leucine (Leu) is similar to isoleucine (Ile), glutamic acid (Glu) is similar to glutamine (Gln), aspartic acid (Asp) is similar to asparagine (Asn), cysteine (Cys) is similar to threonine (Thr), Thr is similar to serine (Ser) and Ala, and lysine (Lys) is similar to arginine (Arg). Further, as alternative method, a person skilled in the art can easily refer to a amino acid matrix method, which represents in what extent can be made substitution of a amino acid, as a matrix, such as PAM (Wilbur, Molecular Biology and Evolution) (USA), 1985, Vol.2, pp.434-447), BLOSUM (Henikoff et al., Proceedings of the National Academy of Sciences of the United States of America) (USA) 1992, Vol.89, pp.10915-10919, and easily

substitute an amino acid, considering height of its score.

Also, among the polypeptides comprising an amino acid sequence in which some of the amino acids have been
5 deleted, inserted or substituted in any of the amino acid sequences set forth in SEQ ID NOs: 13-15, 39-41, 65-73, 101-103 and 107-115, the selection of a polypeptide that has an activity of suppressing food intake and/or
10 suppressing body weight gain can be performed in a manner similar to that described in the selection of the above modified nesfatin. Hereinbelow, such a polypeptide is also referred to as altered nesfatin-1M30.

Furthermore, the present invention also relates to a polypeptide that comprises an amino acid sequence having
15 a homology of at least 60% with any of the amino acid sequences set forth in SEQ ID NOs: 3, 6 and 9; or an amino acid sequence in which some of the amino acids have been deleted, inserted or substituted in any of the amino acid sequences set forth in SEQ ID NOs: 3, 6 and 9, and
20 that has an activity of suppressing food intake and/or suppressing body weight gain. Similarly to the above-mentioned altered products, said polypeptide is also referred to as an altered nesfatin. Said altered nesfatin, similarly to the above-mentioned altered
25 nesfatin-1M30 etc., preferably has a homology of 70% or greater and more preferably 80% or greater with the amino acid sequence set forth in SEQ ID NOs: 3, 6 or 9.

As described above, it was demonstrated by the present invention that for a nesfatin polypeptide to be
30 functional, the step of its being processed by a protease contained in the living body such as prohormone convertase is important. For that purpose, said altered nesfatin preferably is a polypeptide that produces in the living body nesfatin-1, nesfatin-1M30, nesfatin-1M16,
35 nesfatin-1M14, nesfatin-1M10M, NUCB1-M30, NUCB1-M16, NUCB1-M14, or NUCB1-M10M (a polypeptide comprising an amino acid sequence set forth in SEQ ID NOs: 13-15, 39-

41, 65-73, 101-103 or 107-115), or an altered product thereof. It is necessary for such an altered nesfatin to have a recognition site, in its amino acid sequence, for a cleaving enzyme such as a protease contained in the living body. As such a cleaving enzyme, there can be mentioned for example prohormone convertase (proprotein convertase: PC), and as such a prohormone convertase, there can be mentioned for example furin, PC1 (also known as PC3), PC2, PACE4, PC4, PC6 (also known as PC5) and LPC (also known as PC7 or PC8) (The FASEB Journal, 1216, vol. 17, July 2003). As long as the protease produces nesfatin-1 etc. in the periphery or the ventricle of the brain, the type of the protease is not specifically limited.

For similar reasons, the position of the recognition site for said cleaving enzyme is not specifically limited. However, based on the experimental results on mouse-derived nesfatin-1, nesfatin-2, nesfatin-3 and nesfatin-2/3 (Working Example 10), altered nesfatin is preferably established so as to contain at least one recognition site for a cleaving enzyme contained in the living body in between the amino acids 82 and 83 (a recognition site for prohormone convertase that produces nesfatin-1) of SEQ ID NO: 3, 6 or 9 and between the amino acids 163 and 164 (another recognition site for prohormone convertase) of SEQ ID NO: 3, 6 or 9, i.e. in an amino acid sequence corresponding to the amino acid numbers 82-162 of SEQ ID NO: 3, 6 or 9, in that it does not affect the activity of the resulting polypeptide.

Also, the confirmation of whether said altered product have an activity of suppressing food intake and/or suppressing body weight gain including an activity of being cleaved and becoming active in vivo can be made, as for the selection of the above-mentioned altered nesfatin-1M30 etc., by introducing said polypeptide or a nucleic acid molecule encoding said polypeptide locally or systemically into a test animal, and then selecting a

polypeptide that suppresses the amount of food intake and/or the body weight of said animal. In an alternative method, the selection may be performed by introducing an antibody against said polypeptide or an antisense oligonucleotide molecule or a RNAi molecule that can suppress the expression of a gene encoding said polypeptide locally or systemically into a test animal, and then selecting a polypeptide that suppresses the amount of food intake and/or the body weight of said animal.

The nesfatin polypeptide, nesfatin-1 polypeptide, nesfatin-1M30, nesfatin-1M16, nesfatin-1M14, nesfatin-1M10M, NUCB1-M30, NUCB1-M16, NUCB1-M14, or NUCB1-M10M of the present invention include those in which at least one amino acid has been added to the N terminal or C terminal thereof. Such nesfatin polypeptides include those in which a methionine residue, an acetyl residue or a pyroglutamic acid residue or the like has been added to the N terminal of the amino acid sequence set forth in SEQ ID NO: 3, SEQ ID NO: 6 or SEQ ID NO: 9, and those in which a suitable tag sequence (typically a histidine tag or a FLAG tag) has been added to the N terminal or the C terminal. Nesfatin polypeptides etc. having such construction have an advantage that they can be easily purified using a metal chelate carrier or antibody. Also, when nesfatin has been processed in the living body by prohormone convertase, it is thought, a polypeptide in which the recognition site for prohormone convertase has been added to the C terminal of nesfatin-1 is produced, and such a polypeptide is also encompassed by the present invention.

Also, the nesfatin polypeptide, the nesfatin-1 polypeptide, nesfatin-1M30, nesfatin-1M16, nesfatin-1M14, nesfatin-1M10M, NUCB1-M30, NUCB1-M16, NUCB1-M14, or NUCB1-M10M of the present invention include those in which at least one amino acid residue has been modified by a compound or a peptide. Such a nesfatin polypeptide

includes, in addition to the sequence set forth in SEQ ID NO: 3, SEQ ID NO: 6 or SEQ ID NO: 9, polypeptides that are obtained by enzymatically or chemically associating a peptide other than the nesfatin polypeptide or a
5 fluorescent substance etc. by a known method (Hermanson et al., Bioconjugate Techniques, USA, 1996, issued by Academic Press), and includes, for example, one in which the amino acid sequence of an Aequorea-derived
10 fluorescence protein or a secretory alkaline phosphatase has been added (so-called fusion protein). The presence of these fusion proteins can be easily detected; for example, by determining fluorescence intensity for a fusion protein with an Aequorea-derived fluorescence
15 protein, or by determining the intensity of color development, emission or fluorescence resulting from the reaction with said enzyme and its substrate for a fusion protein with a secretory alkaline phosphatase. For such a nesfatin polypeptide, an effect on food intake control and/or body weight control can be identified by
20 investigating the activity by a method described in Working Example 6 etc.

The present invention relates to a pharmaceutical composition for suppressing food intake and/or suppressing body weight gain, said composition comprising
25 as an active ingredient any of the nesfatin polypeptide, the nesfatin-1 polypeptide, nesfatin-1M30, nesfatin-1M16, nesfatin-1M14, nesfatin-1M10M, NUCB1-M30, NUCB1-M16, NUCB1-M14, or NUCB1-M10M, or altered products thereof (hereinafter referred to collectively as "the nesfatin
30 polypeptide etc."), or a peptide comprising some of the amino acid sequences of said nesfatin polypeptide etc. The peptide comprising some of the amino acid sequence of said nesfatin polypeptide refers to a peptide in which some of the amino acid sequence of said polypeptide has
35 been deleted within the extent of retaining the activity of suppressing food intake and/or suppressing body weight gain.

<A pharmaceutical composition for treating or preventing a disease for which enhanced food intake and body weight gain is a problem>

5 The present invention relates to a pharmaceutical composition for treating or preventing a disease for which enhanced food intake and body weight gain is a problem, said composition comprising, as an active ingredient, any of the above nesfatin polypeptide etc., or a peptide comprising some of the amino acid sequence
10 of said polypeptide.

A pharmacological effect of the nesfatin polypeptide etc. of the present invention is demonstrated by the results of the administration of nesfatin polypeptide to the third ventricle of the rat brain (Working Examples 6
15 and 25), the administration of nesfatin-1 polypeptide into the third ventricle of the rat brain (Working Examples 12, 13, 27 and 34), the administration of nesfatin-1 polypeptide into the abdominal cavity or the subcutaneous of mouse (Working Examples 18 and 19), the
20 administration of nesfatin-1M30 polypeptide to the abdominal cavity of mouse (Working Example 20). A pharmacological effect in pathological model animals is illustrated by the administration of nesfatin-1 polypeptide to the third ventricle of the brain in a
25 model animal indicating leptin resistance, i.e., Zucker (fa/fa) rat (Working Example 17), and a pharmacological effect on pathology of leptin resistance, which is also a problem in human adiposis, is illustrated. Further, it is pharmacologically illustrated that food intake may be
30 controlled through the mechanism different from melanocortine system, which is a known factor related to the control of food intake, by the administration experiment in Agouti yellow mouse (Working Example 18). By the illustrated facts, it is demonstrated that
35 nesfatin polypeptide etc. may be used as a pharmaceutical composition for treating or preventing a disease for which enhanced food intake and body weight gain is a

problem.

The diseases for which enhanced food intake and body weight gain is a problem include, for example, obesity, diabetes mellitus, hypertension, hyperlipidemia, hyperuricemia, fatty liver, cardiac diseases, cerebral vascular diseases, sleep apnea syndrome, orthopedic diseases, menstrual disorders and malignant tumors. Obesity includes adiposis, a pathological condition that requires body weight reduction when a health problem associated with obesity is complicated or its complication is clinically predicted. Orthopedic diseases include osteoarthritis due to overweight, lumbar disorders (spondylosis deformans), low back pain (acute low back pain) etc. Also malignant tumors include breast cancer, uterine cancer, colon cancer, kidney cancer, esophageal cancer, pancreatic cancer, liver cancer and gallbladder cancer.

The pharmaceutical composition of the present invention may contain any pharmaceutically acceptable additives. Formulations using pharmaceutically acceptable additives may be prepared by a method described in "Remington: The Science and Practice of Pharmacy, 20th edition, University of the Sciences in Philadelphia, Williams & Wilkins, issued on December 15, 2000". One dosage form of such a pharmaceutical composition is presented as a liquid prepared by dissolving, suspending or emulsifying them in an aqueous or oleaginous solution. Such solvents used include, for example, distilled water, physiological saline etc. for injection as an aqueous liquid, and in addition, an osmoregulatory agent (for example, D-glucose, D-sorbitol, D-mannitol, and sodium chloride), a suitable solubilizing agent such as alcohols (for example, ethanol), polyalcohols (for example, propylene glycol, and polyethylene glycol), nonionic surfactants (for example, polysorbate 80, polyoxyethylenated hydrogenated castor oil 50) and the like may be used. Also, as the solvents,

an oleaginous solution may be used, and said oleaginous solution includes sesame oil, soybean oil etc. and, as the solubilizing agent, benzyl benzoate, benzyl alcohol etc. may be used in combination. In such liquids, there
5 can be used, as appropriate, additives such as buffers (such as phosphate buffers and acetate buffers), soothing agents (such as benzalkonium chloride and procaine hydrochloride), stabilizers (such as human serum albumin and polyethylene glycol), preservatives (such as ascorbic
10 acid, erythorbic acid, and salts thereof), colorants (such as copper chlorophyll, β -carotene, Red No.2, and Blue No.1), preservatives (such as ascorbic acid, erythorbic acid, and salts thereof), antiseptics (such as paraoxybenzoate ester, phenol, benzethonium chloride and
15 benzalkonium chloride), thickeners (such as hydroxypropyl cellulose, carboxymethyl cellulose, and salts thereof), stabilizers (such as human serum albumin, mannitol and sorbitol), and corrigents (such as menthol and citrus fragrances). Another dosage form of pharmaceutical
20 compositions (in order to unify the expression with the above several "pharmaceutical compositions") includes solid forms such as powders, tablets, granules, capsules, pills, suppositories, and lozenges. In the case of solid forms that are administered in the form of oral
25 preparations, additives used include excipients (such as crystalline cellulose, lactose and starch), lubricants (such as magnesium stearate and talc), binders (such as hydroxypropyl cellulose, hydroxypropyl methyl cellulose and macrogol), disintegrants (such as starch and
30 carboxymethyl cellulose calcium), and the like. Also, as needed, there can be used antiseptics (such as benzyl alcohol, chlorobutanol, methyl paraoxybenzoate and propyl paraoxybenzoate), antioxidants, colorants, sweeteners and the like. Furthermore, another form also includes
35 pharmaceutical compositions for application to mucosa, and for this form of formulations, in order to impart adsoptivity to mucosa and retentivity, there can be

contained additives such as tackifiers, tackiness
enhancers, thickner, thickening agent (such as mucin,
agar, gelatin, pectin, carrageenan, sodium alginate,
locust bean gum, xanthan gum, tragacanth gum, gum arabic,
5 chitosan, pullulan, waxy starch, sucralfate, cellulose
and its derivatives (such as hydroxypropyl methyl
cellulose, polyglycerin fatty acid esters, acrylic-
(meth)acrylic alkyl copolymers or salts thereof, and
polyglycerin fatty acid esters). However, dosage forms
10 of pharmaceutical compositions and solvents and additives
to be delivered to the living body are not limited to the
above, and can be selected, as appropriate, by a person
skilled in the art.

For the purpose of ameliorating disease conditions,
15 the above pharmaceutical composition can be administered
orally or parenterally. In the case of oral
administration, dosage forms such as granules, powders,
tablets, capsules, liquids, syrups, emulsions or
suspensions, and elixirs can be selected. In the case of
20 parenteral administration, it can be transnasal agents,
and liquids, suspensions, solid formulations can be
selected. Other agents in the form of parenteral
administration, it can be in the form of injections, and
injections selected may be hypodermic injections,
25 intravenous injections, drip injections, intramuscular
injections, intraventricular injections, or
intraperitoneal injections and the like. Other
formulations for use in parenteral administration
include, for example, transmucosal preparations other
30 than suppositories, sublingual tablets, transdermal
preparations, nasal preparations, and the like.
Furthermore, intravascular local administration can be
performed, in embodiments where they are involved in or
applied to stents or anti-intravascular local embolism
35 agents.

The dosage of the above pharmaceutical composition
may vary depending on the age, sex, body weight of the

patient, condition, therapeutic effect, administration regimen, treatment period, or the types of active ingredients contained in said pharmaceutical composition, but its one dosage is usually in the range of 0.1 - 500
5 mg per person for adults, and preferably in the range of 0.5 mg - 20 mg. The dosage may vary depending on various conditions, and thus a dosage smaller than that described above may sometimes be sufficient, or at other times a dosage greater than the above may be required.

10 Also, gene encoding the nesfatin polypeptide or nesfatin-1 polypeptide may be used in embodiments of gene therapy. Said gene therapy, for example, can attain the therapeutic effect by introducing said gene into the living body. Techniques for introducing a gene encoding
15 a protein that provides therapeutic effect and allowing it to be expressed in order to treat a disease is known (Kaneda, Folia Pharmacologica Japonica, 2001, Vol. 117, pp. 299-306).

20 Furthermore, by administering a transformant in the form in which a gene containing a nesfatin polypeptide or a nesfatin-1 polypeptide has been introduced and that expresses said polypeptide, preferably by administering a transformant that employs a host transplantable into a species into which the gene is to be introduced,
25 treatment with the nesfatin polypeptide or nesfatin-1 polypeptide produced by said transformant can be performed.

30 <A nucleic acid molecule encoding a polypeptide having an activity of suppressing food intake and/or suppressing body weight gain>

The present invention also relates to a nucleic acid molecule encoding any of the above nesfatin polypeptide etc. As the nucleic acid molecule encoding the above nesfatin polypeptide, there can be mentioned a nucleic
35 acid molecule (SEQ ID NO: 1) comprising the nucleotide sequence of a gene encoding the human precursor nesfatin polypeptide, a nucleic acid molecule (SEQ ID NO: 4)

comprising the nucleotide sequence of a gene encoding the mouse precursor nesfatin polypeptide, a nucleic acid molecule (SEQ ID NO: 7) comprising the nucleotide sequence of a gene encoding the rat precursor nesfatin polypeptide, a nucleic acid molecule (SEQ ID NO: 10) comprising the nucleotide sequence of a gene encoding a matured nesfatin polypeptide in which a signal peptide portion has been removed, a nucleic acid molecule (SEQ ID NO: 11) comprising the nucleotide sequence of a gene encoding the mouse matured nesfatin polypeptide, a nucleic acid molecule (SEQ ID NO: 12) comprising the nucleotide sequence of a gene encoding the rat matured nesfatin polypeptide, and the like.

As nucleic acid molecules encoding the above nesfatin-1 polypeptides, there can be mentioned a nucleic acid molecule (SEQ ID NO: 18) encoding the human nesfatin-1 polypeptide, a nucleic acid molecule (SEQ ID NO: 19) encoding the mouse nesfatin-1 polypeptide, a nucleic acid molecule (SEQ ID NO: 20) encoding the rat nesfatin-1 polypeptide, and the like.

As nucleic acid molecules encoding the above nesfatin-1M30 polypeptides, there can be mentioned a nucleic acid molecule (SEQ ID NO: 44) encoding the human nesfatin-1M30 polypeptide, a nucleic acid molecule (SEQ ID NO: 46) encoding the mouse nesfatin-1M30 polypeptide, a nucleic acid molecule (SEQ ID NO: 45) encoding the rat nesfatin-1M30 polypeptide, and the like.

As nucleic acid molecules encoding the nesfatin-1M16 polypeptides, there can be mentioned a nucleic acid molecule (SEQ ID NO: 74) encoding the human nesfatin-1M16 polypeptide, a nucleic acid molecule (SEQ ID NO: 80) encoding the mouse nesfatin-1M16 polypeptide, a nucleic acid molecule (SEQ ID NO: 77) encoding the rat nesfatin-1M16 polypeptide, and the like.

As nucleic acid molecules encoding the nesfatin-1M14 polypeptides, there can be mentioned a nucleic acid molecule (SEQ ID NO: 75) encoding the human nesfatin-1M14

polypeptide, a nucleic acid molecule (SEQ ID NO: 81) encoding the mouse nesfatin-1M14 polypeptide, a nucleic acid molecule (SEQ ID NO: 78) encoding the rat nesfatin-1M14 polypeptide, and the like.

5 As nucleic acid molecules encoding the above nesfatin-1M10M polypeptides, there can be mentioned a nucleic acid molecule (SEQ ID NO: 76) encoding the human nesfatin-1M10M polypeptide, a nucleic acid molecule (SEQ ID NO: 82) encoding the mouse nesfatin-1M10M polypeptide,
10 a nucleic acid molecule (SEQ ID NO: 79) encoding the rat nesfatin-1M10M polypeptide, and the like.

 As nucleic acid molecules encoding the NUCB1-M30 polypeptides, there can be mentioned a nucleic acid molecule (SEQ ID NO: 104) encoding the human NUCB1-M30
15 polypeptide, a nucleic acid molecule (SEQ ID NO: 106) encoding the mouse NUCB1-M30 polypeptide, a nucleic acid molecule (SEQ ID NO: 105) encoding the rat NUCB1-M30 polypeptide, and the like.

 As nucleic acid molecules encoding the NUCB1-M16 polypeptides, there can be mentioned a nucleic acid molecule (SEQ ID NO: 116) encoding the human NUCB1-M16
20 polypeptide, a nucleic acid molecule (SEQ ID NO: 122) encoding the mouse NUCB1-M16 polypeptide, a nucleic acid molecule (SEQ ID NO: 119) encoding the rat NUCB1-M16
25 polypeptide, and the like.

 As nucleic acid molecules encoding the NUCB1-M14 polypeptides, there can be mentioned a nucleic acid molecule (SEQ ID NO: 117) encoding the human NUCB1-M14
30 polypeptide, a nucleic acid molecule (SEQ ID NO: 123) encoding the mouse NUCB1-M14 polypeptide, a nucleic acid molecule (SEQ ID NO: 120) encoding the rat NUCB1-M14 polypeptide, and the like.

 As nucleic acid molecules encoding the above NUCB1-M10M polypeptides, there can be mentioned a nucleic acid
35 molecule (SEQ ID NO: 118) encoding the human NUCB1-M10M polypeptide, a nucleic acid molecule (SEQ ID NO: 124) encoding the mouse NUCB1-M10M polypeptide, a nucleic acid

molecule (SEQ ID NO: 121) encoding the rat NUCB1-M10M polypeptide, and the like.

Also, the nesfatin polypeptides etc. of the present invention include, as described above, those in which at least one amino acid has been added to the N terminal or the C terminal, and those in which at least one amino acid residue has been modified by a compound or a peptide. As nucleic acid molecules encoding such nesfatin polypeptides etc., in the case of the nesfatin polypeptide for example, a nucleic acid molecule encoding a polypeptide in which a polypeptide has been added to the N terminal of the sequence of SEQ ID NO: 3, SEQ ID NO: 6 or SEQ ID NO: 9 can be obtained by adding a nucleotide sequence having the nucleotide sequence ATG and a codon encoding an amino acid sequence desired to be added behind it to the 5'-end of SEQ ID NO: 10, SEQ ID NO: 11 or SEQ ID NO: 12. Also, a nucleic acid molecule encoding a polypeptide in which a recognition (cleaving) sequence of protease such as prohormone convertase has been added to the N terminal and/or the C terminal of the amino acid sequence of the nesfatin polypeptide etc. is encompassed by the present invention, but it is not limited to them.

In addition, a nucleic acid molecule is included that comprises a nucleotide sequence in which a gene sequence encoding an Aequorea-derived fluorescence protein or a secretory alkaline phosphatase has been added to the 5'- or 3'-end of the gene sequence encoding a polypeptide comprising an amino acid sequence set forth in SEQ ID NO: 3, SEQ ID NO: 6 or SEQ ID NO: 9 in such a form that the amino acid sequence of each protein can be translated.

Nucleic acid molecules that encode the above altered nesfatin etc. include nucleic acid molecules encoding a polypeptide that comprises an amino acid sequence having a homology of at least 60% with any of the amino acid sequence set forth in SEQ ID NOs: 3, 6, 9, 13-15, 39-41,

65-73, 101-103 or 107-115 and that has an activity of suppressing food intake and/or suppressing body weight gain, and preferably said homology is 70% or greater and more preferably 80% or greater. As the representative
5 example thereof, there can be mentioned, in the case of the nesfatin-1M30 polypeptide for example, a nesfatin-1M30 polypeptide of a non-human animal species. For example, nucleic acid molecules encoding a polypeptide that comprises an amino acid sequence having a homology
10 of 60% or greater with the amino acid sequence (SEQ ID NO: 39) of human nesfatin-1M30 polypeptide etc. and that has an activity of suppressing food intake and/or suppressing body weight gain include a nucleic acid molecule (SEQ ID NO: 46) encoding the mouse nesfatin-1M30
15 polypeptide, a nucleic acid molecule (SEQ ID NO: 45) encoding the rat nesfatin polypeptide, a nucleic acid molecule (SEQ ID NO: 104) encoding the human NUCB1-M30 polypeptide, a nucleic acid molecule (SEQ ID NO: 106) encoding the mouse NUCB1-M30 polypeptide, and a nucleic
20 acid molecule (SEQ ID NO: 105) encoding the rat NUCB1-M30 polypeptide and the like, but not limited to these nucleic acid molecules.

Also, nucleic acid molecules encoding the above altered nesfatin etc. include those nucleic acid
25 molecules encoding a polypeptide comprising an amino acid sequence in which some of the amino acids have been deleted, inserted or substituted in any of the amino acid sequence set forth in SEQ ID NOs: 6, 9, 13-15, 39-41, 65-73, 101-103 or 107-115 and that has an activity of
30 suppressing food intake and/or suppressing body weight gain.

Furthermore, the present invention also relates to a nucleic acid molecule that hybridizes to a nucleotide sequence set forth in SEQ ID NOs: 4, 7, 11, 12, 18, 19,
35 20, 44-46, 74-82, 104-106 or 116-124, or a partial sequence thereof under a stringent condition, and that encodes a polypeptide having an activity of suppressing

food intake and/or suppressing body weight gain. Such a nucleic acid molecule can be obtained by a hybridization method that employs a nucleotide sequence set forth in SEQ ID NOs: 4, 7, 10, 11, 12, 18, 19, 20, 44-46, 74-82, 104-106 or 116-124, or a partial sequence thereof. Specifically, a library is constructed in which a plasmid vector or a phage vector having inserted therein a cDNA or genomic DNA fragment derived from any type of organism has been introduced into a host such as *Escherichia coli* (E. coli), and then the library is cultured on an agar medium plate containing a suitable selection drug. Then, the resulting recombinant E. coli clone or a phage clone is transferred to a nitrocellulose membrane etc., and then the cells or phage are lysed in an alkali- or surfactant-containing condition so as to immobilize DNA contained therein onto the membrane. To the membrane is reacted a suitable hybridization solution at a suitable temperature in which a linearized probe obtained by labelling DNA comprising a nucleotide sequence set forth in SEQ ID NOs: 4, 7, 10, 11, 12, 18, 19, 20, 44-46, 74-82, 104-106 or 116-124, or a partial sequence thereof with ³²P has been dissolved. After the reaction, the membrane is washed with x 2 SSC to remove the excessive probe, and then washed under a highly stringent condition such as x 0.1 SSC at 65°C, or a medium stringent condition such as x 0.5 SSC at 65°C, and then the membrane is contacted with an X-ray film in the dark for exposure. After an X-ray film that was exposed in a deep freezer for a few hours to a few days is developed to detect the exposed spots, and E. coli or phage clones located at positions corresponding to the original plate from which transfer was made to the membrane are harvested and cultured, the sequence of the genes that have been inserted to the vector is analyzed to obtain genes that are highly homologous to the nesfatin gene, the nesfatin-1 gene or the nesfatin-1M30 gene. Also, by obtaining clones by hybridization of cDNA or a genome library using

the gene sequence or identifying the surrounding sequences by the primer extension method etc., the gene structure encoding the protein can be elucidated to obtain nucleic acid molecules homologous to nesfatin, nesfatin-1, nesfatin-1M30, nesfatin-1M16, nesfatin-1M14, nesfatin-1M10M, NUCB1-M30, NUCB1-M16, NUCB1-M14 or NUCB1-M10M. Furthermore, among the nucleic acid molecules that hybridize under a stringent condition to the nucleotide sequence set forth in SEQ ID NOs: 4, 7, 10, 11, 12, 18, 19, 20, 44-46, 74-82, 104-106 or 116-124 or the partial sequence of said nucleotide sequence, the nucleic acid molecule encoding a polypeptide having an activity of suppressing food intake and/or suppressing body weight gain can be selected by introducing a polypeptide encoded by said nucleic acid molecule or said nucleic acid molecule locally or systemically to a test animal and by selecting a nucleic acid molecule that suppresses food intake and/or suppresses body weight. In alternative method, it can be performed by introducing an antibody that binds to the polypeptide encoded by said nucleic acid molecule or an antisense oligonucleotide molecule or a RNAi molecule that can suppresses the expression of the gene encoded by said nucleic acid molecule locally or systemically into a test animal, and then selecting a nucleic acid molecule that suppresses the amount of food intake by and/or the body weight of said animal.

<Vector>

The present invention also relates to a vector comprising a nucleic acid molecule encoding any of the above nesfatin polypeptide etc. By constructing a recombinant which is a host cell such as a microorganism having introduced therein the nesfatin gene etc. using a vector comprising a nucleic acid molecule encoding such nesfatin polypeptide etc., said gene can be stably conserved or replicated. The nucleic acid molecule of said gene encoding the nesfatin polypeptide integrated into a vector having a function of being replicated in a

host cell can be prepared in large quantities by culturing a recombinant cell in a suitable medium, by cellular growth or by amplifying the copy number of the introduced gene in the cell.

5 Said nucleic acid molecule may be operably linked under the control of a regulatory nucleic acid molecule that controls the expression of said nucleic acid molecule. As the regulatory nucleic acid molecule that controls the expression of said nucleic acid molecule,
10 there can be mentioned a nucleic acid molecule encoding a regulatory sequence for expressing the integrated gene in the host cell such as a promoter sequence or an enhancer sequence, which can be selected as appropriate by a person skilled in the art. By integrating a nucleic acid
15 molecule encoding the nesfatin polypeptide under the control of a nucleic acid molecule encoding such a regulatory sequence, the nesfatin polypeptide etc. can be produced in large quantities in any host cell (such as a microbial cell, a mammalian cell, an insect cell etc.).

20 Also as the vector that can be used in the present invention, there can be mentioned a vector that has a promoter upstream to the gene to be expressed, and a polyadenylation site, transcription termination sequence etc. downstream thereto. As such an expression vector
25 for vertebrates, there can be mentioned pSV2dhfr (Mol. Cell. Biol., 854, 1981) having the SV40 early promoter, pcDNA3.1(+) (Invitrogen Corp.), and pCAGGS (Gene 108:193-200, 1991) and the like, but they are not limiting and can be selected as appropriate by a person skilled in the
30 art. Such a vector can also be introduced into a mammalian cell for the treatment of suppressed food intake and/or suppressed body weight gain.

 When *E. coli* is used as the host cell, pBR322 and an improved vector thereof can be used, but they are not
35 limiting, and various known microbial strains lines and vectors can also be used. As the promoter, there can be mentioned, but not limited to, promoters such as *E. coli*

lactose (lac), E. coli trp and the like. Said promoters are those that have already been characterized and known to a person skilled in the art, and can be assembled synthetically or from known plasmids.

5 Working Example 5 as a specific example demonstrates that by integrating a nucleic acid molecule encoding a nesfatin polypeptide into a vector to construct an E. coli recombinant, a nesfatin polypeptide retaining an activity of suppressing food intake and/or suppressing
10 body weight gain could be prepared. Also, Working Example 16 demonstrates that by allowing the amino acid sequences described in SEQ ID NO: 13 to 15 as fusion proteins with glutathione S-transferase (GST) etc, to be expressed. and purifying using the adsorption to and
15 desorption from a glutathione-immobilized carrier followed by cleavage of the GST portion, the nesfatin-1 polypeptide could be produced by a gene recombinant technology.

 By introducing the vector of the present invention
20 into a mammalian cell using the technology for gene therapy for the purpose of suppressing food intake and/or suppressing body weight gain, the therapeutic effect can be attained. The method of treating diseases by
introducing a gene encoding a protein that provides the
25 therapeutic effect, i.e. the nesfatin polypeptide etc., into a mammalian cell and then allowing it to be expressed is known (Kaneko, Folia Pharmacologica Japonica, issued in 2001, Vol. 117, pp. 299-306).

<Transformant>

30 The present invention also relates to a transformant comprising a nucleic acid molecule encoding any of the above nesfatin polypeptide etc. Such a transformant can be obtained by introducing the gene encoding said polypeptide into a host cell and transforming it.
35 Methods of transformation include biological methods, physical methods, chemical methods and the like. As biological methods, there can be mentioned a method that

employs a virus vector, a method that employs a specific receptor, cell fusion (Sendai virus (HVJ), polyethylene glycol (PEG), electric cell fusion, micronucleate cell fusion (chromosome transfer)), and the like. Physical
5 methods include the microinjection method, an electroporation method, the method that employs a gene particle gun. Chemical methods include the calcium phosphate precipitation method, the liposome method, the DEAE-dextran method, the protoplast method, the
10 erythrocyte ghost method, the erythrocyte membrane ghost method, the microcapsule method, which can be selected and performed as appropriate by a person skilled in the art. The transformant obtained can be cultured according to a standard method, and the nesfatin polypeptide etc.
15 can be produced. As the media used for culturing, various media commonly used can be as appropriate selected depending on the host cell adopted, and culturing can also be performed under a condition suitable for the growth of the host cell.

20 Various means for expressing the protein of interest in an eukaryotic cell are known per se in said field of art. For example, an expression system in yeast includes "The expression of protein in yeast" described in Japanese Unexamined Patent Publication (Kokai) No. 57-
25 159489, an expression system in plant cells includes "An improved method and equipment for introducing a biological substance into a living cell" described in Patent Publication No. 2517813 or "A method of introducing a gene into a plant cell and a plant cell-
30 treatment equipment for gene introduction" described in Japanese Unexamined Patent Publication (Kokai) No. 2003-274953, an expression system in insect cells includes "A method of producing a recombinant baculovirus expression vector" described in Japanese Unexamined Patent
35 Publication (Kokai) No. 60-37988, and an expression system in mammalian cells includes "Improvement in eukaryotic expression" described in Japanese Unexamined

Patent Publication (Kokai) No. 2-171198, but there are many other systems in addition to these.

As host cells for use in transformation, both eukaryotic host cells and prokaryotic host cells can be used. Eukaryotic host cells include vertebrates, yeast, plant cells, insect cells and the like. As plant cells, there can be mentioned tissue sections of dicotyledons and monocotyledons, cells isolated from the tissue, cells derived from callus formed from the tissue and the like. Vertebrate cells include, for example, CHO cells, 293T cells, COS7 cells and the like. Prokaryotic host cells include, for example, E. coli, Bacillus subtilis, Streptomyces and the like, and as E. coli, Escherichia coli strain K12 is often used. When a vertebrate is used as the host cell, the transformant obtained can be introduced into a mammal for cell therapy of suppressing food intake and/or suppressing body weight gain. As such transformants, those that were confirmed to express the gene encoding the introduced nesfatin polypeptide etc. are preferred. In administering said transformant, it is preferred to administer said transformant by dispersing in various buffers, physiological saline etc. (Japanese Unexamined Patent Publication (Kokai) No. 2003-342201)

<Antibody>

The present invention relates to antibody that binds to any of the above nesfatin polypeptide etc. Such an antibody can be obtained by a method known to a person skilled in the art. The antibody for use in the present invention can be polyclonal antibody or monoclonal antibody (Milstein et al., Nature (England), issued on October 6, 1983, Vol. 305, No. 5934, pp. 537-540). For example, a polyclonal antibody against the nesfatin polypeptide, the nesfatin-1 polypeptide, the nesfatin-1M30 polypeptide, nesfatin-1M16, nesfatin-1M14, nesfatin-1M10M, NUCB1-M30, NUCB1-M16, NUCB1-M14 or NUCB1-M10M can be collected from the serum etc. of a mammal sensitized with the antigen. Furthermore, in another example, it

can be collected from the serum etc. of a mammal sensitized with a peptide having a sequence comprising a partial sequence of the nesfatin polypeptide etc. In a more specific example, as shown in Working Example 3, an antibody that binds to the nesfatin polypeptide etc. can be obtained by binding a peptide (SEQ ID NO: 24) having sequence corresponding to amino acids 141-152 of SEQ ID NO: 8 to a carrier protein and using it as the antigen to immunize an animal. When administered to an animal, this antibody exhibits an effect of enhancing food intake and increasing body weight of said animal (Working Example 7). In still another example, a peptide (SEQ ID NO: 32) having a sequence corresponding to amino acids 48-62 of SEQ ID NO: 8 is bound to a carrier protein, which is used as the antigen to immunize an animal so that an antibody that binds to the nesfatin polypeptide etc. can be obtained (Working Example 10). The polypeptide set forth in SEQ ID NO: 32 consists of amino acid sequences common to the nesfatin polypeptide, nesfatin-1 polypeptide and nesfatin-1M30 of human, mouse or rat, and therefore antibodies obtained bind to all of these polypeptides. When administered to an animal, this antibody exhibits an effect of enhancing food intake and increasing body weight of said animal (Working Example 14). In addition to them, it is possible to create antibody by preparing, as appropriate, a peptide to be used as the antigen from the sequence of disclosed nesfatin-1 polypeptide etc.

A monoclonal antibody against the above nesfatin polypeptide etc. can be prepared by collecting immune cells from the animal sensitized with the antigen and subjecting the cells to cell fusion with myeloma cells to collect the antibody from the culture.

Such an antibody can be labelled as appropriate, and can be used to detect the above nesfatin polypeptide etc. Also, instead of labelling this antibody, substances that specifically bind to said antibody, such as Protein A and protein G, can be labelled for indirect detection. As

specific methods for detection, for example, ELISA methods can be mentioned.

5 The antigen used to obtain the antibody of the present invention can be obtained by integrating, for example, a gene encoding the nesfatin polypeptide, the nesfatin-1 polypeptide, nesfatin-1M30, nesfatin-1M16, nesfatin-1M14, nesfatin-1M10M, NUCB1-M30, NUCB1-M16, NUCB1-M14 or NUCB1-M10M mentioned above, or altered products thereof, or part of it into an expression
10 vector, introducing the expression vector into a suitable host cell to construct a transformant, culturing said transformant to express a recombinant protein, and purifying the expressed recombinant protein from the culture or the culture supernatant. Alternatively, an
15 oligopeptide can be chemically synthesized that comprises an amino acid sequence encoded by said gene or a partial amino acid sequence of the amino acid sequence encoded by the full-length cDNA and used as the immunogen. Animals to be immunized include mice, rats, rabbits, goats,
20 horses, hamsters and the like, and can be selected as appropriate by a person skilled in the art.

 <A substance that suppresses the activity or expression of a polypeptide having an activity of suppressing food intake and/or suppressing body weight
25 control>

 In the investigation of the present invention, when an antibody was prepared against a nesfatin polypeptide, a nesfatin-1 polypeptide and a nesfatin-1M30 polypeptide, and administered to the brain of an animal, an increase
30 in the amount of food intake by the animal was noted (Working Examples 7 and 14). Also, when an antisense RNA (oligonucleotide) that suppresses the expression of nesfatin was administered into the brain of an animal, the enhancement of food intake and the increase in the
35 body weight of the animal were noted (Working Example 15). This indicates that the nesfatin polypeptide, the nesfatin-1 polypeptide and the nesfatin-1M30 polypeptide

actually operates in the brain for food intake control and/or body weight control. Furthermore, the increased concentration of the nesfatin polypeptide, the nesfatin-1 polypeptide or the nesfatin-1M30 polypeptide in a
5 peripheral (peripheral blood) or a brain caused the suppression of food intake and/or the suppression of body weight gain, and a substance that suppresses the activity or expression of said polypeptide exhibited an activity of enhancing food intake and/or increasing body weight,
10 and therefore the nesfatin polypeptide, the nesfatin-1 polypeptide and/or the nesfatin-1M30 polypeptide was demonstrated to be a central factor responsible for food intake control and/or body weight control. Accordingly, a substance per se that inhibits the activity or
15 expression of the nesfatin polypeptide, the nesfatin-1 polypeptide and/or the nesfatin-1M30 polypeptide can be used in the treatment, diagnosis and/or screening of therapeutic agents for the suppression of food intake and/or the suppression of body weight gain due to
20 diseases and conditions for which decreased food intake and body weight reduction are a problem, such as cibophobia, functional dyspepsia, cancer, inflammatory diseases, decreased functions of the pituitary, the thyroid, the adrenal gland etc., post-surgery, or
25 excessive stress and the like.

Thus, the present invention relates to a substance that inhibits the activity or the production of the nesfatin polypeptide, the nesfatin-1 polypeptide, the nesfatin-1M30 polypeptide, nesfatin-1M16, nesfatin-1M14,
30 nesfatin-1M10M, NUCB1-M30, NUCB1-M16, NUCB1-M14 or NUCB1-M10M. More preferably, it relates to a substance that exhibits an activity of enhancing food intake and/or enhancing body weight gain. Substances that suppress the activity of the nesfatin polypeptide etc. include those
35 characterized by binding to the nesfatin polypeptide etc. and those that do not require binding to these polypeptides. An example of the former is antibody

against the nesfatin polypeptide etc., and as the antibody those described above can be used. An example of the latter is a dominant negative polypeptide against the nesfatin polypeptide etc. The "dominant negative" is
5 a mutant that dominantly acts on the wild type both quantitatively and qualitatively so as to inhibit the function of the wild type. The dominant negative can be prepared by deleting or converting part of the amino acids of the wild type.

10 The present invention also relates to a substance that suppresses the expression of a gene encoding the nesfatin polypeptide, the nesfatin-1 polypeptide, the nesfatin-1M30 polypeptide, nesfatin-1M16, nesfatin-1M14, nesfatin-1M10M, NUCB1-M30, NUCB1-M16, NUCB1-M14 or NUCB1-
15 M10M. Such substances include, for example, an antisense oligonucleotide, a RNAi molecule and the like. The gene encoding the nesfatin polypeptide etc. as used herein refers to a nucleic acid molecule that comprises the entire nucleotide sequence or part thereof of the nucleic
20 acid molecule encoding the nesfatin polypeptide etc. as a contiguous or non-contiguous unit. Also the expression of gene comprises a step of a nucleic acid molecule encoding the nesfatin polypeptide etc. being transcribed from said gene, a step of stabilizing the transcribed
25 nucleic acid molecule, and a step of the nesfatin polypeptide etc. being produced by translation from the transcribed nucleic acid molecule. Thus, the suppression of gene expression refers to suppress any of the steps of transcription from the gene encoding the nesfatin
30 polypeptide etc.; stabilization; and translation.

The above antisense oligonucleotide can be designed by using, for example, a gene sequence encoding the nesfatin polypeptide. As such an antisense
oligonucleotide, there can be mentioned a morpholino-type
35 antisense oligonucleotide having the structure of SEQ ID NO: 31. As shown in Working Example 15, by administering this to an animal, said antisense oligonucleotide

exhibits an effect of enhancing food intake and enhancing body weight gain. For an antisense oligonucleotides, various modifications or binding formats are known in order to avoid decomposition in the cell, and a person skilled in the art will be able to select the structure of a suitable antisense oligonucleotide (Curreck et al., European Journal of Biochemistry) (UK), 2003, Vol.270, pp.1628-486). As the structure, there can be illustrated the natural form (D-oligo), the phosphorothioate type (S-oligo), the methylphosphonate type (M-oligo), the phosphoroamidate type (A-oligo), the 2'-O-methyl type (D-oligo), the morpholidate type (Mo-oligo), a polyamide nucleic acid and the like. The length used is 10 bases to 70 bases, and preferably 15 bases to 30 bases are used.

RNA interference (RNAi) refers to a phenomenon in which a double-stranded RNA of 21-23 residues decomposes a target RNA containing the same sequence thereby to greatly suppress its expression. Thus, RNA containing a double stranded structure having the same nucleotide sequence as mRNA of the gene encoding the nesfatin polypeptide etc. can be used for suppressing the expression of the gene of the nesfatin polypeptide etc. In order to obtain the RNAi effect, it is preferred to use a double stranded RNA having a sequence of at least 20 contiguous nucleotides. The double stranded structure may be composed of different strands, and of two strands provided by the stem loop structure of one RNA. By adding a two-base overhang to the 3'-end of each strand, the effect of suppressing the expression of the gene can be enhanced. For the sequence, length and the structure used in designing RNAi, a person skilled in the art would be able to optimize RNAi having a potent effect of suppressing gene expression through various modification attempts.

The above antisense oligonucleotide molecule and the RNAi molecule can be produced by integrating a nucleic

acid molecule comprising a nucleotide sequence complementary to the nucleic acid sequence of said molecule into a vector, introducing this into a host cell for transformation, and culturing the transformant. The host cell used and the method of transformation could be selected as appropriate by a person skilled in the art as described in the above <Transformant>.

<A pharmaceutical product for enhancing appetite or enhancing body weight gain>

The present invention also relates to a pharmaceutical composition for enhancing appetite or enhancing body weight gain, said composition comprising as an active ingredient a substance that inhibits the activity or the production of the nesfatin polypeptide etc., or a substance that inhibits the expression of the gene encoding the nesfatin polypeptide etc. Said pharmaceutical composition can be used for diseases and conditions for which the suppression of food intake or body weight gain is a problem. Diseases and conditions for which the suppression of food intake or body weight gain is a problem include, for example, cibophobia, functional dyspepsia, or the suppression of food intake and/or the suppression of body weight gain due to cancer, inflammatory diseases, decreased functions of the pituitary, the thyroid, the adrenal etc., post-surgery, or excessive stress and the like.

The pharmaceutical composition of the present invention may contain any pharmaceutically acceptable additives. Pharmaceutical formulations using pharmaceutically acceptable additives may be prepared by a method described in "Remington: The Science and Practice of Pharmacy, 20th edition, University of the Sciences in Philadelphia, Williams & Wilkins, issued on December 15, 2000". One dosage form of such a pharmaceutical composition is presented as a liquid prepared by dissolving, suspending or emulsifying in an aqueous or oleaginous solution. Such solvents used

include, for example, distilled water for injection, physiological saline etc. as an aqueous liquid, and in addition, an osmoregulatory agent (for example, D-glucose, D-sorbitol, D-mannitol, and sodium chloride), a
5 suitable solubilizing agent such as alcohols (for example, ethanol), polyalcohols (for example, propylene glycol and polyethylene glycol), nonionic surfactants (for example, polysorbate 80, polyoxyethylenated hydrogenated castor oil 50) and the like may be used.
10 Also, as the solvents, an oleaginous solution may be used, and said oleaginous solution includes sesame oil, soybean oil etc., and as the solubilizing agent, benzyl benzoate, benzyl alcohol etc. may be used in combination. In such liquids, there can be used, as appropriate,
15 additives such as buffers (such as phosphate buffers and acetate buffers), soothing agents (such as benzalkonium chloride and procaine hydrochloride), stabilizers (such as human serum albumin and polyethylene glycol), preservatives (such as ascorbic acid, erythorbic acid,
20 and salts thereof), colorants (such as copper chlorophyll, β -carotene, Red No.2, and Blue No.1), preservatives (such as ascorbic acid, erythorbic acid, and salts thereof), antiseptics (such as paraoxybenzoate ester, phenol, benzethonium chloride and benzalkonium
25 chloride), thickeners (such as hydroxypropyl cellulose, carboxymethyl cellulose, and salts thereof), stabilizers (such as human serum albumin, mannitol and sorbitol), and corrigents (such as menthol and citrus fragrances). Another dosage form of pharmaceutical compositions (in
30 order to unify the expression with the above several "pharmaceutical compositions") includes solid forms such as powders, tablets, granules, capsules, pills, suppositories, and lozenges. In the case of solid forms for administration in the form of oral preparations,
35 additives used include excipients (such as crystalline cellulose, lactose and starch), lubricants (such as magnesium stearate and talc), binders (such as

hydroxypropyl cellulose, hydroxypropyl methyl cellulose and macrogol), disintegrants (such as starch and carboxymethyl cellulose calcium), and the like. Also, as needed, there can be used antiseptics (such as benzyl
5 alcohol, chlorobutanol, methyl paraoxybenzoate and propyl paraoxybenzoate), antioxidants, colorants, sweeteners and the like. Furthermore, another form also includes a pharmaceutical composition for application to the mucosa, and for this form of formulations, in order to impart
10 adsoptivity to the mucosa and retentivity, there can be contained additives such as tackifiers, tackiness enhancers, thickeners, thickening agents (such as mucin, agar, gelatin, pectin, caragennan, sodium alginate, locust bean gum, xanthan gum, tragacanth gum,
15 gum arabic, chitosan, pullulan, waxy starch, sucralfate, cellulose and its derivatives (such as hydroxypropyl methyl cellulose), polyglycerin fatty acid esters, acrylic-(meth)acrylic alkyl copolymers or salts thereof, and polyglycerin fatty acid esters). However, dosage
20 forms of pharmaceutical compositions and solvents and additives to be delivered to the living body are not limited to the above, and can be selected, as appropriate, by a person skilled in the art.

For the purpose of ameliorating disease conditions,
25 the above pharmaceutical compositions can be administered orally or parenterally. In the case of oral administration, dosage forms such as granules, powders, tablets, capsules, liquids, syrups, emulsions or suspensions, and elixirs can be selected. In the case of
30 parenteral administration, it can be transnasal agents, and liquids, suspensions or solid formulations can be selected. Other agents in the form of parenteral administration can be in the form of injections, and injections selected may be hypodermic injections,
35 intravenous injections, drip injections, intramuscular injections, intraventricular injections, or intraperitoneal injections and the like. Other

formulations for use in parenteral administration include, for example, transmucosal preparations other than suppositories, sublingual tablets, transdermal preparations, transnasal preparations and the like.

5 Furthermore, intravascular local administration can be performed in embodiments wherein they are contained or applied to stents, or anti-intravascular embolism agents.

The dosage of the above pharmaceutical composition may vary depending on the age, sex, body weight of the
10 patient, condition, therapeutic effect, administration regimen, treatment period, or the types of active ingredients contained in said pharmaceutical composition, but its one dosage is usually in the range of 0.1 - 500 mg per person for adults, and preferably in the range of
15 0.5 mg - 20 mg. The dosage may vary depending on various conditions, and thus a dosage smaller than that described above may sometimes be sufficient, or at other times a dosage greater than the above may be required.

The above antisense oligonucleotide or the RNAi
20 molecule may be integrated downstream to a suitable promoter sequence, and can be administered as the RNA expression vector that provides the effect of antisense oligonucleotide or RNAi. When the expression vector is introduced in a form that can reach the brain of the
25 subject patient, the effect of the antisense oligonucleotide or RNAi effect of the gene allows the expression of a polynucleotide that suppresses the expression of said gene, and the reduced expression level of said gene can attain the therapeutic effect for
30 conditions of suppressed food intake and/or suppressed body weight gain.

<Transgenic animals, animal model of obese/adiposis>

The present invention relates to a transgenic non-human animal comprising a gene encoding the above
35 nesfatin polypeptide etc., or a vector containing them. More specifically, it relates to a transgenic non-human animal that exhibits the state of suppressed food intake

or the state of suppressed body weight gain wherein the expression of said gene is enhanced systemically, preferably, in the hypothalamus. A transgenic non-human animal in which the expression level of the gene encoding the above polypeptide has been artificially enhanced can be used as an animal model that exhibits suppressed food intake and/or suppressed body weight gain.

The present invention relates to a transgenic non-human animal that has introduced therein the above substance (such as antibody, antisense oligonucleotide, and RNAi molecule) that suppresses the activity or the expression of a polypeptide having an activity of suppressing food intake and/or suppressing body weight control, and that exhibits enhanced appetite or enhanced body weight gain. A non-human transgenic animal produced by introducing a gene encoding such a substance can be used as an animal model that exhibits enhanced food intake or enhanced body weight gain. Also, said transgenic non-human animal can be used as an animal model for diseases such as obesity, diabetes mellitus, hypertension, hyperlipidemia, hyperuricemia, fatty liver, cardiac diseases, cerebral vascular diseases, sleep apnea syndrome, orthopedic diseases such as osteoarthritis, menstrual disorders and malignant tumors.

The method of obtaining transgenic animals with a specific gene as the target is known. Thus, transgenic animals can be obtained by a method in which a gene and an egg are mixed and then treated with calcium phosphate, a method in which a gene is directly introduced into the nucleus of the egg in the pronucleus phase with a micropipette under a phase contrast microscope (the microinjection method, U.S. Pat. No. 4873191), a method of using an embryonic stem cell (ES cell), and the like. In addition, there have been developed a method in which a gene is inserted into a retrovirus vector and then infected to the egg, a sperm-vector method in which a gene is introduced into the egg via the sperm, and the

like. The sperm-vector method is a gene recombinant method in which a foreign gene is incorporated into the sperm by adhesion or electroporation and then the sperm is allowed to fertilize the egg to introduce the foreign gene (Lavitrano et M. et al., Cell (1989) 57, 717-723).

Also, if a promoter of which transcription is regulated by a suitable substance such as a drug is used as a promoter for use in the expression vector, the administration of said substance can regulate the expression level of the substance that controls the activity or expression of the gene encoding a foreign nesfatin polypeptide etc. or said polypeptide in a transgenic animal.

Furthermore, the present invention relates to a knock-out animal wherein the entire region or part thereof of the gene encoding the nesfatin polypeptide etc. has been deleted, and a knock-in animal in which said gene has been replaced with another gene is also encompassed in the present invention. For example, a knock-out animal of the nesfatin polypeptide etc. can be used as an animal model that exhibits enhanced food intake and/or enhanced body weight gain.

Furthermore, the present invention also relates to an animal model in which food intake and/or body weight gain has been suppressed comprising a non-human animal in which the above nesfatin polypeptide etc. per se has been administered, or an animal model in which food intake and/or body weight gain has been enhanced comprising a non-human animal in which a substance that suppresses the activity or expression of said polypeptide has been administered. The non-human animal in which food intake and/or body weight gain has been enhanced can be used as an animal model for diseases such as obesity, diabetes mellitus, hypertension, hyperlipidemia, hyperuricemia, fatty liver, cardiac diseases, cerebral vascular diseases, sleep apnea syndrome, orthopedic diseases such as osteoarthritis, menstrual disorders and malignant

tumors.

The animal species of the present invention for use as an animal model can be prepared using any vertebrate other than a human. Specifically, for vertebrates such as mice, rats, rabbits, minipigs, goats, sheep, monkeys, dogs, cats, and cattle, animal models can be prepared by the introduction of a gene or the administration of a substance.

<Method of producing a polypeptide having an activity of suppressing food intake and/or suppressing body weight gain>

The present invention relates to a method of producing the above nesfatin polypeptide etc., using the above transformant that expresses the gene encoding said nesfatin polypeptide etc., or the above transgenic non-human animal and a transgenic plant comprising the gene encoding said polypeptide or a vector containing the gene.

In the above method of producing said polypeptides of the present invention, various modifications and alterations can be made to the above DNA sequence, the plasmid and the virus in order to be compatible with expression, transcription, translation etc. in the transformant or the transgenic non-human animal. For example, due to degeneracy of the genetic code, nucleotides can be substituted through the entire coding region of protein. Such a sequence can be predicted from the amino acid sequence of the nesfatin polypeptide or nesfatin-1 polypeptide, or from the nucleotide sequence of the gene encoding said polypeptide, and can be assembled by a conventional synthetic method described below. Such a synthetic method can be performed by the method of Itakura et al. (Itakura et al., Science 198:1059, 1977) and the method of Crea et al. (Crea et al., Proc. Natl. Acad. Sci. USA 75:5765, 1978). Thus, the gene encoding the above nesfatin polypeptide etc. for use in the method of producing the polypeptide of the

present invention is not limited to those employing the specifically illustrated nucleotide sequence, the plasmid or the virus.

5 The production of the above polypeptide using a transformant can be performed by culturing said transformant. As the culture medium used for culturing, various media commonly used may be selected as appropriate depending on the host cell adopted, and the culturing may be performed under a condition suitable for
10 the growth of the host cell.

Also, the above polypeptide can be produced in the transformant intracellularly or extracellularly, or on the cell membrane. As other methods of producing polypeptides using a gene encoding the nesfatin
15 polypeptide etc., there can be mentioned a method based on cell-free protein synthesis, a representative of which is an in vitro translation reaction system. In this in vitro translation reaction system, 5' upstream to the gene encoding the nesfatin polypeptide etc., a sequence
20 that controls transcription, preferably SP6 promoter, T3 promoter, T7 promoter etc. may be added, and the gene is transcribed into the cell or in vitro to prepare a RNA molecule encoding the nesfatin polypeptide etc., and a cell extract for in vitro transcription prepared from
25 wheat germ, E. coli, reticulocytes etc. to carry out the production. One example of the production can be accomplished by the method described in Sawazaki et al., Protein, Nucleic acid and Enzyme, 2003, Vol. 48, pp. 549-554. Polypeptides produced by such a transformant or
30 cell-free protein synthesis can be separated and purified as desired by various separation processes using the physical properties, chemical properties etc. [see The Japanese Biochemical Society ed., "Biochemistry Databook II", The First edition, the First print, issued by Tokyo Kagaku Dojin Co., Ltd. on June 23, 1980, pp. 1175-1259; Arakawa et al., Biochemistry (USA), issued on December
35 16, 1986, Vol. 25, No. 25, pp. 8274-8277 (1986); Langley

et al., European Journal of Biochemistry (Germany),
issued on March 2, 1987, Vol. 163, No. 2, pp. 313-321].
Said methods specifically include, for example,
conventional reconstitution treatment, treatment with a
5 protein precipitating agent (salting out),
centrifugation, the osmotic shock method, ultrasonic
disruption, ultrafiltration, gel filtration, various
chromatographic methods such as adsorption
chromatography, ion exchange chromatography, affinity
10 chromatography, and high performance liquid
chromatography (HPLC), dialysis, and combinations
thereof, and the like. Purification using affinity with
the above polypeptide can use, for example, the above-
mentioned antibody that binds to the above polypeptide,
15 and can be accomplished by desorption of said polypeptide
and said antibody.

The above method of producing the polypeptide of the
present invention can also be accomplished by producing a
protein in which an affinity tag was fused to said
20 polypeptide in a transformant or a transgenic non-human
animal, and then separating and purifying said affinity
tag-fused protein. By expressing said affinity tag-fused
protein, affinity purification using this tag can be
performed. As said affinity tag, there can be mentioned
25 glutathione S-transferase (GST), poly-Histidine (His tag,
Sisk et al., J. Virol. (USA), issued in February 1994,
Vol. 68, No. 2, pp. 766-775) and FLAG tag (Hopp et al.,
Biotechnology, issued in 1988, Vol. 6, pp. 1204-1210).

In the case of a GST-fused protein, in which the
30 above polypeptide was fused to GST, the above polypeptide
can be produced using a glutathione-immobilized carrier,
i.e. said polypeptide can be purified using absorption
and desorption of GST and the glutathione bound carrier,
and then cleaving the GST portion from the GST-fused
35 protein and purifying it.

In the case of a His tag-fused protein, the above
polypeptide can be produced using a metal ion chelate

carrier, i.e. said polypeptide can be purified using absorption and desorption of the His tag and said metal ion chelate carrier, and then cleaving the His tag portion from the His tag-fused protein and purifying it.

5 In the case of a FLAG tag-fused protein, the above polypeptide can be produced using a carrier to which anti-FLAG tag antibody is bound, i.e. said polypeptide can be purified using absorption and desorption of the FLAG tag and said metal ion chelate carrier, and then
10 cleaving the FLAG tag portion from the FLAG tag-fused protein and purifying it.

 The nesfatin-1 polypeptide can also be prepared by treating the nesfatin polypeptide obtained in the above-mentioned method with a proteolytic enzyme such as
15 protein convertase, fractionating the digest by a reverse phase chromatography, and confirming and collecting fragments containing the nesfatin-1 polypeptide by mass spectrometry etc. The altered nesfatin-1 can also be obtained in a similar manner.

20 The thus obtained nesfatin polypeptide etc. of the present invention can also be modified at the N-terminal after translation, and such modified polypeptide molecules are also encompassed in the present invention. For example, a polypeptide of which N-terminal has been
25 converted to pyroglutamine can be obtained by expressing the above nesfatin polypeptide etc. of the present invention etc. so that the N-terminal becomes a glutamine residue, and treating the polypeptide obtained under an acidic condition of a 5-10% acetic acid solution (Park et
30 al., Proceedings of the National Academy of Sciences of the United States of America (USA), issued in March 1991, pp. 2046-2050). A peptide of which N-terminal has been acetylated can be obtained by expressing the above
35 nesfatin polypeptide etc. of the present invention so that the N-terminal becomes any amino acid having an α -amino group, and treating the polypeptide obtained with sulfo-NHS-acetate or acetic anhydride. Such a method of

modifying the N-terminal of the polypeptide after translation is well known in the art. In addition, the nesfatin polypeptide etc. of the present invention can also be treated with a fluorescent substance to be
5 modified (Hermanson et al., Bioconjugate Techniques (USA) issued by Academic Press in 1996).

A transgenic non-human animal can also be used to produce the above nesfatin polypeptide etc. According to such a method, for example, fractions containing the
10 peptide of interest can be collected as a liquid component separated from the solid component, or as a liquid component extracted with an aqueous solvent or an organic solvent from processed products obtained by cutting, milling, or fractionating an organ, tissue,
15 blood, milk etc. harvested from a transgenic non-human animal having a gene encoding the above nesfatin polypeptide etc. or a vector comprising said gene. The peptide of interest can be separated and purified from said fractions by various separation procedures using the
20 above-mentioned physical properties, chemical properties etc.

It is also possible to use a transgenic plant to produce the above nesfatin polypeptide etc. The method can be performed referring to Japanese Unexamined Patent
25 Publication (Kokai) No. 2003-116385 "A transgenic plant comprising a gene encoding Japanese encephalitis vaccine". Also, a transgenic plant can produced by referring to Japanese Unexamined Patent Publication (Kokai) No. 2002-17186 "Transgenic Plants". From the
30 tissue of leaves, stems, roots, fruits, rinds, sprouts and petals of such a transgenic plant or callus tissue derived from such a tissue, or optionally from processed products obtained by cutting, peeling, milling, or compressing of said tissue, the polypeptide of interest
35 can be collected in an extract with an aqueous solvent or an organic solvent, a squeeze obtained by the compression or an oil, and furthermore said polypeptide can be

separated and purified by various separation procedures using the above-mentioned physical properties, chemical properties etc.

5 The above polypeptide of the present invention can also be obtained by chemical synthesis. In this case, a commonly used peptide synthetic method such as solid-phase synthesis or liquid-phase synthesis can be used. For the condensation and protection of amino acid protecting groups in peptide synthesis and the
10 elimination of the protecting groups after synthesis, a known method can be used (Izumitani et al., "Basis and Experiment of Peptide Synthesis (PEPUTIDO GOSEINO KISOTO JIKKENN)", issued by Maruzen Co., Ltd. in 1975, Hitoshi Yajima - The Japanese Biochemical Society ed.,
15 "Biochemistry Experiment Series (SEIKAGAKU JIKKENN KOUZA) 1, Chemistry of Protein IV", issued by Tokyo Kagaku Dojin Co., Ltd. in 1977). It is also possible to synthesize the entire peptide sequence at one time, while a method can be used in which partial peptides of said protein are
20 synthesized separately and then the partial peptides are condensed (The Japanese Biochemical Society ed., "New Biochemistry Experiment Series (SINSEIKAGAKU JIKKENN KOUZA), Protein IV, Synthesis and Expression", issued by Tokyo Kagaku Dojin Co., Ltd. in 1991).

25 Though the α -amine of amino acids commonly used in peptide synthesis is usually protected with a tBoc group or a Fmoc group, the peptide to be finally obtained may retain the protecting group or may be deprotected. As needed, the deprotected amino terminal of the peptide may
30 be modified with pyroglutamic acid, an acetyl group or a formyl group (Hermanson et al., Bioconjugate Techniques (USA) issued by Academic Press in 1996). In a specific example, the above polypeptide of the present invention having glutamine at the N-terminal is synthesized, and
35 said peptide can be cyclized by treating with a dilute acid such as a 5-10% acetic acid to convert to pyroglutamic acid (Park et al., Proceedings of the

National Academy of Sciences of the United States of America (USA), issued in March 1991, pp. 2046-2050).

Also, as needed, synthetic peptides in which an amide group or a fluorescent substance has been added at the C-terminal of the polypeptide after synthesis can be prepared. For example, in order to obtain a synthetic peptide having introduced therein an amide group at the C-terminal by solid-phase synthesis, a commercial resin that allows the amidation of the C-terminal can be used in a reaction of cleaving the peptide from the solid-phase carrier (resin). Also, modification with fluorescent substances etc. at the C-terminal can be carried out by a known method (Hermanson et al., Bioconjugate Techniques (USA) issued by Academic Press in 1996).

<Assay method of predicting or diagnosing the state of food intake control and/or body weight control>

The present invention relates to a diagnostic method for use in the judgment of diseases associated with food intake control and/or body weight control in mammals, the monitoring and prediction of severity and progress of disease conditions, the judgment of prognosis and the administration of the above pharmaceutical composition and the like. Thus, the present invention relates to a method of predicting or diagnosing the state of food intake control and/or body weight control using a sample derived from a mammal to be tested, which method comprises comparing the amount contained of a nucleic acid molecule (a nucleic acid molecule comprising a nucleotide sequence set forth in any of SEQ ID NOs: 10-12, 18-20, 44-46, 74-82, 104-106 and 116-124) encoding nesfatin, the nesfatin polypeptide etc. (a polypeptide comprising an amino acid sequence set forth in any of SEQ ID NOs: 3, 6, 9, 13-15, 39-41, 65-73, 101-103 and 107-115) in said sample with that in a sample derived from a normal individual. A normal individual as used herein refers to a normal or untreated individual.

Specifically, the present invention relates to an assay method of predicting or diagnosing the state of food intake control and/or body weight control, which method comprises the steps of comparing the amount
5 contained of a nucleic acid molecule comprising a nucleotide sequence set forth in any of SEQ ID NOs: 10-12, 18-20, 44-46, 74-82, 104-106 and 116-124 in a sample derived from a living body of a mammal to be tested with that in a biological sample derived from a normal
10 individual, and detecting the decreased or the enhanced expression of said gene.

As biological materials to be used, there can be mentioned blood, urine, cerebrospinal fluid, saliva, brain tissues harvested from biopsy etc. with blood being
15 most preferred. Blood for use as a sample includes whole blood, or plasma or serum derived from whole blood. Methods of collecting these biological samples are known. Preparations such as lysates of these biological samples can also be used as the sample. Alternatively, mRNA
20 extracted from the preparations may be used as a sample for determining mRNA corresponding to the above gene. For the extraction of lysates of biological samples or mRNA, commercially available kits can be conveniently used. Alternatively, liquid biological samples such as
25 blood and cerebrospinal fluid can be diluted as needed with a buffer etc. to prepare samples for determining protein or gene.

The amount contained of a nucleic acid molecule comprising a nucleotide sequence set forth in any of SEQ
30 ID NOs: 10-12, 18-20, 44-46, 74-82, 104-106 and 116-124 in a biological sample of a mammal can be determined by using a polynucleotide comprising the nucleotide sequence of said nucleic acid molecule or an oligonucleotide with a length of at least 18 bases comprising a nucleotide
35 sequence complementary to the complementary chain as a PCR primer or a probe. In this case, a skilled in the art can design a primer or a probe suitable for an

application, using variety of computer program. Such a polynucleotide or an oligonucleotide may be bound to a suitable label or immobilized to a suitable support depending on the assay format. Such a PCR primer or a probe can be recombinantly or synthetically produced, or may be produced by any means available to a person skilled in the art. For example, the PCR primer that can be used in the present invention include, but not limited to, SEQ ID NO: 22 (forward primer) and SEQ ID NO: 23 (reverse primer). As the probe that can be used in the present invention include, but not limited to, one that is obtained by labelling a fragment amplified using the above primer with the DNA sequence of SEQ ID NO: 21 as the template (see Working Example 2).

The measured values of expression level of a nucleic acid molecule comprising a nucleotide sequence set forth in any of SEQ ID NOS: 10-12, 18-20, 44-46, 74-82, 104-106 and 116-124 can be corrected using a known method. By performing correction, the expression level of a nucleic acid molecule comprising a nucleotide sequence set forth in any of SEQ ID NOS: 10-12, 18-20, 44-46, 74-82, 104-106 and 116-124 can be compared between the biological sample to be tested and the biological sample from a normal individual. The correction of the measured values may be carried out based on the measured value of the expression level of a gene (such as the housekeeping gene) of which expression level does not greatly vary. Examples of the gene of which expression level does not greatly vary include, for example, β -actin and GAPDH.

When mRNA is used in the determination of expression level of a nucleic acid molecule comprising a nucleotide sequence set forth in any of SEQ ID NOS: 10-12, 18-20, 44-46, 74-82, 104-106 and 116-124, a format compatible with the method of diagnosis may be carried out as appropriate. When, for example, mRNA extracted from the biological tissue is used, the RT-PCR method or the Northern blotting method etc. can be selected, and when

tissue specimens are used, the in situ hybridization method etc. can be selected. For example, Working Example 8 discloses that in situ hybridization method indicates that an expression of the nesfatin gene is
5 suppressed in rats enhanced appetite by fasting, and the expression is enhanced in a state of suppressed appetite by food intake.

When the expression of a gene comprising a nucleotide sequence set forth in any of SEQ ID NOs: 10-
10 12, 18-20, 44-46, 74-82, 104-106 and 116-124 in a test sample was detected to be decreased compared to that of a biological sample derived from a normal individual, the state of enhanced food intake or enhanced body weight gain is predicted or diagnosed, and furthermore the onset
15 of a disease selected from obesity, diabetes mellitus, hypertension, hyperlipidemia, hyperuricemia, fatty liver, cardiac diseases, cerebral vascular diseases, sleep apnea syndrome, orthopedic diseases, menstrual disorders and malignant tumors can be predicted or diagnosed. On the
20 other hand, when the expression of said gene in a biological sample was found to be increased, the mammal tested is in the state of suppressed food intake or suppressed body weight gain is predicted or diagnosed, and furthermore, cibophobia, functional dyspepsia, or the
25 disease conditions of suppressed food intake or suppressed body weight gain resulting from cancer, inflammatory diseases, decreased functions of the pituitary, the thyroid, the adrenal etc., post-surgery, or excessive stress and the like can be predicted or
30 diagnosed.

Furthermore, the present invention relates to a method of predicting or diagnosing the state of food intake control and/or body weight control, which method comprises a step of detecting the decreased state or the
35 enhanced state of the amount contained of the nesfatin polypeptide etc. (a polypeptide comprising an amino acid sequence set forth in SEQ ID NOs: 3, 6, 9, 13-15, 39-41,

65-73, 101-103 or 107-115) by comparing the amount contained of said polypeptide in biological sample of a mammal with that in a biological sample derived from a normal individual. Said method can be carried out by
5 determining the nesfatin polypeptide etc.

As biological samples to be used, there can be mentioned blood, urine, cerebrospinal fluid, saliva, brain tissues harvested from biopsy etc. with blood being most preferred. Blood for use as a sample includes whole
10 blood, or plasma or serum derived from whole blood. Methods of collecting these biological samples are known. Preparations such as lysates of these biological samples can also be used as the sample. For the preparation of lysates of biological samples, commercially available
15 kits can be conveniently used. Alternatively, liquid biological samples such as blood and cerebrospinal fluid can be diluted as needed with a buffer etc. to prepare samples for determining protein or gene.

In order to determine the nesfatin polypeptide etc.,
20 an immunological method for said polypeptide can be used in which an antibody that binds to the nesfatin polypeptide etc. can be used. Such an antibody includes those mentioned above, and specifically there can be mentioned an antibody obtained by immunizing a peptide
25 derived from the nesfatin polypeptide obtained in Working Examples 3 and 10, or an antibody obtained by immunizing a peptide derived from the nesfatin-1 polypeptide or the nesfatin-1M30 polypeptide. These antibodies are not limiting, and any polyclonal antibody or monoclonal
30 antibody against the nesfatin polypeptide etc. can be used without specific limitation.

The antibody for use in the present invention may be bound to a suitable label depending on the assay format, or said antibody may be immobilized to a suitable support
35 depending on the assay format.

An immunological method can be carried out as appropriate in a format compatible with the method of

diagnosis. For example, when blood samples or lysates are used, the ELISA method, the RIA method, the Western blotting method etc. can be selected, and when tissue specimens are used, immunohistochemical methods etc. may
5 be selected. In a more specific example, the Western blotting method as shown in Examples, the immunohistochemical method as shown in Working Examples 4 and 11 can be mentioned, and it is possible to construct an assay system for the nesfatin polypeptide etc. using
10 appropriate using antibody. For example, the Western blotting method is shown in Working Example 3, immunohistochemical methods in Working Examples 4 and 9, and the ELISA method (competitive EIA method) in Working Example 21.

15 When the amount contained of the nesfatin polypeptide or the nesfatin polypeptide etc. in a biological sample was found to be decreased compared to that of a biological sample derived from a normal individual, the state of enhanced food intake or enhanced
20 body weight gain is predicted or diagnosed, and furthermore the onset of a disease selected from obesity, diabetes mellitus, hypertension, hyperlipidemia, hyperuricemia, fatty liver, cardiac diseases, cerebral vascular diseases, sleep apnea syndrome, orthopedic
25 diseases, menstrual disorders and malignant tumors is predicted or diagnosed. On the other hand, when the amount contained of said polypeptide in a biological sample was found to be increased, the state of suppressed food intake or suppressed body weight gain is predicted
30 or diagnosed, and furthermore, cibophobia, functional dyspepsia, or the disease conditions of suppressed food intake or suppressed body weight gain resulting from cancer, inflammatory diseases, decreased functions of the pituitary, the thyroid, the adrenal etc., post-surgery,
35 or excessive stress and the like can be predicted or diagnosed.

<Kit for use in the method of predicting or

diagnosing the state of food intake control and/or body weight control>

5 The present invention also relates to a kit for use in the method of predicting or diagnosing the above state of food intake control and/or body weight control. Such a kit includes, for example, a kit for detecting the amount expressed of the gene encoding nesfatin etc. (a gene comprising a nucleotide sequence set forth in any of SEQ ID NOs: 10-12, 18-20, 44-46, 74-82, 104-106 and 116-124) or a kit for detecting the amount contained of the nesfatin polypeptide etc. (a polypeptide comprising an amino acid sequences set forth in any of SEQ ID NOs: 3, 6, 9, 13-15, 39-41, 65-73, 101-103 and 107-115).

10 The kit for detecting the amount expressed of the gene encoding the nesfatin etc. includes at least one of a PCR primer, a probe or a DNA chip for detecting said gene.

15 The PCR primer contained in the kit of the present invention refers to an oligonucleotide with a length of at least 18 bases comprising the nucleotide sequence of a gene encoding nesfatin etc. or a nucleotide sequence complementary to the complementary chain thereof, and would be prepared as appropriate based on the base sequence of a gene encoding nesfatin etc. described herein by a person skilled in the art. For example, the PCR primers contained in the kit of the present invention can include, but are not limited to, SEQ ID NO: 22 (forward primer) and SEQ ID NO: 23 (reverse primer). The probe contained in the kit of the present invention includes, but not limited to, one that is obtained by labelling a fragment amplified using the above primer and the DNA sequence of SEQ ID NO: 21 as the template. The DNA chip contained in the kit of the present invention can be prepared by immobilizing the above probe onto a substrate such as glass.

20 In addition, the kit of the present invention may include, as additional elements, a buffer for diluting

reagents and biological samples, positive controls, negative controls, a substrate for determining the label, a reaction vessel, an instruction describing the assay protocol etc. These elements may be mixed in advance as needed. Also, as needed, preservatives and antiseptics may be added to each element.

The kit for determining the amount produced of the nesfatin polypeptide etc. includes an antibody that recognizes said polypeptide, a standard peptide and at least one modified peptide for competitive binding reaction.

The antibody contained in the kit of the present invention, similarly to the antibody used the above method, may be, but is not limited to, an antibody that recognizes the nesfatin polypeptide etc.

The standard peptide contained in the kit of the present invention is used for constructing a calibration curve that demonstrates a dose dependent binding of the above antibody to the nesfatin polypeptide etc. Such a standard polypeptide includes, for example, a polypeptide recognized by the above antibody such as the nesfatin polypeptide etc.

The modified peptide for competitive binding reaction, contained in the kit of the present invention, refers to a peptide recognized by the above antibody, wherein the peptide has an ability of competing with the nesfatin polypeptide or the like in biological samples to be determined for binding to said antibody. Also, the modified peptide for competitive binding reaction is usually used by labelling it as appropriate.

The kit of the present invention can determine the amount of the nesfatin polypeptide etc. contained in a biological sample by, for example, (1) constructing a calibration curve obtained from the standard peptide and the above antibody in the presence of the above modified peptide for competitive binding reaction, (2) adding the above antibody in the presence of the above modified

peptide for competitive binding reaction in the biological sample, and (3) determining the amount bound of the modified peptide for competitive binding reaction and the antibody using the standard calibration curve.

5 It is also possible to determine the amount of the nesfatin polypeptide etc. using two kinds of antibodies that recognize the nesfatin polypeptide or the like and that do not inhibit each other. In this case, the primary antibody is usually immobilized on a solid-phase,
10 and the secondary antibody is suitably labelled for use.

 The kit of the present invention can determine the amount of the nesfatin polypeptide etc. contained in a biological sample by, for example, (1) constructing a standard calibration curve by adding the standard peptide
15 to the immobilized primary antibody and reacting the peptide with the primer, and then adding a labelled secondary antibody, (2) adding and reacting the biological sample to the similarly immobilized primary antibody and then adding the labelled secondary antibody,
20 and (3) determining the amount bound of the secondary antibody in the reaction with the biological sample using the calibration curve.

 In addition, the kit of the present invention may include, as additional elements, a buffer for diluting
25 reagents and biological samples, positive controls, negative controls, a substrate for determining the label, a reaction vessel, an instruction describing the assay protocol etc. These elements may be mixed in advance as needed. Also, as needed, preservatives and antiseptics
30 may be added to each element.

 <Method of screening a candidate therapeutic compound>

 The present invention relates to a method of screening a therapeutic or preventive candidate compound
35 for diseases or conditions associated with food intake control and/or body weight control, said compound having an effect of food intake control and/or body weight

control.

Thus, the present invention relates to a method of screening a therapeutic or preventive agent having an effect of suppressing food intake and/or suppressing body weight gain, said method comprising the steps of contacting a test substance with a mammalian cell, and detecting the induced expression of the gene (a gene comprising a nucleotide sequence set forth in any of SEQ ID NOs: 10-12, 18-20, 44-46, 74-82, 104-106 and 116-124) encoding nesfatin etc. in said cell or an increase in the amount of the nesfatin peptide etc. (a polypeptide comprising an amino acid sequence set forth in any of SEQ ID NOs: 3, 6, 9, 13-15, 39-41, 65-73, 101-103 and 107-115) contained in said cell or extracelullarly secreted out of the cell. It has been demonstrated that the suppression of food intake or the suppression of body weight gain is required in obesity, diabetes mellitus, hypertension, hyperlipidemia, hyperuricemia, fatty liver, cardiac diseases, cerebral vascular diseases, sleep apnea syndrome, orthopedic diseases, menstrual disorders and malignant tumors. By the above screening method, a therapeutic or preventive agent for said diseases can be obtained.

On the other hand, the present invention also relates to a method of screening a therapeutic or preventive agent having an effect of enhancing food intake and/or enhancing body weight gain, said method comprising the steps of contacting a test substance with a mammalian cell, and detecting the suppressed expression of the gene encoding nesfatin etc. in said cell or a decrease in the amount of the nesfatin peptide etc. contained in said cell or extracellularly secreted out of the cell. It has been demonstrated that the enhancement of food intake or the enhancement of body weight gain is required in cibophobia, functional dyspepsia, or the state of suppressed food intake or suppressed body weight gain resulting from cancer, inflammatory diseases,

decreased functions of the pituitary, the thyroid, the adrenal etc., post-surgery, or excessive stress and the like. By the above screening method, a therapeutic or preventive agent for said diseases can be obtained.

5 Compounds that either increase or decrease the expression level of said polypeptide or a gene encoding it are compounds that act in a promoting or suppressive manner on any step of gene transcription, stabilization or translation, and the secretion, activity expression or
10 stabilization of protein. As used herein, compounds that decrease the expression level of a gene are compounds that have an inhibitory effect on any of these steps.

 The method of screening a candidate therapeutic compound of the present invention for diseases or
15 conditions associated with food intake control and/or body weight control may be carried out in vivo or in vitro. This screening in an in vivo case, for example, may be carried out according to the following steps:

 (1) a step of administering a candidate compound to
20 a test animal;

 (2) a step of determining the expression intensity of the nesfatin polypeptide etc., or the gene encoding said polypeptide in a biological sample from the above test animal; and

25 (3) a step of selecting a compound that enhances, or a compound that decreases, the expression intensity of the nesfatin polypeptide etc., or the gene encoding said polypeptide, as compared to the control that received no candidate compound.

30 A compound that enhances the production of the nesfatin polypeptide etc. and the expression intensity of the gene encoding said polypeptide can be a candidate of a therapeutic agent for suppressing food intake and/or suppressing body weight gain. Substances having such an
35 activity include, but not limited to, a PPAR γ agonist and the like. On the other hand, a compound that suppresses the production of the nesfatin polypeptide etc. or

decreases the expression intensity of the gene encoding said polypeptide can be a candidate of a therapeutic agent for enhancing food intake and/or enhancing body weight gain. Substances having such an activity include, but not limited to, an antibody that binds to the nesfatin polypeptide etc., an antisense oligonucleotide against the gene encoding said polypeptide, a PPAR γ antagonist and the like.

As the test animal for use in the screening method of the present invention, normal animals can be used and pathological animal models for diseases associated with food intake control and/or body weight control can also be used as appropriate. As the example of such pathological animal models, there can be mentioned the C57BL/6L Ham Slc^{AY} obese mice, the Zucker-fa/fa obese rats and the like. Also the transgenic animals of the present invention or animal models that received the nesfatin polypeptide etc., or a substance that suppresses the activity or the expression of said polypeptide can be used.

The determination of expression intensity of the nesfatin polypeptide etc., or the gene encoding said polypeptide can be carried out by an immunological method or by determining mRNA. In the immunological method, a format compatible with the purpose of determination or the biological sample may be used as appropriate. For example, when blood samples or lysates of biological tissues are used as the biological sample, the ELISA method, the RIA method, the Western blotting method etc. can be selected, and when tissue specimens are used, immunohistochemical methods etc. may be selected. When mRNA is to be determined, a format compatible with the purpose of determination or the biological sample may be used as appropriate. When, for example, mRNA extracted from the biological tissue is used, the RT-PCR method or the Northern blotting method etc. can be selected, and when tissue specimens are used, the in situ hybridization

method etc. can be selected.

An example of in vitro screening can be carried out according to the following steps:

5 (1) a step of contacting a candidate compound with a mammal-derived cell or tissue;

(2) a step of determining the expression intensity of the nesfatin polypeptide etc., or the gene encoding said polypeptide in the above cell sample or the tissue sample; and

10 (3) a step of selecting a compound that enhances, or a compound that decreases, the expression intensity of the nesfatin polypeptide etc., or the gene encoding said polypeptide, as compared to the control that received no candidate compound.

15 In this case, a compound that enhances the production of the nesfatin polypeptide etc. and the expression intensity of the gene encoding said polypeptide can be a candidate of a therapeutic agent for suppressing food intake and/or suppressing body weight
20 gain. Substances having such an activity include, but not limited to, a PPAR γ agonist and the like. On the other hand, a compound that suppresses the production of the nesfatin polypeptide etc. or decreases the expression intensity of the gene encoding said polypeptide can be a
25 candidate of a therapeutic agent for enhancing food intake and/or enhancing body weight gain. Substances having such an activity include, but not limited to, an antibody that binds to the nesfatin polypeptide etc., an antisense oligonucleotide against the gene encoding said
30 polypeptide, a PPAR γ antagonist and the like.

As the test cell for use in the screening method of the present invention, a cell separated as appropriate from an animal or an established cell line can be mentioned in the case of cells derived from animals.

35 Their examples include a non-small cell lung cancer cell line, an adipose cell, a brain- or nerve-derived cell, a cell line or the like. As the tissue derived from

animals, organs or tissues harvested as appropriate from the animals can be used, and as an example a brain tissue section in the form containing the hypothalamus can be used.

5 The determination of expression intensity of the nesfatin polypeptide etc., or the gene encoding said polypeptide can be carried out by an immunological method or by determining mRNA. In the immunological method, a format compatible with the purpose of determination or
10 the biological sample may be used as appropriate. For example, when the sample is a cell derived from an animal, flow cytometry or cytoimmunological staining etc. can be used, and when tissue specimens derived from animals are used, immunohistochemical methods etc. may be
15 selected. When preparations such as lysates from samples are used, the ELISA method, the RIA method, the Western blotting method etc. can be selected. When mRNA is to be determined, a format compatible with the purpose of determination, or the biological sample, may be used as
20 appropriate. When, for example, animal-derived cell samples or tissue samples are used as they retain the morphology, the in situ hybridization method etc. can be selected, and when mRNA extracted from said samples is used, the RT-PCR method, the Northern blotting method
25 etc. can be selected.

 The present invention discloses that the PPAR γ agonist enhances the expression of the nesfatin polypeptide etc. and the gene encoding it. Thus, In the above step of "contacting a candidate compound with a
30 mammal-derived cell or tissue", the compounds that suppresses the expression of the nesfatin polypeptide etc., or the gene encoding of said polypeptide of which expression is enhanced can be screened by contacting PPAR γ such as troglitazone with the sample simultaneously with,
35 before or after contact of the candidate compound with the sample, and the compounds that enhance expression of said polypeptide or the gene encoding it, in a mechanism

that does not involve PPAR γ , can be screened.

The compound can also be screened by determining the reaction of the cell that occurs when the nesfatin polypeptide etc. acts on the cell. As an example of such screening, there can be mentioned those that include the following steps:

(1) a step of contacting a candidate compound with a mammal-derived cell or tissue;

(2) a step of contacting the nesfatin polypeptide etc. with said cell or tissue sample; and

(3) a step of determining the presence or absence of the reaction of the cell, or intensity, by the nesfatin polypeptide etc., as compared to the control that was not contacted with the candidate compound.

In this case, a compound that enhances the reactive intensity of the cell by the nesfatin polypeptide etc. can be a candidate of a therapeutic agent for suppressing food intake and/or suppressing body weight gain. Also, a compound that decreases the reactive intensity of the cell by the nesfatin polypeptide etc. can be a candidate of a therapeutic agent for enhancing food intake and/or enhancing body weight gain. Substances having the latter activity include, but are not limited to, an antibody that binds to the nesfatin polypeptide etc.

As the test cell, a cell separated as appropriate from an animal or an established cell line can be mentioned in the case of cells derived from animals. The examples include a non-small cell lung cancer cell line, an adipose cell, a brain- or nerve-derived cell, a cell line or the like. As the tissue derived from animals, organs or tissues harvested as appropriate from the animals can be used, and as an example brain tissue section in a form containing the hypothalamus can be used.

The reaction in the above cell refers to a physical and chemical change induced by the action of the nesfatin polypeptide etc. on the cell, and include, for example,

changes in cell morphology, membrane potential, cell growth, intracellular calcium concentration, migration reaction, intracellular secondary messenger molecule (cAMP, dGMP etc.) concentration and the like.

5 Furthermore, in the screening method of the present invention, a transcription regulatory region that is in a genome of the nesfatin and that controls the expression of said gene is obtained, and can be used in a reporter assay system that employs the nucleic acid molecule of
10 said transcription regulatory region. The reporter assay system refers to an assay system in which a transcription regulatory factor that acts on said transcription regulatory region is screened with the amount expressed of the reporter gene disposed downstream to the
15 transcription regulatory region as an index.

As an example of such screening, there can be mentioned those that include the following steps of (1) to (3):

(1) a step of contacting the candidate compound with
20 a cell having introduced therein a vector comprising the transcription regulatory region of the gene encoding the nesfatin polypeptide and the reporter gene under the control of the transcription regulatory region;

(2) a step of determining the activity of said
25 reporter gene; and

(3) a step of selecting a compound that decreases, or a compound that increases, the expression level of said reporter gene as compared to the control that was not contacted with the candidate compound.

30 In this case, a compound that enhances the expression intensity of the reporter gene can be a candidate of a therapeutic agent for suppressing food intake and/or suppressing body weight gain. Substances having such an activity include, but are not limited to,
35 a PPAR γ agonist and the like. Also, a compound that decreases the expression intensity of the reporter gene can be a candidate of a therapeutic agent for enhancing

food intake and/or enhancing body weight gain. Substances having such an activity include, but are not limited to, an antibody that binds to the nesfatin polypeptide etc., an antisense oligonucleotide against
5 the gene encoding said polypeptide, a PPAR γ antagonist and the like.

As the transcription regulatory region, there can be mentioned a promoter, an enhancer, a CAAT box commonly seen in the promoter region, or a TATA box. As the
10 reporter gene, there can be used the chloramphenicol acetyltransferase (CAT) gene, the luciferase gene, the growth hormone gene and the like.

As the cell into which a reporter gene vector is introduced, there can be mentioned a cell line separated
15 or established from animals, non-mammalian cells such as yeast, and the like.

As the method of introducing the reporter gene vector into the host, there can be illustrated the biological method, the physical method, the chemical
20 method and the like. As the biological method, there can be illustrated a method that employs a virus vector, a method that employs a specific receptor, cell fusion (Sendai virus (HVJ)), polyethylene glycol (PEG), electric cell fusion, micronucleate cell fusion (chromosome
25 transfer)), and the like. The physical method includes a microinjection method, an electroporation method, a method that employs a gene particle gun. The chemical method includes a calcium phosphate precipitation method, a liposome method, a DEAE-dextran method, a protoplast
30 method, an erythrocyte ghost method, an erythrocyte membrane ghost method, and a microcapsule method.

Polynucleotides, antibodies, cell lines, or model animals required for various screening methods of the present invention can be assembled in advance to make
35 kits. These kits may contain, as additional elements, a substrate compound for use in labelling, a medium or a vessel for culturing cells, a positive or negative

standard sample, and instructions describing the method of using the kit. The elements may be mixed in advance as needed, and each element may contain preservatives and/or antiseptics as needed.

5 The present invention will now be explained in more detail hereinbelow with reference to Working Examples, but it should be noted that these Examples do not limit the present invention in any way. In the following
10 Working Examples, unless otherwise specified, each procedure was performed in accordance with the methods described in "Molecular Cloning" (Sambrook, J., Fritsch, E.F. and Maniatis, T., issued by Cold Spring Harbor Laboratory Press in 1989), or when commercially available reagents or kits are used, they were used as described in
15 the instructions attached in the commercially available products. In experiments using animals, unless otherwise specified, animals (male rats) were kept under an illumination cycle of 12 hours of the light period from 6 a.m. to 6 p.m. and 12 hours of the dark period from 6
20 p.m. to 6 a.m. the next morning at 22°C, and the experiment was carried out under the authority of the animal experiment facility of Gunma University.

WORKING EXAMPLES

Working Example 1

25 **Cloning of the gene induced by stimulation with the PPAR γ agonist**

 In order to identify the gene that acts on food intake control and/or body weight control among the genes of which expression is regulated by PPAR γ , genes that are
30 specifically induced when SQ-5 cells (mainly derived from humans) are stimulated with the PPAR γ agonist were cloned, and from them, genes that are likely to function as a secretory factor were selected.

 The method of obtaining genes of which expression is
35 specifically induced by PPAR γ in non-small cell lung cancer was carried out according to the method of Satoh

et al., Oncogene (England), 2002, Vol. 21, pp. 2171-2180. The method is briefly described below. From each of SQ-5 (Riken BioResource Center RBC0110) cultured for 48 hours under stimulation with 10^{-4} M of troglitazone (Sankyo Co., Ltd.), a PPAR γ agonist, and SQ-5 cells not stimulated with troglitazone, poly(A)+RNA (mRNA) was prepared, and was subjected to a reverse transcription reaction to prepare double stranded cDNA. A subtraction method was carried out in which the gene expressing in common cDNA (driver) using the cells being expressed in the non-stimulated cells is subtracted from cDNA (tester) prepared from the cells stimulated with troglitazone using the double stranded cDNA obtained. The subtraction method was carried out using fragments obtained by cleaving the tester and driver cDNA with a restriction enzyme RsaI and by the Clontech's PCR-based cloning kit according to the attached protocol. The residual cDNA after subtraction was subjected to electrophoresis on a 1% agarose gel in TAE buffer (40mM Tris-acetate, 1mM EDTA), and then cDNA with a length corresponding to 0.5-2.0 kb was collected and ligated to the PGEM^(R)-T Easy vector using the pGEM^(R)-T Easy Vector System I (Promega Corp., Cat. No. A1360) according to the attached protocol. The ligated vector was introduced into a E. coli strain DH5 α and allowed to form colonies on an agar medium prepared with a LB medium containing 50 μ g/ml ampicillin, and the colonies obtained were picked up to obtain clones containing cDNA of the gene of which expression is specifically induced by troglitazone stimulation.

Each clone was identified by analyzing the nucleotide sequence of the cloned cDNA. E. coli of each clone obtained was cultured overnight in a 10 mL of the LB medium containing 50 μ g/ml ampicillin, and a plasmid was prepared from the cells using the QIAGEN's QIAprep^(R) Spin Miniprep kit according to the attached protocol.

For the plasmid of each clone obtained, a reactant

for nucleotide sequence analysis was prepared using the T7 primer (Promega Corp., Cat. No. Q5021) and the BigDye Terminator Cycle Sequencing FS Ready Reaction kit (Cat. No. 4303149) of Applied Biosystems according to the
5 attached protocol, and its nucleotide sequence was analyzed using the ABI337 type DNA Sequencer (Perkin-Elmer).

By subjecting the nucleotide sequence of cDNA of each clone as a query to the BLAST method with the
10 sequences on the EMBL and the Genbank nucleic acid database, a sequence was obtained for which the full-length cDNA sequence was known.

From the full-length cDNA sequence obtained in the above process, cDNA encoding a protein having a signal
15 peptide was analyzed by the Signal P software in order to select secretory factors.

<Result>

Clones obtained by the subtraction of cDNA of the SQ-5 cells not stimulated with troglitazone using the
20 cDNA obtained from the SQ-5 cells stimulated with troglitazone were 596 clones. For 213 clones among them, remarkable homology was noted with the registered sequences in the nucleic acid sequences in EMBL and Genbank. Furthermore, by the analysis of signal
25 peptides, 9 clones were suggested to have a signal peptide sequence. One of the 9 clones was the human NEFA gene for which a function had not been identified (SEQ ID NO: 1).

Working Example 2

30 Induction of the NEFA gene expression in a cultured cell line by PPAR γ agonist stimulation

In order to confirm the induction of the NEFA gene expression by troglitazone, cell lines HTB185 and SQ5 expressing PPAR γ and an adipose cell line 3T3-L1 were
35 analyzed for the expression of the NEFA gene by Northern blotting.

SQ-5 (Riken BioResource Center RBC0110) was

subcultured in a RPMI 1640 medium (Invitrogen-BRL, Cat. No. 11875-085) containing 10% bovine fetal serum (Invitrogen-GIBCO). Human cerebrospinal blastocytoma cell line HTB185 cells (D283 Med: ATCC HTB185) and mouse fetal fibroblast cell line (precursor adipose cell line) 3T3-L1 cells (ATCC[®] CL-173) of which differentiation was induced with insulin, dexamethasone and IBMX were subcultured in a DMEM medium (Invitrogen-GIBCO, Cat. No. 11955-040) containing 10% bovine fetal serum. 10⁶ cells from each cell suspended in 10 ml of the medium were plated in a dish with a diameter of 10 cm. After the cells adhered to the substrate, DMSO alone (control) or troglitazone (Sankyo Co., Ltd.) were added to a concentration of 10⁻⁴ M, 10^{-4.5} M or 10⁻⁵ M, and cultured under the condition of 5% CO₂ at 37 °C for 24 hours or 48 hours. For each experiment group, cells treated in 5 dishes were scraped from the substrate after a certain period, and total RNA was extracted and collected using ISOGEN (Nippon Gene, Cat. No. 317-02503) according to the method described in the protocol.

A probe for detecting the NEFA gene was prepared using a 565 bp DNA fragment set forth in SEQ ID NO: 21 by labelling with in vitro transcription using a SP6 polymerase. Using a plasmid extracted from the mouse brain cDNA library (Invitrogen Corp., Cat. No. 10655-25) as the template and the following primers at a concentration of 0.2 μM, amplified DNA was prepared by 35 cycles of reaction comprising denaturation at 94°C for 1.5 minute, annealing at 58°C for one minute and amplification at 72°C for 2 minutes (Takara Bio Inc., Takara Ex Taq[™] polymerase, Cat. No. RR001A 2.5U was used).

PCR primers for preparing the NEFA probe:

Forward primer: 5-CCAGTGGAAAATGCAAGGAT-3 (SEQ ID NO: 22)

Reverse primer: 5-TCTTTGCTTCCGGGATGATTA-3 (SEQ ID NO: 23)

After the purity of the amplified DNA product obtained was confirmed by a 2% agarose gel electrophoresis using the TAE buffer, it was subcloned into the pGEM^(R)-T Easy vector using the pGEM^(R)-T Easy Vector System I (Promega Corp., Cat. No. A1360) according to the attached protocol, and introduced into the E. coli DH5 α to form colonies on an agar medium prepared with the LB medium containing 50 μ g/ml ampicillin. Several colonies obtained were each picked up, and the E. coli was cultured overnight in 10 ml of the LB medium containing 50 μ g/ml ampicillin. From the cells, plasmid was prepared using the QIAGEN's QIAprep(R) Spin Miniprep kit according to the attached protocol. A reactant for nucleotide sequence analysis was prepared using the T7 primer (Promega Corp., Cat. No. Q5021) or the SP6 primer (Promega Corp., Cat. No. Q5011) and the BigDye Terminator Cycle Sequencing FS Ready Reaction kit (Cat. No. 4303149) of Applied Biosystems according to the attached protocol, and its nucleotide sequence was analyzed using the ABI337 DNA Sequencer (Perkin-Elmer). A clone that has a sequence corresponding to the predicted sequence and in which the 5'-end of the amplified NEFA-derived gene faces the T7 promoter side of the vector was made a clone for obtaining a plasmid for preparing the probe. The clone obtained was cultured overnight in a 10 mL of the LB medium containing 50 μ g/ml ampicillin, and a plasmid was prepared from the cells using the QIAGEN's Plasmid Mini kit according to the attached protocol. The prepared plasmid was cleaved with a restriction enzyme NcoI, extracted with phenol, purified with phenol/CIAA extraction, and after precipitating with ethanol, it was dissolved at 1 μ g/ml in a ribonuclease-free TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) to prepare a template plasmid DNA for preparing the probe. A labelled RNA probe was prepared from 1 μ g of the template plasmid DNA using Promega's Riboprobe^(R) System-SP6 with 2.5 μ M [α -

³²P]UTP (Amersham Biosciences Inc., Cat. No. PB10203, 3000 Ci/mM, 10 mCi/ml) and 20U of RNA synthase SP6 (contained in the kit) according to the protocol attached to the kit.

5 For electrophoresis for Northern blotting, total RNA obtained from each cell was subjected to electrophoresis using a 1.2% agarose gel prepared in a MOPS buffer (20 mM 3-(N-morpholino)-propanesulfonic acid, 5 mM sodium acetate, 0.5 mM EDTA) containing 0.66 M deionized
10 formaldehyde at 100 V for 2 hours. Using a 10-fold concentration of SSC (150 mM NaCl, 15 mM sodium acetate), RNA was transferred from the gel after electrophoresis to a nylon membrane (Perkin-Elmer Inc., Gene Screen Plus^(R) Hybridization Transfer Membrane). To the transferred
15 membrane, RNA was immobilized by UV using Stratagene's Stratalinker. Then, the nylon membrane was subjected to prehybridization in a prehybridization solution (0.25 M NaCl, 20 mM Tris-HCl, pH 7.5, 2.5 mM EDTA, 1% SDS, 0.5% bovine serum albumin, 0.5% polyvinyl pyrrolidone, 0.5%
20 Ficoll, 50% deionized formamide) at 43°C for 3 hours, and to hybridization in a hybridization solution (0.25 M NaCl, 20 mM Tris-HCl, pH 7.5, 2.5 mM EDTA, 1% SDS, 0.5% bovine serum albumin, 0.5% polyvinyl pyrrolidone, 0.5% Ficoll, 50% deionized formamide, 10% dextran sulfate)
25 containing 1,000,000 cpm/ml of the labelled RNA probe at 43°C overnight. After rinsing the nylon membrane after hybridization with a two-fold concentration of SSC, it was washed twice under a stringent condition of 0.2-fold concentration of SSC containing 0.1% SDS at 60°C for 30
30 minutes. The nylon membrane after washing was exposed overnight to an X-ray film (Kodak, XAR film) in the presence of an intensifying screen (Cronex, Lightning Plus), and the labelled RNA probe that hybridized was detected.

35 <Result>

The result of Northern blotting analysis of the

induction of the NEFA gene with troglitazone (TGZ) in a cell line is shown in Fig. 1. Lanes 1-4 represent results for the human medulloblastoma cell line HTB185 cells that are expressing PPAR γ . Lanes 1 and 3 show the detection of the NEFA gene expression with the RNA of cells cultured (24 hours and 48 hours, respectively) without the TGZ stimulation of the HTB185 cells, while lanes 2 and 4 show the detection of the NEFA gene expression with the RNA of cells stimulated 10^{-4} M troglitazone for 24 and 48 hours, respectively. As a result, only a slight expression of the NEFA gene was noted in the cells not stimulated with TGZ (lanes 1 and 3), whereas marked induction was noted in the cells stimulated with TGZ for 48 hours (lane 4). Lanes 5 and 6 in the Figure represent the result obtained with SQ-5 that is expressing PPAR γ . Lane 5 shows the detection of the NEFA gene expression with the RNA of the SQ-5 cells cultured without TGZ stimulation, while lane 6 shows the detection of the NEFA gene expression with the RNA of cells stimulated 10^{-4} M TGZ for 24 hours. As a result, a low level of expression was noted in the cells not stimulated with TGZ (lane 5), whereas marked induction of expression of the NEFA gene was noted in the cells stimulated with TGZ for 24 hours (lane 6). Furthermore, lanes 7-10 in Fig. 1 represent results for the mouse fetal fibroblast cell line (precursor adipose cell line) 3T3-L1 cells that are expressing PPAR γ . Lane 7 shows the detection of the NEFA gene expression with the RNA of the 3T3-L1 cells cultured without TGZ stimulation, while lanes 8-10 show the detection of the NEFA gene expression with the RNA of the 3T3-L1 cells stimulated with 10^{-5} M, $10^{-4.5}$ M and 10^{-4} M of TGZ, respectively, for 48 hours. As a result, a considerable amount of expression of the NEFA gene was noted even in the absence of TGZ stimulation (lane 7), and similar expression was obtained when stimulated with 10^{-5} M to 10^{-4} M of TGZ, indicating that

the NEFA gene is being constantly expressed irrespective of the stimulation of PPAR γ .

Working Example 3

5 Preparation of antibody against NEFA and the demonstration of expression in the rat brain

The secretory protein expressed in the adipose cells is also expressed in the brain, and thus has been proposed as the Brain-Adipose Axis that is likely to have an effect of controlling food intake (Masatomo Mori: 10 Adipose Research (Shibou Kenkyu), 2004, Vol. 10, pp. 117-119, Shimizu H. and Mori M.: Nutritional Neurosci 8:7-20, 2005). Thus, an antibody against NEFA that is expressed in the adipose cells and the brain tumor cells to investigate the localized expression of NEFA in the 15 hypothalamus.

For the preparation of the antibody against NEFA, a synthetic peptide (SEQ ID NO: 24: synthesized at ATP K.K.) in which Cys was attached to the C-terminal of a sequence from His at position 141 to Ser at position 152 20 of the amino acid sequence (SEQ ID NO: 8) of the rat precursor NEFA polypeptide (said peptide is hereinafter referred to as the NAP peptide) was used as the antigen. NAP peptide: N-HisLeuAsnHisGlnAsnProAspThrPheGluSerCys-C (SEQ ID NO: 24).

25 Said synthetic NAP peptide was conjugated to keyhole limpet hemocyanin (KLH) using the Imject^(R) Maleimide Activated Mariculture Keyhole Limpet Hemocyanin of Pierce Inc. according to the attached protocol. 0.2 mg of the peptide obtained was used for one immunization per 30 rabbit. For immunization, 0.25 ml of the conjugate solution (conjugate concentration: 1 mg/ml) and an equal amount of Freund's complete adjuvant H-37 Ra (Wako Pure Chemical - Difco, Cat. No. 528-00031) were mixed, and 50 μ l each was intradermally injected at 8 different places 35 on the shaved back of New Zealand White rabbits (purchased from Imai Experimental Animal Testing Site). Similar immunization was performed further 4 times every

two weeks. One week after the last immunization, part of the blood was collected, and antibody titer in the serum was confirmed by the ELISA method employing the immobilized peptide conjugate. On the next day, the animals were sacrificed to collect whole blood. From the blood obtained, serum was prepared. From the serum rabbit IgG was purified using the DEAE Sepharose FF (Amersham Biosciences Inc., Cat. No. 17-0709-10) according to a standard method. The purified rabbit IgG was affinity-purified using a peptide-immobilized column that was prepared from 1 mg of the NAP peptide using the SulfoLink kit (Pierce Inc., Cat. No. 44895), according to the protocol attached to the kit.

Using the obtained antibody (anti-NAP polyclonal antibody) against the NAP peptide, the expression of the NEFA polypeptide in the rat brain was analyzed by Western blotting.

Protein extracts from the rat brain was prepared by homogenizing the hypothalamus (1.7 g) of 8 week-old Wistar rats (Nippan Charles River) in 5 ml of the extraction buffer (50 mM Tris-HCl, pH8.0, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40). The rat brain lysate obtained was mixed with an equal amount of the Laemmli sample buffer (Bio-Rad Inc., Cat. No. 161-0737) containing 5% β -mercaptoethanol for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and heated to 100°C for 5 minutes, and then centrifuged in a microcentrifuge at an ordinary temperature at 15,000 rpm for 10 minutes to collect the supernatant. 20 μ l of the supernatant was electrophoresed on a polyacrylamide gel (Bio-Rad Inc., Ready gel 10-20%, Cat. No. 161-J390V) using the Tris-glycine/SDS buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3) at 100V for 1 hour, and then transferred to a nitrocellulose membrane. The membrane was blocked in 3% gelatin/TBS at room temperature for 1 hour, and washed three times in TBS-0.05% Tween and once in TBS. It was

then allowed to react overnight at room temperature in a 1% gelatin/TBS solution containing 1 μ g/ml of anti-NAP polyclonal antibody. On the next day, it was washed three times in TBS-0.05% Tween and once in TBS, and then
5 was reacted with anti-rabbit IgG goat polyclonal antibody-peroxidase conjugate (Cappel Inc., Cat. No. 674371) diluted to about 1 μ g/ml in 1% gelatin/TBS solution at room temperature for 1 hour. It was then washed for three times in TBS-0.05% Tween and twice in
10 TBS, and was allowed to develop color using the HRP color development kit (Bio-Rad Inc., Cat. No. 170-64631) at room temperature for five minutes, and the bands were detected

<Result>

15 Fig. 2 A shows the schematic diagram of the structure of the NEFA polypeptide and the position of the peptide, which was used for preparing the antibody, on NEFA, and Fig. 2 B shows the result of Western blotting of the protein extracts from the rat brain with a
20 polyclonal antibody prepared by immunization with the NAP peptide. In the Western blotting of the protein extracts from the rat brain with a polyclonal antibody prepared against the NAP peptide, a single band was observed at a molecular weight of about 47.5 kd (Fig. 2 B). This
25 revealed that the antibody against NAP recognizes the full-length NEFA and that NEFA is being expressed in the rat brain.

Working Example 4

**Study on the expression site of nesfatin in the rat
30 brain hypothalamus**

In order to analyze the expression site of NEFA in the rat brain hypothalamus associated with the control of food intake, an immunohistochemical analysis of the sections of the rat brain was carried out. Eight week-
35 old Wistar rats (purchased from Nippon SLC) were anesthetized by an intraperitoneal injection of 40 mg/kg of pentobarbital sodium, and then thoracotomy was

performed to inject 50 ml of physiological saline (0.85% NaCl) into the heart, and then PBS containing 4% paraformaldehyde (20 mM phosphate buffer, 150 mM NaCl, pH 7.2) was injected and circulated using a 400 ml peristaltic pump to perfuse and fix the brain. The brain was then extracted, and the portion containing the hypothalamus was excised, and was fixed overnight at 4°C in PBS containing 4% paraformaldehyde. The fixed brain sample was immersed in PBS containing 10% sucrose for 4 hours, PBS containing 10% sucrose for 4 hours, and PBS containing 20% sucrose overnight at 4°C. Then the brain sample was immersed in an OCT compound and frozen in dry ice-acetone to prepare the embedded block. From the block prepared, sections of 6 µm in thickness were prepared at -20°C using a cryostat, and then were air-dried on a silane-coated slide (MAS coat slide prepared by Matsunami). Tissue sections on the slide were treated with 3% hydrogen peroxide for 5 minutes, and then washed twice in PBS for 5 minutes. Furthermore, the tissue sections were blocked by treating with 10% goat normal serum (Nichirei Corp., Cat. No. 426042) at room temperature for 10 minutes, and then were reacted to 1 µg/ml of a polyclonal antibody (Working Example 3) against the NAP peptide diluted in PBS containing 0.5% BSA at room temperature for 1 hour (Ab group). After washing the slide in PBS, the slide was reacted with the Histofine Simple Stain Rat MAX PO (Nichirei Corp., Cat. No. 414181), and after three times-washing in PBS for 5 minutes per each, color was developed using the Simple Stain DAB solution (Nichirei Corp., Cat. No. 415172). The slide after color development was washed in water, and then stained with the Meyer-Hematoxylin (Muto Pure Chemicals Co., Ltd., Cat. No. 3000), washed in water, dehydrated in alcohol and cleared in xylene, and mounted using a non-aqueous mounting agent (Nichirei Corp., Cat. No. 415141) with a cover slip. The brain section on the

slide after mounting was examined with a microscope.

<Result>

Fig. 3 shows a micrograph (200 × magnification) of
histoimmunochemical staining with the anti-NAP peptide in
5 the tissue section at the hypothalamus and the third
ventricle (3V) regions of the rat's brain. Specific
staining was seen in Arc: arcuate nucleus, PVN:
paraventricular nucleus, LH: lateral hypothalamic area,
SON: supraoptic nucleus of the hypothalamus in the
10 figure, indicating the expression of NEFA in these sites.
From the foregoing, it was demonstrated that the NEFA
polypeptide is expressed in the rat hypothalamus,
specifically in the arcuate nucleus, the paraventricular
nucleus, the lateral hypothalamic area and the supraoptic
15 nucleus.

Since the expression of the NEFA polypeptide encoded
by the NEFA gene was noted at sites associated with the
control of food intake in the hypothalamus, said
polypeptide was named nesfatin.

20 **Working Example 5**

Preparation of recombinant nesfatin

In order to confirm the properties of nesfatin
expressed in the nerve nucleus of the hypothalamus
associated with food intake, recombinant nesfatin was
25 prepared in E. coli. The recombinant matured mouse
nesfatin (SEQ ID NO: 26) was purified by expressing a
fusion protein (recombinant mouse GST-NEFA: SEQ ID NO:
25) in which glutathione S-transferase (GST) was bound to
the N-terminal of mouse matured mouse nesfatin (SEQ ID
30 NO: 6), and treating the fusion protein with a protease.
The level of homology on the amino acid level of the rat
nesfatin and the mouse nesfatin is as high as 96.5%.

A gene for expressing recombinant mouse GST-NEFA was
prepared using a plasmid extracted from the mouse brain
35 cDNA library (Invitrogen Corp., Cat. No. 10655-25) as the
template and using the primers described below (0.25 μM

each) in a denaturation reaction at 94°C for 1.5 minute, followed by five cycles of reaction comprising denaturation at 94°C for 0.5 minute, annealing at 60°C for 0.5 minute, and extension at 72°C for 2 minutes and then
5 30 cycles of reaction comprising denaturation at 94°C for 0.5 minute, annealing and extension at 72°C for 2 minutes, to prepare the amplified DNA (Stratagene's PfuTurbo^(R) polymerase, Cat. No. 600250 was used).

10 PCR primers for preparing recombinant matured mouse NEFA cDNA

Forward primer: 5-TTGGATCCGTTCTATCGATGTGGACAAGAC-3 (SEQ ID NO: 27)

Reverse primer: 5-TTGC GGCCGCTTATGTGTGTGGCTCAA ACTTCAG-3 (SEQ ID NO: 28)

15 The PCR product obtained was purified by QIAGEN's MinElute PCR Purification Kit, cleaved with restriction enzymes BamHI and NotI, and an about 1.3 kb band was excised by a 2% agarose gel-TAE electrophoresis and the DNA fragment was collected (QIAGEN's MinElute Gel
20 Extraction Kit, Cat. No. 28604 was used). The 1.3 kb DNA fragment was ligated to pGEM-11Zf(+) vector (Promega, P2411) that had been cleaved with restriction enzymes BamHI and NotI, which was transformed to E. coli strain DH5 α (Takara Bio Inc., Cat. No. 9057) and was allowed to
25 form colonies on an agar medium comprising an LB medium containing 50 μ g/ml ampicillin.

Several of the colonies obtained were picked up, and for the plasmid prepared from each clone the nucleotide sequence was analyzed using pUC/M13 forward primer
30 (Promega Corp., Cat. No. Q5391), pUC/M13 reverse primer (Promega Corp., Cat. No. Q5401), two kinds of primers mSQ1 (5-CCTGAACCAACCAGAAATCC-3: SEQ ID NO: 29) and mSQ2 (5-AGACTGATGGATTGGACC-3: SEQ ID NO: 30) in the sequence of the mouse NEFA gene, and using the BigDye Terminator
35 Cycle Sequencing FS Ready Reaction kit (Cat. No. 4303149) of Applied Biosystems and the ABI337 type DNA Sequencer

(Perkin-Elmer). Clones that were shown to have a nucleotide sequence corresponding to SEQ ID NO: 11 by the nucleotide sequence analysis were cultured overnight in 10 ml of the LB medium containing 50 µg/ml ampicillin, and plasmid was prepared from the cells using the QIAGEN's QIAprep^(R) Spin Miniprep kit according to the attached protocol.

The plasmid was cleaved with restriction enzymes BamHI and NotI, was electrophoresed on a 2% agarose TAE gel, and the 1.2 kb band was excised to collect DNA. The collected DNA fragments were ligated to the pGEX-4T-1 vector (Amersham Biosciences Inc., Cat. No. 27-4580-01) that had been cleaved with restriction enzymes BamHI and NotI, and which was transformed to E. coli strain BL21 (Amersham Biosciences Inc., Cat. No. 27-1542-01) using the Gene Pulser Xcell electroporation system (Bio-Rad Inc., Cat. No. 165-2660J1) so as to allow colonies to be formed on an agar medium prepared with the LB medium containing 50 µg/ml ampicillin. Recombinant E. coli obtained by transformation was confirmed for the presence or absence of an insert by PCR using the pGEX5' Sequencing Primer and the pGEX3' Sequencing Primer (Amersham Biosciences Inc., Cat. No. 27-1410-01 and 27-1411-01), and the insertion of the NEFA gene was confirmed by PCR using the PCR primer set for preparing the NEFA probe used in Working Example 2. Clones for which the matured NEFA cDNA was confirmed to be integrated into the pGEX-4T-1 vector were used to express the recombinant mouse GST-NEFA protein. The clones were cultured overnight at 37°C with 10 ml of a LB medium containing 100 µg/ml ampicillin, and 5 ml of it was added to 500 ml of a LB medium containing 100 µg/ml ampicillin and cultured by shaking at 37°C until the absorbance at a wavelength of 600 nm became 0.6 (some of the bacteria were sampled out: Preinduced bacteria). To the medium was added isopropyl-β-D-thiogalacto-pyranoside (IPTG:

Gibco BRL) to a final concentration of 1 mM, and was further cultured by shaking at 37°C for 3 hours. After culturing, the medium containing the cells were centrifuged at 5,000g at 4°C for 15 minutes to collect the cells, which were suspended in 25 ml of PBS. The suspension was frozen at -80°C for about 1 hour, and then thawed quickly at 37°C, and the cells were subjected to sonication for 30 seconds x five times to disrupt the cells. It was followed by centrifugation at 10,000g at 4°C for 30 minutes to collect the supernatant (the precipitated fraction was sampled out: Post-sonicated pellet).

The supernatant was applied to a 5 ml-scale GSTrapFF column (Amersham Biosciences Inc., Cat. No. 17-5131-01) equilibrated with PBS using the AKTA-FPLC liquid chromatography system (Amersham Biosciences Inc.), and then the column was washed with 50 ml of PBS (flow rate: 1 ml/min). Then the elution buffer (10 mM reduced glutathione, 0.4 M Tris-HCl, pH 8.0, 0.2 M NaCl) was run at a flow rate of 1 ml/min to collect 1 ml fractions, and fractions containing protein as determined by absorbance at a wavelength of 280 nm were collected (the fraction having the highest absorbance at 280 nm: Purified GST-NEFA). A portion of each of the Preincubation bacteria fraction, the Post-sonicated pellet fraction, and the Purified GST-NEFA fraction was dissolved in the Laemmli sample buffer (Bio-Rad Inc., Cat. No. 161-0737) containing 5% β -mercaptoethanol, treated at 100°C for 5 minutes, and centrifuged in a microfuge at 15,000 rpm for 5 minutes to obtain a supernatant, which was subjected to a polyacrylamide gel electrophoresis (Bio-Rad Inc., Ready gel 10-20%, Cat. No. 161-J390V) at 100 V for 1 hour using a Tris-glycine/SDS buffer and then stained in a Coomassie brilliant blue stain (Bio-Rad Inc., Bio-Safe CBB G-250, Cat. No. 161-0786). For most of the Purified GST-NEFA fraction, reduced glutathione was removed from the sample

using liquid chromatography with the HiTrap Desalting column (Amersham Biosciences Inc., Cat. No. 17-1408-01) equilibrated with PBS, and the sample was applied again to a 5 ml-scale GStrapFF column equilibrated with PBS.

5 After the column was washed with 25 ml of PBS at a flow rate of 1 ml/min, 15 ml of the protease reaction buffer (50 mM Tris-HCl, pH7.5, 150 mM NaCl, 1 mM EDTA, 1 mM DTT) was run into the column, and 4.6 ml of the protease reaction buffer containing 20 U/ml thrombin (Amersham Biosciences Inc., Cat. No. 27-0846-01) was applied to the
10 column. When the entire volume entered the column, the pump of the liquid chromatography was stopped, and reaction with protease was carried out at room temperature (22°C) overnight. On the next day, 15 ml of
15 PBS was run through the column at a flow rate of 1 ml/min to collect fractions, and the fractions collected were run through the HiTrap Benzamidine FF column (Amersham Biosciences Inc., Cat. No. 17-5143-02) to obtain a fraction in which thrombin was removed (Prepurified
20 sample). The fraction was fractionated by chromatography with an anion exchange resin to purify the recombinant matured mouse NEFA polypeptide. The Prepurified sample (20 ml) was diluted 5-fold with the Buffer A (20 mM bis-Tris, pH 6.5, 0.1 M NaCl, 0.1% CHAPS), and applied to the
25 HiTrap Q HP column (Amersham Biosciences Inc., Cat. No. 17-1153-01) equilibrated with the Buffer A, and then washed with the Buffer A at a flow rate of 1 ml/min (1 ml fractions were collected: Washed sample). The washed column was eluted using the Buffer A and the Buffer B (20
30 mM bis-Tris, pH 6.5, 1M NaCl, 0.1% CHAPS) in a linear concentration gradient of 0-50%/60 minutes of the Buffer B at a flow rate of 0.5 ml/min. At this time, 0.5 ml fractions were collected, and fractions containing protein as determined by absorbance at a wavelength of
35 280 nm were collected (Purified sample). A portion of each of the samples (Prepurified sample, Washed sample, Purified sample) obtained in this purification process

was taken, and was mixed with an equal amount of the Laemmli sample buffer containing 5% β -mercaptoethanol followed by heating at 100°C for 5 minutes, and then was subjected to Western blotting in a manner similar to that described in Working Example 3. The fraction was dialyzed against PBS to obtain a purified matured mouse NEFA polypeptide.

<Result>

A of Fig. 4 shows a stained image of SDS-PAGE of a sample obtained by expressing the GST-NEFA fusion protein in recombinant E. coli and purifying the GST-NEFA fusion protein with the GStrapFF column. B shows the result of Western blotting using anti-NAP polyclonal antibody of a sample obtained by excising the matured mouse NEFA polypeptide from the GST-NEFA fusion protein with thrombin and purifying with the HiTrap Q HP column. In A of Fig. 4, a marked increase in the product with a molecular weight 65 kd was noted in the Post-sonicated pellet wherein the cells were disrupted after the expression induction with IPTG in lane 3 relative to the Preincubated bacteria sample in lane 2. As the major 65 kd product was also noted in the fraction (Purified sample) purified with the GStrapFF column, it was demonstrated that the recombinant mouse GST-NEFA fusion protein was expressed in the recombinant E. coli. In B of Fig. 4, several bands were noted in the sample before purification (Prepurified sample) in addition to the band of the matured mouse NEFA polypeptide with a molecular weight of 47.5 kd cleaved with thrombin and the band of the recombinant mouse GST-NEFA fusion protein with a molecular weight of 65 kd. On the other hand, in the Purified sample, a major band with a molecular weight of 47.5 kd and a weaker band with a molecular weight of 65 kd were noted, indicating that an almost pure recombinant matured mouse NEFA polypeptide was obtained.

Working Example 6

Study on the effect of intraventricular

administration of nesfatin on feeding behavior

In order to demonstrate the effect of recombinant nesfatin obtained on the feeding behavior of animals, an experiment was carried out in which unanesthetized rats
5 received intra-cerebroventricular administration into the third ventricle.

Rats used were Wistar rats (purchased from Nippon SLC), and the animals were housed under a cycle of 12 hours of the light period from 6 a.m. to 6 p.m. and 12
10 hours of the dark period from 6 p.m. to 6 a.m. the next morning and fed a powder chew (Nippon Clea, CE-2), and kept at 22°C, and housing was continued under a similar condition during the experiment. For the experiment, among 8-9 week-old male Wistar rats, those individuals
15 weighing 200-250 g were used. For administration, 5 µl of saline (control) or recombinant nesfatin prepared in Working Example 5 dissolved to 0.2 pmol, 0.6 pmol and 3.0 pmol in 5 µl of PBS was administered per individual (N=5 for each group). The rats received intraperitoneal
20 administration of 40 mg/kg body weight of pentobarbital sodium for anesthesia. After the hair was removed from the head, they were fixed in a brain stereotaxic apparatus (David Kopf Instruments, Model 962) and the scalp was incised and a 23G needle (guide cannula) was
25 implanted so as to reach the third ventricle (2.5 mm behind the bregma, 9.5 mm from the surface). One week after implanting the guide cannula, the animals were subjected to the experiment. After recovering from the surgery, a 29G cannula for injection was inserted into
30 the guide cannula under no anesthesia, and 5 µl of PBS or the matured mouse nesfatin solution in PBS was administered at a rate of 1 µl/second. After fixing the cannula as it was for 2-3 minutes after the
administration, the cannula was extracted, and the
35 animals were returned to the individual cages. One, three, and six hours thereafter, the weight of the food

was determined to record the amount of food intake, which was used as an index of feeding behavior. For the test of significant difference, analysis of variance was used.

<Result>

5 The amount of food intake at 0-1 hour after the administration of 0 (PBS alone), 1, 4 and 20 pmol of recombinant nesfatin into the third ventricle is graphically shown in A of Fig. 5, the amount of food intake at 1-3 hours after administration is graphically
10 shown in B of Fig. 5, and the amount of food intake at 3-6 hours after administration is graphically shown in C of Fig. 5. For any of 0-1, 1-3 and 3-6 hours, a reduction in the amount of food intake was noted in a dose dependent manner in the recombinant nesfatin
15 administration group compared to the rats that only received saline. Specifically, in the 20 pmol recombinant nesfatin-administration group, a significant activity of suppressing food intake was noted in any of 0-1 hour/1-3 hours/3-6 hours (each $P<0.01$) as compared to
20 the control group. These data suggested that nesfatin in the brain has an effect of suppressing feeding behavior.

Workig Example 7

**Study on the effect of intraventricular
administration of anti-nesfatin antibody on feeding
25 behavior**

In order to demonstrate the effect of nesfatin on the control of food intake, the effect of administration of an antibody against nesfatin into the third ventricular on feeding behavior was investigated.

30 The antibody used was the anti-NAP peptide antibody (NAP IgG) prepared in Working Example 4, and 5 μ l of antibody diluted in saline was administered to rats (dose 5 μ g). As the control, the same amount and concentration of IgG (control IgG) purified from non-immunized rabbits
35 was administered. Rats used were Wistar rats (purchased from Nippon SLC), and were housed as in Working Example 6. Among 8-9 week-old male Wistar rats, those

individuals weighing 200-250 g were selected. The time of administration was before beginning the light period when there is little feeding behavior, and the method of administration was similar to that in Working Example 6. During the period of 0 -3 hours, 3-6 hours, or 6-9 hours after the intra-celebroventricular administration, the weight of the powder food was determined. For the test of significant difference, analysis of variance was used.

<Result>

For rats that received the intraventricular administration of control IgG or the anti-nesfatin antibody (NAP IgG), the amount of food intake at 0-3 hours after the administration is graphically shown in A of Fig. 6, the amount of food intake at 3-6 hours after administration is graphically shown in B of Fig. 6, and the amount of food intake at 6-12 hours after administration is graphically shown in C of Fig. 6. It was demonstrated that in individuals that received the intra-celebroventricular administration of anti-nesfatin antibody, food intake during 0-3 hours (Fig. A of 6) and 3-6 hours (B of Fig. 6) was promoted in a statistically significant manner (each $P < 0.001$). The feeding amount of the rat from the anti-nesfatin antibody-administration group increased about 9 times during 0-3 hours (A of Fig. 6), and about 10 times during 3-6 hours, respectively, compared to that of the control IgG-administration group during the corresponding periods. However, since the period of 6-9 hours was in the dark period and food intake was started in the control IgG-administration group, no significant difference was noted. Thus, as the administration of the anti-nesfatin antibody promoted food intake by rats, it was demonstrated that the anti-nesfatin antibody suppresses the effect of endogenous nesfatin and that nesfatin is involved in feeding behavior.

Working Example 8

Study on the expression of the NEFA gene in the rat

hypothalamus and the effect of fasting on the expression

An in situ hybridization was carried out to determine the effect of fasting on changes in the expression of endogenous NEFA gene.

5 The probe used for in situ hybridization in the brain tissue was the plasmid for preparing the NEFA probe that was prepared in Working Example 2. Using 1 µg of said plasmid cleaved with a restriction enzyme NcoI and purified, a DIG-labelled cRNA probe was prepared by a
10 reaction of the SP6-RNA transcriptase using the digoxigenin (DIG) RNA labelling kit (Roche Diagnostics K.K., Cat. No. 1175025). The reaction with the SP6-RNA transcriptase was carried out at 40°C for 2 hours. After the reaction, 2 µl of 0.2 M EDTA (pH 7.0) was added to 20
15 µl of the reaction mixture to stop the reaction. After 2.5 µl of 3M LiCl and 75 µl of ethanol cooled to -20°C were added thereto and mixed, it was allowed to stand at -20°C overnight. On the next day, the reaction mixture was centrifuged in a microfuge at 4°C and 15,000 rpm for
20 20 minutes and the supernatant was removed. After adding 50 µl of 70% ethanol to the precipitate and centrifuging again, the supernatant was removed and the precipitate was dried. The precipitate was dissolved in the DEPC-treated water (Nippon Gene K.K., Cat. No. 314-90205), and
25 the amount of RNA was determined by agarose gel electrophoresis, and was diluted in the DEPC-treated water to a RNA concentration of about 0.1 mg/ml. In order to shorten the length (about 450 bases) of the cRNA probe obtained, 10 µl of the DEPC-treated water and 40 µl
30 of carbonate buffer (60 mM sodium carbonate, 40 mM sodium bicarbonate, pH 10.2) were added to 10 µl of the RNA solution at 0.1 mg/ml, and reacted at 60°C for 30 minutes. To the reaction mixture, 60 µl of the neutralization solution (3 M sodium acetate, 1% acetic acid, pH 6.0) was
35 added, and 360 µl of ethanol that had been cooled to -20°C

was further added and mixed, and then centrifuged in a microfuge at 4°C and 15,000 rpm for 20 minutes, which was precooled at -70°C for 30 minutes to be set at 4°C, and the supernatant was removed to obtain the precipitate.

5 After adding 100 µl of 70% ethanol (-20°C) to the precipitate and centrifuging again, the supernatant was removed and the precipitate was dried. The dried precipitate was dissolved in 100 µl of the DEPC-treated water, and the concentration was determined by agarose
10 gel electrophoresis. The concentration of the probe obtained was about 50 µg/ml. The DIG-labelled NEFACRNA probe thus obtained was used for in situ hybridization.

About 8 week-old male Wistar rats (purchased from Nippon SLC) (body weight 220-250 g) were grouped into the
15 individuals (Normal) that can take the powder food ad libitum, the individuals (Starvation) that were kept 48 hours with drinking water alone without any food, the individuals (Re-feeding) that were kept without food for 36 hours and then were given the food ad libitum for 12
20 hours, and each brain was perfusion fixed with PBS containing 4% paraformaldehyde in a method similar to that described in Working Example 5 and the brain was extracted. From the extracted brain, a portion that contains the hypothalamus was excised, which was further
25 fixed in PBS containing 4% paraformaldehyde at 4°C overnight. The fixed brain tissue was treated with a cryostat in a method similar to that described in Working Example 4 to prepare sections of 10 µm in thickness, which were placed on a silane-coated glass slide. The
30 slide glass having a brain tissue section placed thereon was placed in an incubator and treated at 50°C for 2 minutes, and then the sections were dried at room temperature for 30 minutes. The sections were fixed again in PBS containing 4% paraformaldehyde at room
35 temperature for 7 minutes, and then washed in PBS for 3 minutes and in 2-fold concentrated SSC twice for 5

minutes.

An in situ hybridization solution (4-fold concentrated SSC, 10% dextran sulfate, 1-fold concentrated Denhardt's solution, 2 mM EDTA, 50% deionized formamide, 500 µg/ml trout sperm DNA) was delivered so as to cover the brain tissue section on the slide glass, and was prehybridized at 37°C for 1 hour. After the completion of prehybridization, the delivered liquid was discarded, and an in situ hybridization solution containing 200 ng/ml of DIG-labelled NEFA cRNA probe was delivered so as to cover the tissue, and was hybridized in a humid chamber at 37°C for 16 hours. The slide glass having the brain tissue section placed thereon after hybridization was washed in 2-fold concentrated SSC at 37°C for 5 minutes, and then washed at 37°C three times for 5 minutes each in 0.2-fold concentrated SSC containing 60% formamide, and then at room temperature twice for 5 minutes each in 2-fold concentrated SSC.

The probe that hybridized in the brain tissue after washing was detected using the DIG Nucleic Acid Detection kit (Roche Diagnostics K.K., Cat. No. 11175041), and the outline of the method is as follows. The slide glass having the brain tissue placed thereon was washed in 100 mM Tris-HCl (pH 7.5) buffer containing 150 mM NaCl at room temperature for 5 minutes, and blocked using the same buffer (blocking buffer) saturated with the blocking reagent contained in the kit at room temperature for 30 minutes. On the slide glass was placed anti-DIG-alkaline phosphatase conjugate contained in the kit diluted to a concentration of 1/200 in the blocking buffer, and it was reacted at room temperature for 2 hours. The slide glass after the reaction was washed twice in 100 mM Tris-HCl (pH 7.5) buffer containing 150 mM NaCl for 5 minutes, and then washed in the detection buffer (100 Tris-HCl, pH 7.5, 100 mM NaCl, 50 mM MgCl₂) for 10 minutes. Then, the

detection buffer containing 0.18 mg/ml BCIP and 0.34 mg/ml NBT was placed and was reacted at room temperature for 16 hours to develop color. The slide glass was washed in 10 mM Tris-HCl (ph 8.0) containing 1mM EDTA for 5 minutes to stop the color development reaction.

Then the slide glass was washed in distilled water for 5 minutes, and stained in 1% methylene green for 5 minutes, washed in distilled water, dehydrated in alcohol and cleared in xylene, and mounted using a non-aqueous mounting agent (Nichirei Corp., Cat. No. 415141) with a cover slip. It was then examined with a microscope.

<Result>

Relative to the control group shown in A of Fig. 7, there was a marked decrease in the expression of nesfatin mRNA in the paraventricular nucleus (PVN) in the case of 48-hour fasting shown in B of Fig. 7, and as shown in C of Fig. 7, it was demonstrated that the expression of the nesfatin mRNA gene was restored by re-feeding after fasting. These results revealed that endogenous nesfatin is involved in the control of food intake.

Working Example 9

Study on nesfatin expression in the brain of starvated rats

Changes in endogenous nesfatin at fasting were studied by the immunohistological stain method.

About 8 week-old male Wistar rats (purchased from Nippon SLC) (body weight 220-250 g) were grouped into the control group that can take the powder food ad libitum and the fasting group that was fasted for 24 hours, and the brain tissue section of these rats were prepared for the analysis by immunohistological chemistry using anti-nesfatin antibody. The preparation of tissue sections and the immunohistological stain method were carried out in a manner similar to that described in Working Example 4. Furthermore, in order to study the state of activation of nerve cells in the regions of the brain related to food intake, the immunohistochemical analysis

was carried out at the brain tissue of the fasting group using an antibody against c-Fos. The method was carried out using a 500-fold diluted c-Fos (K-25) antibody (Santa Cruz Biotechnology, sc-253) instead of anti-nesfatin antibody in a manner similar to that described above.

<Result>

A micrograph (200 × magnification) of immunohistochemical staining with the anti-nesfatin antibody (NAP Ab) at the hypothalamus of the control group is shown in A of Fig. 8, a micrograph (the same magnification as A) of immunohistochemical staining with the anti-nesfatin antibody (NAP Ab) at the hypothalamus of the fasting group is shown in B of Fig. 8, and a micrograph (the same magnification as A) of immunohistochemical staining with the anti-c-Fos antibody at the hypothalamus of the fasting group is shown in C of Fig. 8. The upper half of the figure shows the image of the paraventricular nucleus, and the lower half shows the image of the arcuate nucleus. In the hypothalamus of the fasting group as compared the control group, staining properties at the paraventricular nucleus (upper half of the figure) and the arcuate nucleus (the lower half of the figure) are markedly decreased, indicating that the expression of nesfatin is decreased in fasting. In a state in which appetite is enhanced, the expression of the c-Fos protein was noted. From this, it is believed, in a state in which appetite is enhanced due to fasting, the expression of nesfatin, thought to exhibit the effect of a suppressed appetite, is decreased and is responsible for the control of appetite.

Working Example 10

Preparation of nesfatin-1, nesfatin-2, nesfatin-3 and nesfatin-2/3 polypeptide, and preparation of antibody against nesfatin-1, nesfatin -3 and nesfatin-2/3 peptide

In Working Examples 3-9, it was demonstrated that nesfatin has an effect the expression in the brain hypothalamus and on food intake control and body weight

control. Also, as the precursor of nesfatin has a signal peptide, nesfatin is likely to be secreted extracellularly out of the cell. Some active proteins are known to be cleaved with the prohormone convertase (PC) in a post-translational process. Thus, it was investigated whether or not the Arg-Arg sequence or the Lys-Arg sequence that are the sequence at the cleavage site of the prohormone convertase are present in the mouse, rat and human NEFA sequence. As a result, it was found that in common with mouse, rat and human NEFA sequence, the Lys-Arg sequence was present at the amino acid positions 107 and 108 from the N-terminal and the positions 199 and 200, and the Arg-Arg sequence is present at positions 188 and 189 (Fig. 9A). Thus, among the nesfatin sequence, a peptide comprising 82 residues corresponding to amino acid Nos. 25 to 106 was named nesfatin-1 (SEQ ID NO: 15), a peptide comprising 79 residues corresponding to amino acid Nos. 109 to 187 was named nesfatin-2 (SEQ ID NO: 16), and a peptide comprising 231 residues corresponding to amino acid Nos. 190 to 420 was named nesfatin-3 (SEQ ID NO: 17). In this case, the DNA-binding region (amino acid Nos. 171-223) which is a known domain structure, is divided into nesfatin-2 and nesfatin-3, the nectin-binding region (amino acid Nos. 213-420), the calcium-binding region (amino acid Nos. 254-265 and 306-317) and Asp/GLu-rich region (amino acid Nos. 306-317) are contained in the sequence of nesfatin-3, but no known domains are contained in the sequence of nesfatin-1 (Fig. 9B). Therefore, the portion of the sequence consisting of the nesfatin-2 and the nesfatin-3 was named nesfatin-2/3 (SEQ ID NO: 47). The synthesis of the rat nesfatin-1 peptide (SEQ ID NO: 15) and the nesfatin-2 peptide (SEQ ID NO: 16) was referred to Yanaihara Institute Inc., where they were synthesized by solid-phase synthesis and purified by reverse phase liquid chromatography. The cDNA prepared from rat brain was amplified, using primers set forth in

SEQ ID NO: 48 and SEQ ID NO: 49, to obtain DNA (SEQ ID NO: 50), and then the DNA was used to prepare nesfatin-3 peptide (SEQ ID NO: 17), by Post Genome Institute Co., Ltd. (Shimizu Y. et al.: Nature Biotech 19: 751-755, 2001), by the cell-free protein synthesis.

The PCR primers used:

Forward Primer: 5'-ATGGAGTATTTAAAAACGCTGAGTGAG-3' (SEQ ID NO: 48)

Reverse Primer: 5'-TTATGTGTGTGGCTCAAACCTCA-3' (SEQ ID NO: 49)

The nesfatin-3 prepared in this manner has a sequence in which Met is added to the N-terminal (SEQ ID NO: 51).

By the same manner, the cDNA derived from rat brain was amplified, using primers set forth in SEQ ID NOs: 52 and 53 to obtain DNA (SEQ ID NO: 50), to obtain DNA (SEQ ID NO: 54), and then the DNA was used to prepare nesfatin-2/3 (SEQ ID NO: 47), by the cell-free protein synthesis.

The PCR primers used:

Forward Primer: 5'-ATGGAAGAAGTAGGAAGACTGAGAA-3' (SEQ ID NO: 52)

Reverse Primer: 5'-TTATGTGTGTGGCTCAAACCTCA-3' (SEQ ID NO: 53)

Also, in order to prepare antibodies for regions other than those for anti-NAP peptide antibodies prepared in Working Example 3, a sequence in which Cys was added to the C-terminal of a sequence corresponding to the amino acid Nos. 24-38 (corresponding to the amino acid Nos. 48-62 of the mouse precursor NEFA polypeptide of SEQ ID NO: 5) of NAP-1 peptide (NAP-1Ab: SEQ ID NO: 32), a sequence corresponding to the amino acid Nos. 1-9 (corresponding to the amino acid Nos. 190-198 of the mouse precursor NEFA polypeptide of SEQ ID NO: 5) of NAP-3, and a sequence in which Cys was added to the C-terminal of a sequence corresponding to the amino acid Nos. 136-149 (corresponding to the amino acid Nos. 325-

338 of the mouse precursor NEFA polypeptide of SEQ ID NO: 5) of NAP3 peptide (NAP-1 Ab: SEQ ID NO: 33 and NAP-1 Ab: SEQ ID NO: 34) were prepared.

NAP-1 Ab: N-

5 ProAspThrGlyLeuTyrTyrAspGluTyrLeuLysGlnValIleCys-C (SEQ ID NO: 32)

NAP-C1 Ab: N-GluTyrLeuLysThrLeuSerGluGluCys-C (SEQ ID NO: 33)

NAP-C2 Ab: N-

10 LysGluPheLeuGluProAspSerTrpGluThrLeuAspGlnCys-C (SEQ ID NO: 34)

The preparation of each peptide of NAP-1 Ab, NAP-C1 Ab and NAP-C2 Ab was referred to ATP Co., Ltd. in which they were synthesized by solid-phase synthesis and
15 purified by reverse phase liquid chromatography. Each peptide of NAP-1 Ab, NAP-C1 Ab and NAP-C2 Ab thus obtained was conjugated to KLH in a manner similar to that described in Working Example 3, and then immunized to rabbits to prepared serum containing antibody to each
20 peptide. From the serum, rabbit IgG was purified using the DEAE column, and named NAP-1 IgG (nesfatin-1 IgG), NAP-C1 IgG (nesfatin-C1 IgG), and NAP-C2 IgG (nesfatin C2 IgG).

Each of the prepared nesfatin-1 and nesfatin-3
25 peptide were subjected to SDS-polyacrylamide gel electrophoresis (12 %), and then Western blotting was carried out for each peptide, by in a manner similar to that described in Working Example 3, using the nesfatin-1 IgG antibody and the nesfatin-C2 IgG antibody,
30 respectively.

<Result>

The nesfatin-1 peptide, nesfatin-2 peptide, nesfatin-3 peptide and nesfatin-2/3 peptide that were synthesized by solid-phase synthesis and purified by
35 reverse phase liquid chromatography were purified by an analytical C18 reverse phase chromatography to a purity of almost a single peak. By collecting a portion of the

peaks and analyzing with mass spectrometry (nesfatin-1 and nesfatin-2) or SDS-polyacrylamide gel electrophoresis (12 % gel) (nesfatin-3 and nesfatin-2/3), it was demonstrated that peptides estimated to be approximately the molecular weight of each peptide of predicted nesfatin-1, nesfatin-2, nesfatin-3 and nesfatin-2/3 was synthesized. In rabbits that were immunized with the conjugate of each peptide of NAP-1 IgG, NAP-C1 IgG and NAP-C2 IgG and KLH, increased in the titer of antibody against these 3 peptides were observed, and antibody (IgG) against each peptide was obtained.

As the results of the Western blotting with the nesfatin-1 IgG after electrophoresis of the nesfatin-1 peptide, a 9.7 kd single band was observed, and as the results of the Western blotting with the nesfatin-C2 IgG after electrophoresis of the nesfatin-3 peptide, a 27.9 kd single band was observed (Figure 9C). Herefrom, it is shown that the nesfatin-1 IgG antibody and the nesfatin-C2 IgG antibody, bond to the nesfatin-1 peptide, the nesfatin-3 (nesfatin-2/3), respectively.

Working Example 11

Immunohistochemical analysis with NAP-1 IgG and anti-PC antibody

In order to demonstrate that nesfatin is processed to produce nesfatin-1, the localization of nesfatin and PC was first analyzed by a double immunohistological stain of the rat hypothalamus with NAP-1 IgG (nesfatin-1 IgG) prepared in Working Example 10 and anti-prohormone convertase antibody (anti-PC).

Brain tissue sections were prepared in the following method. Eight week-old Wistar rats (purchased from CHARLES RIVER LABORATORIES Japan, Inc.) were anesthetized by an intraperitoneal injection of 40 mg/kg of pentobarbital sodium, and then thoracotomy was performed to inject 50 ml of physiological saline (0.85% NaCl) into the heart, and then PBS containing 4% paraformaldehyde (20 mM phosphate buffer, 150 mM NaCl, pH 7.2) was

injected and circulated using a 400 ml peristaltic pump to perfusion fix the brain. The brain was then extracted, and the portion containing the hypothalamus was excised, and was fixed overnight at 4°C in PBS containing 4%
5 paraformaldehyde. The fixed brain sample was immersed in PBS containing 10% sucrose for 4 hours, PBS containing 15% sucrose for 4 hours, and PBS containing 20% sucrose overnight at 4°C. Then the brain sample was immersed in an OCT compound and frozen in dry ice-acetone to prepare
10 the embedded block. From the block prepared, sections of 6 µm in thickness were prepared at -20°C using a cryostat, and then were air-dried on a silane-coated slide (MAS coat slide prepared by Matsunami). Tissue sections on the slide were treated with 3% hydrogen peroxide for 5
15 minutes, and then washed twice in PBS for 5 minutes.

The slide glass having a brain tissue section placed thereon was blocked by treating with 10% goat normal serum (Nichirei Corp., Cat. No. 426042) at room temperature for 10 minutes, and then was reacted to NAP-1
20 IgG (Nesfatin-1 IgG: Working Example 10) diluted 1/500 in PBS containing 5% BSA at room temperature for 1 hour. The slide glass to which antibody was reacted was washed in PBS three times for 5 minutes, and the Histofine Simple Stain Rat MAX PO (Nichirei Corp., Cat. No. 414181)
25 was reacted thereto, and after washing in PBS, color was developed using 4-chloro-1-naphthol (ICN, Cat. No. 980611: 4-chloro-1-naphthol stabilized chromogen). The slide glass after color development was washed in PBS three times for 5 minutes, and then immersed in 0.1M
30 glycine buffer (pH 2.2) for 1.5 hours (said buffer was changed every 30 minutes), and washed in PBS three times for 5 minutes. The slide glass was further blocked with 10% goat normal serum (Nichirei Corp., Cat. No. 426042) at room temperature for 10 minutes. The slide glasses
35 that were treated to this point were divided into two groups: one was reacted to an antibody (PC-1/3: Chemicon,

Cat. No. AB1260) that recognizes prohormone convertase subtypes PC1 and PC3, and the other was reacted to antibody (PC-2: Chemicon, Cat. No. AB1262) that recognizes prohormone convertase subtype PC2 as an
5 antibody solution diluted in PBS containing 0.5% BSA at room temperature for 1 hour. The slide glass was then washed in PBS three times for 5 minutes, and to the slide glass was reacted to goat anti-rabbit IgG-Alexa594 conjugate (Molecular Probes, Cat. No. A11008) diluted
10 1/200 in PBS containing 0.5% BSA at room temperature for 30 minutes. Then the slide glass was washed in PBS three times for 5 minutes and slightly air-dried, and then mounted with glycerol and a cover slip, and the perimeter was sealed with clear manicure to prepare a specimen.
15 The specimen was examined as a phase contrast image of 4-chloro-1-naphthol stain by a cofocal laser microscope (Bio-Rad Inc. MRC-1024 cofocal laser scanning equipment + Nikon's Eclipse E800 upright microscope) and fluorescence with Alexa 594 was examined as a fluorescent image by an
20 excitation beam with the Krypton-Argon laser using a 605±30 nm band filter.

<Result>

In the immunohistochemical image for the rat hypothalamus tissue, a stained image with nesfatin-1 IgG
25 is shown in an upper panel and a lower panel in A of Fig. 10, a fluorescence image with PC-1/3 in the upper panel in B of Fig. 10, and a fluorescence image with PC-2 in the lower panel in B of Fig. 10. The upper panel in A of Fig. 10 and the lower panel in B of Fig. 10, and lower
30 panel in A of Fig. 10 and the lower panel in B of Fig. 10 represent a color image and a fluorescence image at the same field. As shown in the upper panel and the lower panel in A of Fig. 10, cells stained with nesfatin-1 IgG was observed in the neural cells in the rat brain. Many
35 of cells which were stained with anti-PC-1/3 and anti-PC-2 antibody also corresponded to the nesfatin-1 IgG positive cells. This indicated that in cells expressing

nesfatin prohormone convertases PC1/3 and PC2 are simultaneously expressed suggesting the possibility that nesfatin undergoes processing by prohormone convertase.

Working Example 12

5 Study on the effect of intracerebral administration of nesfatin-1, nesfatin-2, nesfatin-3 and nesfatin-2/3 on food intake control

The site of nesfatin of which the activity of food intake suppression is present was investigated.

10 Samples used for intracerebral administration were PBS alone (vehicle group), or nesfatin-1, nesfatin-2, nesfatin-3 or nesfatin-2/3 prepared in Working Example 10 each dissolved in PBS, and 25 pmol was administered per individual into the third ventricle immediately before
15 the dark period. Rats used were Wistar rats (purchased from Nippon SLC) and were housed as in Working Example 6, and among 8-9 week-old male Wistar rats, those individuals weighing 200-250 g were selected and used (N=5). The technique of intraventricular administration and the measurement of the amount of food intake in rats
20 were carried out in a method similar to those in Working Example 6. In the measurement of the amount of food intake, the reduced amount of food at 1 hour after administration (0-1 hr) and 2 hours after administration (1-3 hrs) was determined as food intake.

25 In the same condition above, 1, 5 or 25 pmol of nesfatin-1 as a sample was administered per rat individual into the third ventricle immediately before the dark period. In this experiment, the amount of food
30 intake was determined at 1 hour after administration (0-1 hr).

<Result>

Compared to the control group (Cont), as shown in A of Fig. 11, the intraventricular administration of
35 nesfatin-2, nesfatin-3 and nesfatin 2/3 did not exhibit any significant changes in the amount of food intake (0-1 hr: a-1 in A of Fig. 11; 1-3hr: a-2 in A of Fig. 11A).

On the contrary, the intra-celebroventricular administration of 25 pmol of nesfatin-1 exhibits marked effect on the suppression of feeding. As shown in B of Fig. 11, the intraventricular administration of 1, 5 or 25 pmol of nesfatin-1 per rat individual did exhibit the decreased amount of food intake, with the increased amount of the administered nesfatin-1, compared to the saline group (0). In particular, a significant decreased amount of food intake was observed in the administration of 5 pmol or 25 pmol of nesfatin-1. These results revealed that the activity of suppressing food intake by nesfatin is localized in nesfatin-1.

Working Example 13

Changes in the amount of food intake and body weight in the intraventricular continuous administration of nesfatin-1

Effect of intraventricular continuous administration of nesfatin-1 on changes in the amount of food intake and body weight was investigated.

The administration of nesfatin-1 was carried out into the ventricle by an osmotic pump for 10 consecutive days. Among 8 week-old male Wistar rats (purchased from Nippon SLC), those individuals weighing 200-250 g were used. The rats received intraperitoneal administration of 40 mg/kg body weight of pentobarbital sodium for anesthesia. After the hair was removed from the head, they were fixed in a brain stereotaxic apparatus (David Kopf Instruments, Model 962) and the scalp was incised and a 23G needle (guide cannula) was implanted so that the tip reaches the third ventricle (2.5 mm behind the bregma, 9.5 mm from the surface). One week after implanting the guide cannula, the animals were subjected to the experiment. The osmotic pump used was Alzet's Model 2002. Nesfatin-1 (nesfatin-1 administration group) dissolved in physiological saline that was filter-sterilized with a 0.22 μ m Millex GV filter (Millipore Co. Ltd.), or sterilized physiological saline alone (control

group) were injected into the osmotic pump immediately before use, and were primed in sterilized physiological saline from the day before use. The osmotic pump to which each sample was connected to a tube connected to the injection cannula, and through the guide cannula previously implanted the injection cannula was fixed so that the tip of the cannula reaches the third ventricle of the rat. In this form of use, the sample is injected to the third ventricle at a flow rate of 12 μ l per 24 hours, and in the nesfatin-1 administration group, it is equivalent to 5 pmol of nesfatin-1 per day administered to the ventricle. After confirming the rats into which the osmotic pump was implanted recovered from anesthesia, they were placed in individual cages where they were kept under the condition that they can take the powder chew and water at libitum. The measurement of the amount of food intake started from the day after the cannula and the osmotic pump were implanted, and the weight of the residual food was measured at 9 a.m. every day, and by determining the difference from the weight of the food on the previous day, the amount of food intake of the day (24 hours) was calculated. From day 6 after the start of administration, body weight was measured at the time of measuring the amount of food intake, and changes in body weight were also recorded.

<Result>

As shown in A of Fig. 12, in the nesfatin-1 administration group, a decrease in the amount of food intake was noted from day 1 after the start of administration, and the decrease was maintained during the period of measurement as compared to the control group. Also as shown in B of Fig. 12, the rate of increase in body weight in the nesfatin-1 administration group has significantly declined as compared to the control group.

Working Example 14

Study on food intake control by the intraventricular

administration of anti-nesfatin-1 antibody or anti-nesfatin-3 antibody

In Working Examples 12 and 13, the effect of decreasing the amount of food intake by the intraventricular administration of nesfatin-1 peptide was found. In order to further confirm this fact, NAP-1 IgG (nesfatin-1 IgG) that recognizes nesfatin-1, NAP-C1 IgG (nesfatin-C1 IgG), or NAP-C2 IgG (nesfatin-C2 IgG) that recognizes nesfatin-3 were intraventricularly administered to rat to study its effect on the amount of food intake. To study the relationship between the nesfatin cleaved by prohormone convertase to generate nesfatin-1, and the food intake control, a mutant (KR-AA mutant: Mut) in which a Lys-Arg sequence corresponding to amino acid numbers 84 and 85, among a sequence set forth in SEQ ID NO: 26, is replaced with a Ala-Ala sequence was prepared and investigated effect on the intraventricular administration in rats.

Samples used for intraventricular administration were IgG (control IgG group) purified from unimmunized rabbits with PBS, NAP-1 IgG (nesfatin-1 IgG), NAP-C1 IgG (nesfatin-C1 IgG) or NAP-C2 IgG (nesfatin-C2 IgG) prepared in Working Example 11, which was dissolved in PBS, and 5 μ l (5 μ g) of them was administered to the third ventricle. The rats used were Wistar rats (purchased from Nippon SLC) and were housed as in Working Example 6, and among 8-9 week-old male Wistar rats, those individuals weighing 200-250 g were selected and used. The technique of intraventricular administration and the measurement of the amount of food intake were carried out in a method similar to those in Working Example 7. In the measurement of the amount of food intake, the reduced amount of food at 3 hours after administration (0-3 hr) and 3 hours over 3 hours after administration (3-6 hrs) was determined as food intake.

The preparation of the KA-AA mutant was carried out by altering a nesfatin gene from the mature mouse

prepared in Working Example 5 and by expressing the gene. Two fragments were prepared using the plasmide in which the nesfatin gene from the mature mouse obtained in Working Example 5 was cloned to pGEM-11zf(+), by PCR method. The first fragment was prepared using 5 ng of the plasmid and by carrying out PCR reaction with a set of mNucB2-F360[Sac2Thr] primer and mNucB2[KR-AA] R583 primer.

mNucB2-F360[Sac2Thr]: 5'-

GGTTCCGCGGGTCTGGTTCCGCGTGGTTCTGTTTCCTATCGATGTGGACAAGACCAA-3' (SEQ ID NO: 56)

mNucB2[KR-AA] R583: 5'-

CTTCTTGAGCAGCCAGCTCATCCAGTCTCGTCCTCA-3' (SEQ ID NO: 57)

The PCR reaction comprised a denaturation reaction at 90°C for 1 minute, followed by 20 cycles of reaction comprising denaturation at 98°C for 10 seconds, annealing at 60°C for 30 seconds, and extension at 68°C for 1 minute, to prepare the amplified DNA (Stratagene's PfuTurbo^(R) polymerase, Cat. No. 600250 was used).

The second fragment was prepared using 5 ng of DNA fragment of the plasmid and by carrying out PCR reaction with a set of mNucB2[KR-AA] F612 primer and mNucB2-R1527[NotI] primer.

mNucB2[KR-AA] F612: 5'-

GAGCTGGCTGCTCAAGAAGTAGGAAGACTGCGGGATGCT-3' (SEQ ID NO: 58)

mNucB2-R1527[NotI]: 5'-

GGTTGCGGCCGCACTTTATGTGTGTGGCTCAAAC-3' (SEQ ID NO: 59)

The PCR condition is the same as the amplification condition for the first DNA fragment.

To prepare 2 DNA fragments, the samples obtained by two PCR reactions were added to a reaction mixture so that a concentration of the reaction mixture is 5 µl each per 50 µl. Using 0.25 µM each of His-Thr-For[SpeI] primer and the above mNucB2-R1527[NotI] primer, and a reaction mixture of 1 x concentrated Pfu buffer, 2.5

units of PfuTurbo DNA polymerase (stated above: Strategene) and 0.2 mM of dNTPs (Promega: C1141), a denaturation reaction was carried out at 90°C for 1 minute, followed by 20 cycles of reaction comprising
5 denaturation at 98°C for 10 seconds, annealing at 60°C for 30 seconds, and extension at 68°C for 1 minute, to prepare the amplified DNA.

His-Thr-For[SpeI]: 5'-
GGTTACTAGTGGTTCTGGTCATCACCATCACCATCACTCCGCGGGTCTGGTTCCGCG
10 T-3' (SEQ ID NO: 60).

The PCR product obtained was subjected to 1% agarose gel electrophoresis to excise an amplified band, which was purified with QIAEX-II kit (QIAGEN). The amplified DNA excised was cleaved with restriction enzymes SpeI and
15 NotI, and ligated to pET41a(+) plasmid vector (Novagen) cleaved with restriction enzymes SpeI and NotI, using the Quick DNA ligase kit (New England Biolabs). From the cloned DNA, a clone, which has no errors in mutant
20 positions introduced after analyzing DNA sequences in a similar manner to Working Example 5 and other nucleotide sequences, can be selected to obtain a vector for expressing the KR-AA mutant. The expression and
25 purification were carried out in a similar manner to Working Example 5. The amino acid sequence of the KR-AA mutant obtained is set forth in SEQ ID NO: 61. Wt (normal nesfatin), which was prepared in Working Example 5, was used. 5 pmol each of the KR-AA mutant (Mut) peptide and the normal nesfatin (Wt) peptide were
30 administered to the third ventricle of rats in a similar manner to Working Example 6. One, three, six and 12 hours thereafter, the weight of the food was determined to record the amount of food intake, which was used as an index of feeding behavior.

<Result>

35 In each of the administration into the third intraventricular administration group of control IgG,

NAP-1 IgG (nesfatin-1 IgG), NAP-C1 IgG (nesfatin-C1 IgG) or NAP-C2 IgG (nesfatin-2 IgG), the amount of food intake during 0-1 hour after the administration is graphically shown in a-1 of Fig. 13A, the amount of food intake during 3-6 hours is graphically shown in a-2 of Fig. 13A, and the amount of food intake during 6-9 hours is graphically shown in a-3 of Fig. 13A. In 0-3 hour (a-1 of Fig. 13A) and 3-6 hours (a-2 of Fig. 13A), as compared to the control IgG administration rats, individuals that received NAP-1 IgG (nesfatin-1 IgG) exhibited a significant increase in the amount of food intake ($P < 0.01$ for 0-3 hour, $P < 0.05$ for the 3-6 hours), but in the groups that received NAP-C1 IgG (nesfatin-C1 IgG) or NAP-C2 IgG (nesfatin-C2 IgG), no significant difference was noted as compared to the vehicle group. In the results for 6-9 hours, no significant difference was noted in each of the control IgG group, the NAP-1 IgG (nesfatin-1 IgG) group, the NAP-C1 IgG (nesfatin-C1 IgG) group and NAP-C2 IgG (nesfatin-C2 IgG) group. This result indicated that the function of endogenous nesfatin-1 is inhibited by antibody against nesfatin-1, resulting in the increase of amount of food intake. This demonstrated that a substance that inhibits the function of nesfatin-1 has an effect of enhancing the amount of food intake, in addition to the effect of suppressing food intake by the intraventricular administration of the nesfatin-1 polypeptide as demonstrated in Working Example 13.

Further, the results of an effect on the amount of food intake in rats, in which 5 pmol each of a recombinant mouse nesfatin (Wt) and the mutant (KR-AA mutant: Mut) in which a Lys-Arg sequence corresponding to amino acid numbers 84 and 85 was replaced with an Ala-Ala sequence, was administered to intraventricular in the rats, are shown in Fig. 13B. In Fig. 13B, the amount of food intake at 0-1 hour, 1-3 hour and 3-6 hours after the administration were graphically shown in b-1, b-2, b-3, respectively. As shown in b-1 to b-3, in the rats in

which 5 pmol of Wt (normal nesfatin) was administered, a significant control activity of food intake was observed at each of the period, while no control activity of food intake was observed in the nesfatin (Mut) in which a mutation was introduced into the site cleaved by prohormone convertase. Herefrom, it was demonstrated that the step of nesfatin-1 processed by prohormone convertase is important for nesfatin to functionate.

Working Example 15

Study on the effect of continuous intraventricular administration of antisense RNA against the NEFA gene on food intake and body weight

In order to further investigate the relationship between the expression of the nesfatin (NEFA) gene and the control of food intake and body weight, antisense RNA that suppresses the expression of nesfatin gene was continuously administered into the third ventricle and its effect was investigated.

As the antisense RNA against nesfatin-1 gene, was used a morpholino RNA which sandwiches a translational start site, as follows.

Nesfatin-1 antisense RNA:

5-ATGGTCCTCCACCTCATCTTCAGAG-3 (SEQ ID NO: 31)

The administration of nesfatin antisense RNA was carried out into the ventricle by an osmotic pump for 12 consecutive days using an osmotic pump. Among 8 week-old male Wistar rats (purchased from Nippon SLC), those individuals weighing 200-250 g were selected and used. The rats received intraperitoneal administration of 40 mg/kg body weight of pentobarbital sodium for anesthesia. After the hair was removed from the head, they were fixed in a brain stereotaxic apparatus (David Kopf Instruments, Model 962) and the scalp was incised and a 23G needle (guide cannula) was implanted so that the tip reaches the third ventricle (2.5 mm behind the bregma, 9.5 mm from the surface). One week after implanting the guide cannula, the animals were subjected to the experiment.

The osmotic pump used was Alzet's Model 2002 that was primed in sterilized physiological saline from the day before use. Nesfatin antisense RNA (antisense administration group) dissolved in physiological saline that was filter-sterilized with 0.22 μ m Millex GV filter, or missense RNA (ATcGTgCTCCACgTCATCTaCacAG) sterilized in the same manner that dissolved in sterilized physiological saline (control group) were injected into the osmotic pump immediately before use. The osmotic pump to which each sample was connected to a tube connected to the injection cannula, and through the guide cannula previously implanted the injection cannula was fixed so that the tip of the cannula reaches the third ventricle of the rat. In this form of use, the sample is injected to the third ventricle at a flow rate of 12 μ l per 24 hours, and 40 μ g each of antisense RNA and missense RNA were injected. After confirming the rats into which the osmotic pump was implanted recovered from anesthesia, they were placed in individual cages where they were kept under the condition that they can take the powder food and water at libitum. The measurement of the amount of food intake started from the day after the cannula and the osmotic pump were implanted, and the weight of the residual food was measured at 9 a.m. every day and, by determining the difference from the weight of the food on the previous day, the amount of food intake of the day (24 hours) was calculated. From day 6 after the start of administration, body weight was measured at the time of measuring the amount of food intake, and changes in body weight were also recorded.

On day 12 after the start of administration, the individuals in each group were sacrificed, and the hypothalamus in the brain was removed. Using the sample extracted, the expression of nesfatin was confirmed by Western blotting with nesfatin-1 Ab. Western blotting was carried out in a manner similar to those described in

Working Examples 13 and 12. The concentration of bands stained in Western blotting was determined by densitometry.

<Result>

5 Changes in the amount of food intake during 24 hours for the control group and the antisense administration group to which nesfatin antisense RNA was administered are shown in A of Fig. 14 and changes in body weight are shown in B of Fig. 14. As compared to the control group, 10 an increase in the amount of food intake (A of Fig. 14) was noted in the antisense administration group from the day 1 after the start of administration, and the amount of food intake was always greater than the control group during the measurement period (until day 12 after the 15 start of administration). For changes in body weight (B of Fig. 14), no difference was noted on day 6 after the start of administration between the control group and the antisense administration group, but on day 7 and after a significant increase in body weight ($P<0.05$) was noted in 20 the antisense administration group as compared to the control group, and the difference expanded from day 9 to day 11 of the measurement period. In the Western blotting analysis of nesfatin-1 using the rat hypothalamus sacrificed on day 12, the expression of 25 nesfatin-1 was significantly decreased in the antisense administration group (the band intensity by densitometry was 8.5 ± 0.7 AU) compared to the control group (the band intensity indicated 14.3 ± 1.2 AU, which was determined using densitometry). From the result, it was revealed 30 that the intraventricular administration of nesfatin antisense RNA has an effect of suppressing the expression of nesfatin-1 and of enhancing the amount of food intake and body weight gain.

Working Example 16

35 **Production of recombinant nesfatin-1 by a recombinant**

In order to prepare nesfatin-1 in large quantities,

a method of producing recombinant nesfatin-1 using a recombinant was investigated.

A gene encoding mouse nesfatin-1 was obtained, and an expression vector was constructed by binding the gene of glutathione S-transferase (GST) and histidine tag to the N-terminal of the nesfatin-1 gene, so that a cleavage site (-Leu-Val-Pro-Arg-Gly-Ser-) cleaved using thrombin mediates the amino acid sequence of histidine tag and the amino acid sequence of mouse nesfatin-1 in the protein after translation. The gene of mouse nesfatin-1 was obtained using mouse brain cDNA (Clontech) by two runs of PCR (nested PCR). The first PCR used a forward primer (mNucB2-F337: SEQ ID NO: 35) and a reverse primer (mNucB2-R712: SEQ ID NO: 36) at 100 pM each, Pyrobest DNA polymerase (Takarabio K.K. R005A), the reaction buffer attached and dNTP and was carried out according to the attached protocol. The PCR reaction comprised, after reaction of 90°C for 1 minute, a temperature condition of 30 cycles of 98°C for 10 seconds and 68°C for 1 minute, and then a reaction at 68°C for 2 minutes.

Forward primer (mNucB2-F337): 5'-GCACGCTGAC CGCTC TGGAAG-3' (SEQ ID NO: 35)

Reverse primer (mNucB2-R712): 5'-CAAATGTGTT AGGAT TCTGGTGGTTCA-3' (SEQ ID NO: 36)

Using 0.5 µl of the PCR product obtained, the second run of PCR was carried out using a forward primer (mNucB2-N3[SacI-Thr]) and a reverse primer (mNucB2-R389[NotI]) at 100 pM each, and using Pyrobest DNA polymerase as in the first PCR. The PCR reaction comprised, after reaction of 90°C for 1 minute, a temperature condition of 20 cycles of 98°C for 10 seconds, 60°C for 30 seconds and 68°C for 1 minute, and then a reaction at 68°C for 2 minutes.

Forward primer (mNucB2-N3[SacI-Thr]): 5'-GGTTCCGCGGGTCTGGTTCCGCGTGG TTCTCCTATCGATGTGGACAAGACCAA-3' (SEQ ID NO: 37)

Reverse primer (mNucB2-R589[NotI]): 5'-
GGTTGCGGCCGCTTACCTCT TCAGCTCA TCCAGTCTCG-3' (SEQ ID NO:
38)

5 PCR reaction samples that were subjected to two runs
of PCR were purified with phenol/chloroform extraction,
cleaved with restriction enzymes SacII and NotI, and then
subjected to agarose gel electrophoresis to excise a band
corresponding to a length of about 300 bp, which was
purified with QIAEX-II kit (QIAGEN). The purified PCR
10 product of about 300 bp was ligated to pET41a(+) plasmid
vector (Novagen) cleaved with restriction enzymes SacII
and NotI using the Quick DNA ligase kit (New England
Biolabs). The ligated vector was introduced into an E.
coli strain J409 and the 8 transformants thus obtained
15 were subjected to plasmid extraction on a small scale.
The plasmids thus obtained were analyzed for the
nucleotide sequence of the sequence of the nesfatin-1
gene integrated, using the BigDye Terminator Cycle
Sequencing FS Ready Reaction kit and ABI377 type DNA
20 sequencer (Perkin-Elmer Inc.). As a result, an
expression vector having a gene in which the correct
sequence of nesfatin-1 has been integrated was obtained,
and this was named pET41a(+)GST-His-LVPRGS-mNAP1.

By introducing the pET41a(+)GST-His-LVPRGS-mNAP1
25 into E. coli BL21 (DE3) Codon Plus RIPL and expressing, a
fusion protein (GST-His-LVPRGS-mNAP1) of GST-Histidine
tag-thrombin cleavage sequence-nesfatin-1 was expressed.
Clones obtained by introducing the pET41a(+)GST-His-
LVPRGS-mNAP1 into E. coli BL21 (DE3) Codon Plus RIPL and
30 selecting in a LB medium containing kanamycin was
cultured in 10 ml of the LB medium containing kanamycin
at 37°C. Culturing was stopped when the absorbance at a
wavelength of 600 nm became 0.8. 3 ml of the culture
liquid was inoculated into 100 ml of the LB medium
35 containing kanamycin, which was cultured at 37°C. When
the absorbance at a wavelength of 600 nm became 0.8, 1 ml
of 100 mM IPTG was added to induce the expression of

protein. After adding IPTG, a shaking culture was carried out at 37°C for 3 hours. The culture liquid was centrifuged at 8000 rpm for 20 minutes (4°C) to collect the cells of *E. coli*.

5 From the cells of *E. coli* thus obtained, the GST-His-LVPRGS-mNAP1 fusion protein was extracted, and purified with a nickel chelate column (Ni-NTA agarose). The cells were suspended in 20 ml of the Sonication buffer (50 mM KH₂PO₄, 50 mM NaCl, 2 mM DTT, pH 7.5)
10 containing one-fold concentration of Complete-EDTA free (Roche Diagnostics K.K.) and 0.5-fold concentration of BugBuster (Merck Ltd., Novagen, Cat. No. 70584), and were disrupted by sonication in ice water for 10 minutes. The sonicated sample was centrifuged at 15,000 rpm for 20
15 minutes, and the supernatant was collected. 10 ml of the supernatant obtained was applied to 1 ml Ni-NTA agarose column equilibrated with the Lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, pH 8.0), and washed twice
20 in 10 ml of the Wash buffer (50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole, pH 8.0). The column after washing was eluted twice with 2.5 ml of the Elution buffer (50 mM Na₂HPO₄, 300 mM NaCl, 250 mM imidazole, pH 8.0), and a fraction containing the eluted GST-His-LVPRGS-mNAP1
25 fusion protein was collected. The extraction supernatant from the residual cells was similarly treated, and a fraction containing GST-His-LVPRGS-mNAP1 fusion protein was collected.

 From the GST-His-LVPRGS-mNAP1 fusion protein, the portion of GST and histidine tag was removed, and further
30 purification, GST-His-LVPRGS-mNAP1 fusion protein bound to the GST resin was subjected to thrombin treatment and to purification with reverse phase chromatography in order to remove *E. coli*-derived lipopolysaccharide (LPS) that may act as an inflammatory substance. The buffers
35 at this stage and after were those confirmed to be LPS-free. 7.2 ml of the fraction containing the GST-His-LVPRGS-mNAP1 fusion protein was washed one-fold

concentration of GST Bind/Wash buffer (Merck Ltd.,
Novagen, Cat. No. 70571), which was finally added to the
GST resin (Merck Ltd., Novagen, Cat. No. 70541)
(equivalent to 7.2 ml) suspended in 3 ml of the GST
5 Bind/Wash buffer, and gently stirred at 20°C for 1 hour.
After collecting the resin by centrifugation, said resin
was washed twice in 36 ml of the GST Bind/Wash buffer.
To the washed resin, 3.6 ml of a solution of 20 units/ml
of thrombin in PBS was added and suspended, and reacted
10 at 20°C for 20 hours under gentle stirring. The resin
after the reaction was delivered in 1.8 ml in a filter-
attached cup (Millipore) with a pore size of 0.22 µm,
centrifuged at 3,000 rpm for 2 minutes, and the filtered
thrombin-treated sample was collected. To 450 µl of the
15 thrombin-treated sample was added 50 µl of acetic acid to
prepare a sample for C18 reverse phase chromatography.
The reverse phase chromatography comprised the elution
with a gradient of acetonitrile in 0.1% trifluoroacetic
acid, and the gradient was set at 10% acetonitrile:10
20 minutes/10-20% acetonitrile gradient:60 minutes/30-40%
acetonitrile gradient:40 minutes/40-60% acetonitrile
gradient:5 minutes. Protein eluted from the column was
monitored by measuring absorbance at a wavelength of 280
nm. By examining the fractions eluted with acetonitrile
25 gradient by SDS-PAGE and Western blotting, nesfatin-1 was
found to be eluted at an acetonitrile concentration of
36.2%. Thus, this fraction was collected, lyophilized,
and dissolved again in distilled water for injection,
which was used to determine protein concentration by
30 absorbance and LPS content by Endospacy assay (Seikagaku
Kogyo).

<Result>

From 100 ml of the culture liquid, about 7 mg of
crude GST-His-LVPRGS-mNAP1 was purified with Ni-NTA-
35 agarose, and the recovery of nesfatin-1 that was
thrombin-treated or highly purified by C18 reverse phase

chromatography was 472.5 µg. The amount of LPS contained in the highly purified nesfatin-1 was about 4 pg relative to 1 µg of nesfatin-1. Furthermore, when the highly purified nesfatin-1 was intraventricularly administered to rats in a method similar to that in Working Example 13, the effect of suppressing food intake and/or suppressing body weight gain was noted. This indicated that the production of active nesfatin-1 is possible by expression and purification using a recombinant.

Working Example 17

Study on the effect of nesfatin-1 administration into the third ventricle of Zucker fa/fa rats on food intake control

As described in the conventional technology, many of obese people or patients with adiposis exhibit resistance to leptin, which poses a problem in the pathology and treatment. Thus, using Zucker fa/fa rats (Michael et al., Nature Genetics, Vol. 13, pp. 18-19, 1996), an animal pathological model of leptin-resistance, the effect of nesfatin-1 on the control of the amount of food intake was investigated.

As rats, 8 week-old male Zucker fa/fa (Zucker) rats and Zucker +/+ (Lean) rats as the control animal were purchased from Nippon Charles River, and were housed in a cycle of 12 hours of the light period from 6 a.m. to 6 p.m. and 12 hours of the dark period from 6 p.m. to 6 a.m. the next morning and fed a powder food (Nippon Clea, CE-2), and kept at 22°C, and throughout the experiment period, a similar condition was continued in housing. After preliminary housing of the purchased rats for over one week, individuals weighing 200-250 g were selected from among 9 to 10 week-old individuals.

The sample used for administration was one in which the recombinant mouse nesfatin-1 prepared in Working Example 16 was dissolved to 5 pmol in 5 µl of PBS, and as the control sample physiological saline (Saline) was

used. Five μ l of the samples prepared were administered into the third ventricle of each rat for the group of five each of Zucker rats and the Lean rats per group (the Zucker/nesfatin-1 group, the Lean/nesfatin-1 group, the Zucker/Saline group, the Lean/Saline group). The timing of administration was immediately before beginning the dark period when food intake behavior is enhanced, and the method of administration was similar to that described in Working Example 6.

After intraventricular administration, the amount of food intake was determined by measuring the amount decreased of the powder food for each rat during 0-1 hour, 1-3 hours and 3-6 hours. For test of significant difference, analysis of variance was used.

<Result>

A of Fig. 15 shows the result measured of food intake for the group (the Lean/nesfatin-1 group) in which nesfatin-1 was administered and for the group (the Lean/Saline group) in which physiological saline was administered to the Lean rats, and B of Fig. 15 shows the result measured of food intake for the group (the Zucker/nesfatin-1 group) in which nesfatin-1 was administered and the group (the Zucker/Saline group) in which physiological saline was administered to the Zucker rats. In a result (A of Fig. 15) for the control animal, Lean rats, the amount of food intake during 0-1 hour and during 1-3 hours was decreased in the nesfatin-1 administration group relative to the Saline administration group ($P < 0.001$). No difference was noted in the amount of food intake during 3-6 hours. In Zucker, a leptin-resistant animal, as in Lean, a significant reduction in the amount of food intake during 0-1 hour and during 1-3 hours was noted in the nesfatin-1 administration group relative to the Saline administration group ($P < 0.001$). During 3-6 hours, a significant reduction ($P < 0.05$) was also noted (B in Fig. 15). The above suggests that the effect of suppressing

food intake by nesfatin-1 was exhibited without the effect of leptin and it is considered effective under a leptin-resistant condition.

Working Example 18

5 **Study on the effect of intraperitoneal
administration of nesfatin-1 on the control of the amount
of food intake in mice**

10 It was demonstrated in the experiment of
intraventricular administration in rats that nesfatin and
nesfatin-1 are involved in controlling the amount of food
intake. In order to study the effect on other animal
species, the administration experiment on mice was
carried out. Considering practical utility as a
pharmaceutical, it was thought to be important that the
15 peripheral administration is also effective, and thus an
intraperitoneal administration was selected as an
administration route. Furthermore, an administration
experiment was also carried out on the Agouti-yellow
(c57BL/6J-A^y/a) mice which are animal model of obesity in
20 which the function of suppressing food intake by
MC3R/MC4R has been inhibited by the excessive expression
of the Agouti protein.

 The experimental animal was 7 week-old male ICR mice
purchased from Japan SLC, Inc., and were housed after
25 purchase in a cycle of 12 hours of the light period from
6 a.m. to 6 p.m. and 12 hours of the dark period from 6
p.m. to 6 a.m. the next morning with free access to a
pellet food (Nippon Clea, CE-2), kept at 22°C, and housing
was continued in a similar condition during the
30 experiment period. The mice purchased were subjected to
preliminary housing for over one week, individuals
weighing 35-40 g were selected among 8 to 9 week-old
individuals and were used in the experiment.

 The sample used in the administration was the
35 recombinant mouse nesfatin-1 prepared in Working Example
16 dissolved in 200 µl of physiological saline so as to
contain 2 nmol, 10 nmol or 50 nmol, and as the control

sample, physiological saline (Saline) alone was used. Using a tuberculin syringe equipped with a 25G needle, 200 μ l each of the sample was administered once into the abdominal cavity of each mouse (5 animals per group), and
5 the time of administration was immediately before the start (6 p.m.) of the dark period.

In the experiment on the animal model of obesity, C57BL/6J mice were used as the control group and Agouti-yellow mice were used as the animal model of obesity, and
10 these animals were purchased from Nippon Charles River (Jackson Lab.). The housing condition and the week age of the mice used were similar to those for the ICR mice, whereas individuals weighing 25-28 g for the control group mice and individuals weighing 31-38 g for the
15 Agouti-yellow mice were selected and used. The sample used in the administration was recombinant mouse nesfatin-1 dissolved in 200 μ l of physiological saline so as to contain 10 nmol, and as the control sample, physiological saline (Saline) alone was used (16 animals
20 per group). Other conditions were similar to those in the above.

Each mouse that received administration was placed in an individual cage, and during 0-3 hours after the administration the weight decreased of the pellet food
25 was measured to determine the amount of food intake. For testing of significant difference, analysis of variance was used.

<Result>

The result measured of the amount of food intake during 0-3 hours after administration when nesfatin-1 or
30 physiological saline was intraperitoneally administered into ICR mice is shown in A of Fig. 16. In the result of A of Fig. 16, a decreases in the amount of food intake was noted in the mice that received 2 nmol, 10 nmol and
35 50 nmol of nesfatin-1 per mouse relative to the control (Saline group), and statistically significant decreases in the amount of food intake were noted in 10 nmol

($p < 0.05$) and 50 nmol ($p < 0.005$). This indicated that since nesfatin-1 exhibits an activity of suppressing food intake in mice as well as in rats, it has an effect of controlling food intake in many species, and
5 administration from not only via the brain but via the periphery such as the abdominal cavity is effective in suppressing food intake. Also, it was demonstrated that the intraperitoneally administered nesfatin-1 is has an effect of suppressing food intake in the early stage
10 after administration.

Also, in the experiment on the mouse model of obesity, the result measured of the amount of food intake when physiological saline (Cont) or nesfatin-1 (10 pmol) was administered to the mice (c57BL/6J) of the control
15 group is shown in B of Fig. 16, and the result measured of the amount of food intake when physiological saline (Cont) or nesfatin-1 (10 pmol) was administered to the Agouti-yellow mice, the mouse model of obesity, is shown in C of Fig. 16. As a result, both in the mice of the
20 control group and the mice of the Agouti-yellow mice, the amount of food intake was significantly decreased in the mice that received the intraperitoneal administration of nesfatin-1 relative to the control. Since Agouti-yellow mice is a model of obesity in which the function of
25 suppressing food intake by melanocortin, a ligand of MC3R/MC4R, does not work due to the over expression of the Agouti protein, it was suggested, a pharmacological activity was demonstrated in a leptin-resistant model in the result for the Zucker (fa/fa) rats in Working Example
30 17, and similarly food intake was controlled by a mechanism independent of that of food intake suppression in the existing Agouti/melanocortin system.

Working Example 19

**Study on the effect of the intraperitoneal and
35 subcutaneous administration of nesfatin-1 on controlling the amount of food intake in mice**

In Working Example 18, it was disclosed that the

intraperitoneal administration of nesfatin-1 into mice is also effective in suppressing food intake, and thus as an example of another peripheral administration, the effect of subcutaneous administration of nesfatin-1 was also examined.

The sample used in the administration was the recombinant mouse nesfatin-1 prepared in Working Example 16 dissolved in 200 μ l of physiological saline so as to contain 10 nmol, and as the control sample, physiological saline (Saline) alone was used. Using a tuberculin syringe equipped with a 25G needle, 200 μ l each of the samples was administered once into the abdominal cavity or the hypodermis of the back of each mouse, and the time of administration was immediately before the start (6 p.m.) of the dark period. Other conditions were similar to those in Working Example 18.

<Result>

The result measured of the amount of food intake during 0-3 hours after administration when nesfatin-1 (10 nmol) or physiological saline (0) was intraperitoneally (ip) or subcutaneously (sc) administered into the mice is shown in A of Fig. 17, and that during 0-14 hours is shown in B of Fig. 17. In A of Fig. 17, the amount of food intake tended to decrease in the groups in which 10 nmol of nesfatin-1 was intraperitoneally (ip) and subcutaneously (sc) administered relative to the physiological saline-administration group (0), and specifically for the intraperitoneal administration, a statistically significant decrease ($P < 0.05$) was noted. In B of Fig. 17 as well, the amount of food intake tended to decrease in the group in which 10 nmol of nesfatin-1 was intraperitoneally (ip) and subcutaneously (sc) administered relative to the physiological saline-administration group (0), but in the intraperitoneal administration group (ip) the decrease in the amount of food intake was not significant and in the subcutaneous administration group (sc) the decrease was significant

($P < 0.005$) relative to the physiological saline-administration group. In the results of both A and B of Fig. 17, the intraperitoneal administration of nesfatin-1 tended to exhibit the effect of suppressing food intake early and the effect by the subcutaneous administration tended to lag behind. The above suggests that for the peripheral administration, nesfatin-1, whether intraperitoneally or subcutaneously administered, is effective in suppressing food intake. For drugs that act in the brain, the presence of effect by the peripheral administration is important in practical use, and in this regard nesfatin-1 was shown to be useful as a pharmaceutical based on the results of Working Examples 18 and 19.

Working Example 20

Study on the effect of the intraperitoneal administration of a partial peptide (nesfatin-1N23, nesfatin-1M30, nesfatin-1C29) of nesfatin-1 on the control of food intake in mice

In Working Examples 10 and 12, nesfatin-1 was found from the cleavage sites in nesfatin of prohormone convertase. In the analyzing the function of nesfatin-1, however, it is important to identify the functional site of said peptide, and also in its application into pharmaceuticals, the short amino acid length of the peptide is considered advantageous in terms of production, dosage, antigenicity and the like. Thus, in order to examine in further detail the sites having an activity of suppressing food intake, partial peptides were prepared from the structure of nesfatin-1 comprising a 82-amino acid length, and an experiment was carried out to measure the amount of food intake when they were intraperitoneally administered to mice.

Of the amino acid sequence (SEQ ID NO: 14) of mouse nesfatin-1 derived from the sequence of mouse nesfatin, a sequence of amino acid numbers 1 to 23 from the amino terminal was termed as nesfatin-1N23, that of amino acid

numbers 23 to 53 as nesfatin-1M30, and that of amino acid numbers 54 to 82 as nesfatin-1C29.

nesfatin-1N23:

ValProIleAspValAspLysThrLysValHisAsnThrGluProValGluAsnAla
5 ArgIleGluPro (SEQ ID NO: 42)

nesfatin-1M30:

ProAspThrGlyLeuTyrTyrAspGluTyrLeuLysGlnValIleGluValLeuGlu
ThrAspProHisPheArgGluLysLeuGlnLys (SEQ ID NO: 41)

nesfatin-1C29:

10 AlaAspIleGluGluIleArgSerGlyArgLeuSerGlnGluLeuAspLeuValSer
HisLysValArgThrArgLeuAspGluLeu (SEQ ID NO: 43)

Each peptide of nesfatin-1N23, nesfatin-1M30 and nesfatin-1C29 used was a synthetic peptide of which production was referred to Biologica Co., Ltd. and was
15 purified by HPLC to a purity of 95% or higher. Each peptide was prepared in physiological saline so as to contain 50 nmol per 200 µl, which was used as the sample, and as the control sample physiological saline (Vehicle) alone was used. Using a tuberculin syringe equipped with
20 a 25G needle, 200 µl each of the samples was administered once into the abdominal cavity of each mouse (5 animals per group), and the time of administration was immediately before the start (6 p.m.) of the dark period. Mice used were male ICR mice (Nippon SLC) and the housing
25 condition was similar to those in Working Example 18.

Each mouse that received administration was placed in an individual cage, and during 0-3 hours after the administration the weight decreased of the pellet food was measured to determine the amount of food intake. For
30 testing of significant difference, analysis of variance was used.

Comparison by the alignment of the amino acid sequences of human, rat and mouse nesfatin-1 was also carried out. Using the amino acid sequence of human
35 nesfatin-1 (SEQ ID NO: 13), mouse nesfatin-1 (SEQ ID NO: 14) and rat nesfatin-1 (SEQ ID NO: 15), alignment was carried out using the CLUSTAL-W method (Higgins et al.,

Nucleic Acids Research, Vol. 22, pp. 4673-4680, 1994).

<Result>

The result measured of the amount of food intake during 0-3 hours after administration when physiological saline (Vehicle), nesfatin-1N23 (-N23), nesfatin-1M30 (-M30) and nesfatin-1C29 (-C29) was intraperitoneally administered into mice is shown in A of Fig. 18. In the nesfatin-1M30-administration group (N-1b) relative to the control group (Vehicle) that received physiological saline, a significant decrease ($P<0.02$) in the amount of food intake was noted. However, in the nesfatin-1N23-administration group (N-1a) and the nesfatin-1C29-administration group (N-1c), no significant decrease or enhancement in the amount of food intake was noted. The above indicated that nesfatin-1M30 is the most important functional site for the activity of nesfatin-1 (and nesfatin) of suppressing food intake. Also, since the intraperitoneal administration of nesfatin-1M30 exhibited an activity of suppressing food intake, the possible use of said polypeptide as a pharmaceutical was indicated.

The result of amino acid alignment of human, mouse and rat nesfatin-1, and the sites of nesfatin-1N23, nesfatin-1M30 and nesfatin-1C29 are shown in B of Fig. 18. It was shown that the amino acid sequences of the sites of nesfatin-1M30 are highly conserved among species.

Working Example 21

Construction of an EIA system and the detection of the nesfatin-1 peptide in the hypothalamus tissue

From the result with an antibody (nesfatin-1 IgG) against nesfatin-1 and an antibody (PC1/3 and PC2) against prohormone convertase in the cells of the rat hypothalamus in Working Example 11, it was demonstrated, nesfatin and prohormone convertase were expressed in the same cells and nesfatin-1 is likely to be produced therein. In order to further investigate this, a competitive EIA system that detects nesfatin or nesfatin-

1 was constructed, and a fractionation pattern by the reverse phase HPLC of a sample extracted from the tissue of the rat hypothalamus and a pattern of nesfatin-1 prepared by synthesis were compared.

5 The competitive EIA system was created using nesfatin-1 IgG prepared in Working Example 10 and a biotin-labelled nesfatin-1 polypeptide. Nesfatin-1 IgG dissolved in PBS at 10 µg/ml was aliquoted into a 96-well ELISA plate (SUMITOMO BAKELITE Co., Ltd.: MS-8596F) at 50
10 µl/well, which was sealed with a plate seal and allowed to stand at 4°C overnight in order to immobilize the antibody. After washing each well of the antibody-immobilized plate with PBS, PBS containing 10% bovine serum albumin (BSA) was dispensed at 250 µl/well and the
15 plate was allowed to stand at room temperature for 2 hours. Then, after each well of the plate was washed three times with PBS, the antibody-immobilized plate was prepared.

 In order to prepare a labelled nesfatin-1, a
20 cysteine residue was added to the C terminal of nesfatin-1 (nesfatin-1 Cys: SEQ ID NO: 62). Though the method of preparation was similar to that of Working Example 16, PCR in order to obtain a nucleic acid encoding nesfatin-1 Cys was carried out using the following primer set:

25 Forward primer: 5'-
GGTTCCGCGGGTCTGGTTCCGCGTGGTTCTCCTATCGATGTGGACAAGACCAA-3'
(SEQ ID NO: 63)

 Reverse primer: 5'-
GGTTGCGGCCGCTTAACACCTCTTCAGCTCATCCAGTCTCG-3' (SEQ ID NO:
30 64)

 Nesfatin-1 Cys expressed and purified as in the method of Working Example 16 was dissolved in 0.1M phosphate buffer (pH 6.0) containing 50 mM 2-
35 mercaptoethanolamine and 1 mM EDTA and treated at 37°C for 90 minutes. Then trifluoroacetic acid (TFA) was added to 0.1%, which was placed on a Sep-Pak C18 column (Waters).

After washing the column with 10 ml of aqueous solution of 0.1% TFA and 10% acetonitrile, it was eluted with 3 ml of an aqueous solution of 0.1% TFA and 60% acetonitrile. After the eluate was lyophilized, it was dissolved in 0.1 M phosphate buffer (pH 7.0) to 5 mg/ml, and 20 mg/ml of biotin (Long Arm) maleimide (VECTRO Lab.) dissolved in 1/40 volume of dimethylformamide (DMF) was added and reacted at room temperature for 3 hours. To biotin (Long Arm) maleimide-reacted nesfatin-1 Cys, TFA was added to 0.1%, and was applied to HPLC equipped with a reverse phase C18 column (Nacalai Tesque Inc.: COSMOSIL (trade mark) 5C18-AR-300 20.0 mm I.D. × 150 mm). While monitoring absorbance at a wavelength of 210 nm, it was washed with an aqueous solution of 0.1% TFA and then an aqueous solution of 20% acetonitrile containing 0.1% TFA until the absorbance of the eluate cannot be observed. Subsequently, an aqueous solution of acetonitrile at a 20-60% gradient was run to obtain a fraction having the highest peak of absorbance at 210 nm. The fraction obtained was lyophilized and then dissolved in PBS, which was used as the labelled nesfatin-1.

The labelled nesfatin-1 was dissolved to 1 µg/ml in PBS containing 2% BSA. For the creation of a standard curve, the recombinant nesfatin-1 prepared in Working Example 16 was diluted to a concentration of 6000 ng/ml in PBS containing 2% BSA, which was then diluted in PBS containing 2% BSA at a common ratio of 2 and used (standard samples: 6000, 3000, 1500, 750.0, 375.0, 187.5, 93.8 ng/ml). Fifty µl each of the prepared labelled nesfatin-1 and the standard samples were placed into a microtest tube and mixed, and 50 µl each of them was dispensed in a well of the antibody-immobilized plate. As an example of a biological sample, 50 µl of the cerebrospinal fluid as it is collected or the sample diluted 2-fold in PBS containing 2% BSA was mixed with 50 µl of the labelled nesfatin-1 solution in a microtest

tube, and 50 μ l thereof was aliquoted in wells of the antibody-immobilized plate (test sample). After the aliquoted antibody-immobilized plate was allowed to stand at room temperature for 1 hour, the reaction sample in the well was discarded, and washed three times in PBS containing 0.2% Tween 20. Then 50 μ l of avidin-peroxidase (Sigma, A7419-2ML) diluted 1/1,000 with PBS containing 2% BSA and 0.2% Tween 20 was dispensed in each well and was allowed to stand at room temperature for 30 minutes. After the reaction, the solution in each well was removed, and after washing four times in PBS containing 0.2% Tween 20, it was washed twice in TBS (50 mM Tris-HCl, 0.15 M NaCl, pH 8.0). To each well of the antibody-immobilized plate after washing, 50 μ l of a peroxidase substrate, TMB (PIERCE: 1-Step (TM) Turbo TMB), was added and reacted at room temperature for 30 minutes. Then to each well, 50 μ l of 0.5 N sulfuric acid was added to stop the reaction, absorbance at a wavelength of 450 nm and that at a wavelength of 620 nm were measured by an absorbance plate reader, and the absorbance at a wavelength of 620 nm was subtracted from that at a wavelength of 450 nm ($\Delta 620$ nm) to obtain the measured value.

The analysis of nesfatin-1 expression at the hypothalamus was carried out by fractionation using HPLC with the peptide extracted from the tissue as the sample. The hypothalamus was excised from the brains of eight rats, homogenized in 4 ml of an aqueous solution of 0.1% TFA by a Teflon (trade mark) homogenizer, and centrifuged at 10,000 rpm for 10 minutes to collect the supernatant. After the collected supernatant was filtered with a filter (Millipore) having a pore size of 0.45 μ m, it was run through the Sep-Pak C18 column (Waters), and the column was washed in 5 ml of an aqueous solution of 0.1% TFA. Then, to the column 3 ml of an aqueous solution of 60% acetonitrile was added for elution, and the eluate

was dried in an evaporator. The dried product was dissolved in 0.8 ml of an aqueous solution of 0.1% TFA, centrifuged at 10,000 rpm for 10 minutes, and 500 μ l of the supernatant was injected to a HPLC instrument
5 equipped with a C18 reverse phase chromatocolumn (Nacalai Tesque Inc.: COSMOSIL μ C18-AR-II, 4.6 mm I.D. $\times$ 250 mm). After injecting the sample, an aqueous solution of 0.1% TFA was run through the column at a flow rate of 1 ml/min while monitoring the absorbance of the eluate at a
10 wavelength of 224 nm. After washing, while keeping the flow rate, acetonitrile at a 0-60% concentration gradient (Δ 1%/min) was run in the presence of 0.1% TFA to collect the eluate as 1 ml fractions. After freezing the fractions obtained at -80°C , they were lyophilized, and
15 the samples after drying were each dissolved in 200 μ l of PBS containing 2% BSA, and nesfatin and nesfatin/nesfatin-1 were measured by a competitive EIA method. Similarly, from the samples obtained by drying 600 μ l of the cerebrospinal fluid collected from eight
20 rats, peptide samples were prepared, fractionated by HPLC, and the eluted fractions were examined by the competitive EIA system. Also, as the control, 80 μ g of the recombinant nesfatin-1 peptide prepared in Working Example 16 was dissolved in 100 μ l an aqueous solution of
25 0.1% TFA, which was injected into HPLC, and the fractions in which nesfatin-1 was eluted were detected.

<Result>

A standard reaction curve obtained by measuring the standard samples in the competitive EIA system is shown
30 in Fig. 19 A-1. It was demonstrated that with increased concentrations of the reacted nesfatin-1, the binding of the labelled nesfatin-1 is competitively inhibited and the absorbance at 450 (Δ 620) decreases, indicating that nesfatin-1 concentrations in samples can be determined in
35 this system. The sensitivity of this system corresponds to 4.6 ng/tube (93 ng/ml) of nesfatin-1. The result of

the concentration of nesfatin-1 (nesfatin) in the cerebrospinal fluid determined by this competitive EIA system is shown in Fig. 19 A-2. The values obtained by measuring the cerebrospinal fluid as it is or after
5 diluting 1/2 (converted by the dilution factor after measurement) are almost the same and indicated that about 230 ng/ml of nesfatin-1 (nesfatin) is present.

b-1 of Fig. 19B shows a result in which a peptide sample extracted from the rat hypothalamus was
10 fractionated by HPLC and the presence of nesfatin-1 in the fractions were determined, and b-2 of Fig. 19B shows a result in which a similar study was carried out using a peptide sample extracted from the rat cerebrospinal fluid. In both b-1 of Fig. 19B and b-2 of Fig. 19B, a
15 reaction peak which is likely to be nesfatin-1 was noted in the fractions from No. 44 and 45. In a result when the recombinant nesfatin-1 was fractionated by HPLC under a similar condition, nesfatin-1 was eluted at No. 44 fraction, the factor of which presence was indicated from
20 HPLC fractions of the hypothalamus and the cerebrospinal fluid by the competitive EIA system is believed to be nesfatin-1.

Working Example 22

**Study on the effect of the intraperitoneal
25 administration of the partial peptide (nesfatin-1M16, nesfatin-1M14, nesfatin-1M10M) of nesfatin-1M30 on food intake control in mice**

In Working Example 20, the study on the effect of suppressing food intake of a partial peptide derived from
30 nesfatin-1 led to the invention of nesfatin-1M30. Furthermore, in order to examine in further detail the sites having an activity of suppressing food intake, a partial peptide of nesfatin-1M30 was prepared from the structure of nesfatin-1M30 comprising a 30-amino acid
35 length, and an experiment was carried out to measure the amount of food intake when it was intraperitoneally administered to mice.

For nesfatin-1M16 comprising a 16-amino acid length, nesfatin-1M14 comprising a 14-amino acid length and nesfatin-1M10M comprising a 10-amino acid length which are partial peptides of mouse nesfatin-1M30, the
5 preparation of synthetic peptides having the following sequences was referred to Biologica Co., Ltd. and were purified by HPLC to a purity of 95% or higher.

nesfatin-1M16: N-
ProAspThrGlyLeuTyrTyrAspGluTyrLeuLysGlnValIleGlu-C (SEQ
10 ID NO: 71)

nesfatin-1M14: N-
ValLeuGluThrAspProHisPheArgGluLysLeuGlnLys-C (SEQ ID NO:
72)

nesfatin-1M10M: N-LysGlnValIleGluValLeuGluThrAsp-C
15 (SEQ ID NO: 73)

Each peptide was prepared in physiological saline so as to contain 10 pmol per 100 μ l, which was used as the sample for administration, and as the control sample physiological saline (Vehicle) alone was used. The
20 physiological saline as the control and the peptide samples prepared were intraperitoneally administered at 100 μ l per mouse (6 animals per group). Mice used were male ICR mice (Nippon SLC) and the housing condition and the experimental condition were similar to those in
25 Working Example 18.

Each mouse that received administration was placed in an individual cage, and during 0-3 hours after the administration the weight decreased of the pellet food was measured to determine the amount of food intake. For
30 testing of significant difference, analysis of variance was used.

<Result>

Fig. 20 shows the amount of food intake during 0-3 hours after administration when a partial peptide of
35 nesfatin-1M30, nesfatin-1M16 (M16), nesfatin-1M10M (M10M) or nesfatin-M14 (M14), was intraperitoneally administered into mice. In all of the group that received nesfatin-

1M16 (M16), nesfatin-1M10M (M10M) or nesfatin-M14 (M14) relative to the control group (Cont.) that received physiological saline alone, a significant effect of suppressing food intake was noted.

5 **Working Example 23**

Study on the effect of human nesfatin-1M30 and mouse NUCB1-M30 on food intake control

 Working Example 20 demonstrated that mouse nesfatin-1M30 has an effect of suppressing food intake. Based on this, human nesfatin-1M30 was prepared and studied on its effect on the food intake behavior when administered on mice. Nucleobindin-1 (NUCB1) is a factor that forms a family having a high homology with NEFA/nesfatin in the amino acid sequence of peptides and the nucleotide sequence of the genes. Thus, NUCB1-M30 which is a site corresponding to nesfatin-1M30 of NUCB1 was created and was administered to mice to examine the effect on food intake.

 The preparation of human nesfatin-1M30 (SEQ ID NO: 39) and mouse NUCB1-M30 by chemical synthesis was referred to Biologica Co., Ltd. and was purified by HPLC to a purity of 95% or higher.

 Human nesfatin-1M30:

N-

25 ProAspThrGlyLeuTyrTyrAspGluTyrLeuLysGlnValIleAspValLeuGlu
ThrAspLysHisPheArgGluLysLeuGlnLys-C (SEQ ID NO: 39)

 Mouse NUCB1-M30:

N-

30 ProAspThrGlyLeuTyrTyrHisArgTyrLeuGlnGluValIleAsnValLeuGlu
ThrAspGlyHisPheArgGluLysLeuGlnAla-C (SEQ ID NO: 103)

 Each of the prepared human nesfatin-1M30 and mouse NUCB1-M30 was prepared in physiological saline so as to contain 10 pmol per 100 µl, which was used as the sample for administration. For nesfatin-1M30 that exhibited an activity of suppressing food intake, one prepared in Working Example 20 was used as the comparative sample at similar amounts as nesfatin-1M30 and NUCB1-M30, and as

the control sample physiological saline (Vehicle) alone was used.

5 The physiological saline as the control and the peptide sample prepared were intraperitoneally administered at 100 μ l per mouse (n=6 per group). Mice used were male ICR mice (Nippon SLC) and the housing condition and the experimental condition were similar to those in Working Example 18.

10 Each mouse that received administration was placed in an individual cage, and during 0-3 hours after the administration the weight decreased of the pellet food was measured to determine the amount of food intake. For testing of significant difference, analysis of variance was used.

15 The alignment of amino acid sequence was performed for human, rat and mouse nesfatin and human, rat and mouse NUCB1. The method used the amino acid sequences of human, mouse and rat nesfatin of SEQ ID NO: 2, SEQ ID NO: 5 and SEQ ID NO: 8, respectively, and the amino acid
20 sequences of human, mouse and rat NUCB1 of SEQ ID NO: 84, SEQ ID NO: 88 and SEQ ID NO: 92, respectively, and analyzed by the Clustal-W method.

<Result>

25 The result measured of the amount of food intake during 0-3 hours after administration when human nesfatin-1M30 (human/nesfatin-1M30), mouse nesfatin-1M30 (mouse/nesfatin-1M30) or mouse NUCB1-M30 (mouse NUCB1) was administered is shown in Fig. 21A. Fig. 21A indicates that in all of the groups that received human
30 nesfatin-1M30 (human/nesfatin-1M30), mouse nesfatin-1M30 (mouse/nesfatin-1M30) or mouse NUCB1-M30 (mouse NUCB1) relative to the control group (Vehicle) that received physiological saline alone, a significant effect of suppressing food intake was noted.

35 Also, the results of alignment of amino acid sequence for human, rat and mouse nesfatin and for human, rat and mouse NUCB1 and sites corresponding to nesfatin-1

and nesfatin-1M30 are shown in Figs. 21B to 21C. It was shown that amino acid sequences are highly conserved in sites corresponding to nesfatin and nesfatin-1 of NUCB2, in particular the site corresponding to nesfatin-1M30.

5 **Working Example 24**

Study on the expression site of the nesfatin gene in the rat hypothalamus

 The expression of nesfatin mRNA in the brain hypothalamus was analyzed by an in situ hybridization method in Working Example 8. In order to further analyze the site where the nesfatin gene is being expressed, a study by an in situ hybridization method using radioisotope was carried out.

 Using eight week-old male Wistar rats (purchased from Nippon SLC) (body weight: 220-250 g) that were housed with free access to the food, the rats were deeply anesthetized by pentobarbital in the light period, and the brain was fixed by perfusing 4% paraformaldehyde dissolved in ice-cold 0.1M borate buffer (pH 9.5) from the heart. The brain was extracted, and was immersed in 0.1M borate buffer (pH 9.5) containing 10% sucrose and 4% paraformaldehyde for 2 days. The fixed brain was frozen in dry ice-acetone, and sliced with a cryostat to sections 20 μ m thick, which were placed on a slide glass (MAS coat slide S-9116 prepared by Matsunami Glass).

 For the preparation of the radioisotope-labelled probe, a plasmid was used which was obtained by cleaving a plasmid for the preparation of the NEFA probe that was used in Working Example 2 with a restriction enzyme NcoI and then purifying it. To 0.1 μ g of the plasmid, 20 U (1 μ l) of SP6 RNA polymerase (Promega, P1085) was added at a condition of 19 μ l of a solution containing 36 mM Tris-HCl buffer (pH 7.5), 6 mM magnesium chloride, 2 mM spermidine, 8 mM dithiothreitol, 25 mM adenosine triphosphate/guanosine triphosphate/cytosine triphosphate, 5 mM uracil triphosphate and 5 mM [α -³⁵S]-

uracil triphosphate, and 1U of RNAsinTM ribonuclease inhibitor (Promega, N2111), reacted at 37°C for 60 minutes to prepare a 35S-labelled NEFA cRNA probe. After the reaction, 20 µl of the TNE buffer (10 mM Tris-Cl, pH 8.0, 0.5 M NaCl, and 0.25 mM EDTA, pH 8.0) was added to stop the reaction, and then the probe was purified using the NENSORBTM PREP Nucleic Acid Purification Cartridges (Perkin-Elmer, Inc., NLP028001EA) according to the attached protocol.

The slide glass of the prepared section sample was dried overnight under vacuum before hybridization, treated with PBS containing 10 µg/ml of Protease K (Sigma, P2308) at 37°C for 30 minutes, and washed twice with PBS, and furthermore treated by immersing in 0.1 M triethylamine-hydrochloric acid buffer (pH 8.0) containing 0.25% acetic anhydride at room temperature for 10 minutes, followed by washing twice with 2x concentrated SSC. The washed section sample was dehydrated by immersing in 75% ethanol, 95% ethanol and 99% ethanol in this order, dried in the air, and further dried under vacuum.

On to the dried slide glass of the section sample, an in situ hybridization solution (10 mM Tris-HCl buffer, pH 8.0, 30 mM NaCl, 10% dextran sulfate, 1x concentrated Denhardt's solution, 12 mM EDTA, 50% deionized formamide, 0.5 mg/ml yeast rRNA) was placed so as to cover the brain tissue section, and prehybridized at 65°C for 1 hour. After discarding the prehybridization solution, 80 µl, per slide glass, of a hybridization solution containing 10⁶ cpm/ml 35S-labelled NEFA cRNA probe and 10 mM dithiothreitol was placed, and a cover slip was placed thereon, which was placed in a wet chamber and hybridized overnight at 65°C. After the slide glass after hybridization was washed four times with 4x concentrated SSC, it was treated with the TNE buffer (10 mM Tris-HCl,

pH 8.0, 0.5 M NaCl, and 0.25 mM EDTA, pH 8.0) containing 20 µg/ml of RNAase A at 37°C for 30 minutes, washed twice with 2x concentrated SSC at room temperature, and further washed twice with 0.1x concentrated SSC at 65°C for 30 minutes. The slide glass after washing was dehydrated by immersing in 75% ethanol, 95% ethanol and 99% ethanol in this order, and then dried in the air. After the slide glass having the tissue section thereon was exposed to X-ray film for 7 days, it was immersed in a 2x diluted light-sensitive emulsion (Kodak, NTB3) and exposed to light for 3 weeks. After exposure the slide glass was washed with water, stained with Thionine, and black spots resulted from the exposure were examined under microscope.

The position of each part in the rat brain was identified according to The Rat Brain in Stereotaxic Coordinates by Paxinos G. and Watson C. (Academic Press) (USA) 1986.

<Result>

A of Fig. 22 shows an image of in situ hybridization of a tissue section containing paraventricular nucleus (PVN) and supraoptic nucleus (SON), B of Fig. 22 shows that of a tissue section containing zona incerta (Zi) and arcuate nucleus (Arc), and C of Fig. 22 shows that of a tissue section containing lateral hypothalamic area (LHA). At each area of PVN, SON, Zi, Arc and LHA, spots that were light-sensitized by the hybridization of the radioisotope-labelled NEFA cRNA probe, indicating the expression of the NEFA gene.

Working Example 25

Study on the effect of intraventricular administration of nesfatin on food intake behavior

While the amount of food intake during 0-1 hour/1-3 hours/3-6 hours after the administration of nesfatin to rats was studied in Working Example 6, the effect on food intake behavior during 6-12 hours after the

administration was further studied herein.

In order to demonstrate the reproducibility of Working Example 6, the amount of food intake during 0-1 hour after administration was determined when 0 (PBS alone), 1, 4 and 20 pmol of recombinant nesfatin was administered to the third ventricle of brain, as in Working Example 6. Also, in the same manner as in Working Example 6, after 5 pmol of nesfatin was administered into the rat ventricle, the amount of food intake was determined during 0-1 hour/1-3 hours/3-6 hours after the administration. The control group used was the one that only received physiological saline (0 pmol). For testing of significant difference, analysis of variance was used.

<Result>

As shown in A of Fig. 23, the amount of food intake at 0-1 hour after the administration of 4 pmol or 20 pmol of nesfatin into the brain ventricle was significantly reduced as compared to the control group (0 pmol) ($p < 0.01$). Also, as shown in B of Fig. 23, a significant reduction in the amount of food intake was noted in the individual that received the intraventricular administration of 5 pmol of nesfatin during 0-1 hour ($p < 0.01$), 1-3 hours ($p < 0.05$) and 3-6 hours ($p < 0.001$) as compared to the control group (0 pmol). However, in the amount of food intake during 6-12 hours, no difference was noted between the group that received 5 pmol of nesfatin and the control group.

Working Example 26

Study on the amount expressed of nesfatin mRNA and the nesfatin polypeptide in the rat hypothalamus during starvation

In order to study the expression of the nesfatin gene during starvation, in situ hybridization was carried out in the brain hypothalamus region of the rats that had free access to the food and the fasted rats, and increases or decreases in the amount of nesfatin mRNA at

arcuate nucleus, paraventricular nucleus, lateral hypothalamic area and supraoptic nucleus from the tissue were determined. Also, in order to study the amount expressed of the nesfatin-1 peptide at paraventricular nucleus, the hypothalamus region of the rats that had free access to the food and the fasted rats was excised, and the extracted peptides were determined by competitive EIA system.

As in Working Example 8, rats used were individuals (the control group) that were allowed free access to the powder food, and individuals (the fasting group) that were housed on water alone without any food for 48 hours. For the determination of the amount expressed of nesfatin mRNA at each region of the hypothalamus, tissue sections were prepared in a manner similar to Working Example 24, which was subjected to in situ hybridization, and the image obtained by exposing to X-ray film was measured using an image analyzer (Imaging Research Inc., MCIDTM Elite) (Imaki et al., Brain Research, Netherlands, 1993, Vol. 623, pp. 223-228). Density of the exposed image at each region of arcuate nucleus, paraventricular nucleus, lateral hypothalamic area and supraoptic nucleus was determined, and the values were changed into numerical values as relative optical values on a 256-stage gray scale from white to black, which were then changed into numerical values as relative optical densities according to the following equation to obtain the absolute values for mRNA expression:

Relative optical density = $\log_{10}(256/\text{relative optical value})$.

Each region of arcuate nucleus, paraventricular nucleus, lateral hypothalamic area and supraoptic nucleus in the rat brain was identified according to The Rat Brain in Stereotaxic Coordinates by Paxinos G. and Watson C. (Academic Press) (USA) 1986.

The nesfatin-1 peptide at the paraventricular nucleus of the rat brain was determined as follows. Rats

that were allowed free access to the food and that were fasted as described above were sacrificed by decapitation, and the brain was immediately frozen in dry ice-ethanol, and sliced by a cryostat into sections 60 μ m thick. The tissue was Nissl stained, and the parts corresponding to paraventricular nucleus on both sides of the brain were excised and recovered. The recovered tissue was homogenized in 100 μ l of 0.1 N hydrochloric acid in a 1.5 ml microtube using the microtube pestle (Scientific Specialties, 1005-39). The homogenized solution was centrifuged in a microfuge at 15,000 rpm for 20 minutes, the supernatant was collected and the solvent was removed by lyophilization. The lyophilized sample was dissolved in 100 μ l of PBS, and the amount of the nesfatin-1 peptide was determined at a condition of 50 μ l/well by the competitive EIA described in Working Example 21.

<Result>

A of Fig. 24 shows the result of image analysis by in situ hybridization of nesfatin mRNA expression at various hypothalamus regions of arcuate nucleus (Arc), paraventricular nucleus (PVN), lateral hypothalamic area (LHA) and supraoptic nucleus (SON) in the rat brain in the feeding ad libitum group (control group) and the fasting group. In arcuate nucleus (Arc), lateral hypothalamic area (LHA) and supraoptic nucleus (SON), no difference in the value (relative optical density) of nesfatin mRNA expression was noted in the fasting group relative to that in the feeding ad libitum group (control group). In contrast, in the result for paraventricular nucleus (PVN), the amount expressed of nesfatin mRNA in the fasting group was significantly reduced relative to the feeding ad libitum group (control group).

B of Fig. 24 shows the result of image analysis by a competitive EIA method of the expression of the nesfatin-1 peptide at paraventricular nucleus (PVN) among the

hypothalamic regions of the rat brain in the feeding ad libitum group (control group) and the fasting group. It was demonstrated that the expression of the nesfatin-1 peptide at paraventricular nucleus is significantly
5 decreased in the fasting group relative to the feeding ad libitum group (control group).

The above result shows that fasting markedly reduces the expression of the nesfatin (NEFA) gene and nesfatin-1 at paraventricular nucleus of the hypothalamus that is
10 important in the control of food intake.

Working Example 27

Study on the effect of intraventricular administration of nesfatin-1 into the rat brain on food intake behavior

15 It was shown in Working Example 12 that the part of nesfatin-1 alone had an activity of suppressing food intake in the experiment of the administration of partial peptides of nesfatin. In order to further validate the experiment, changes in the activity of suppressing food
20 intake with time after administration was investigated.

The experimental condition was similar to that in Working Example 12, and 5 pmol of nesfatin-1 was administered into the third ventricle of the rat brain immediately before the start of the dark period, the
25 amount decreased of the food (the amount of food intake) during 1 hour immediately after administration (0-1 hr), during 2 hours from 1 hour after the administration (1-3 hr), during 3 hours from 3 hours after the administration (3-6 hr), and during 6 hours from 6 hours after the
30 administration (6-12 hr) was measured as the amount of food intake. As the control group, the amount of food intake by individuals that received physiological saline alone was measured.

<Result>

35 Fig. 25 shows the amount of food intake during 0-1 hr, 1-3 hr, 3-6 hr and 6-12 hr when nesfatin-1 was administered to the third ventricle of the rat brain.

Significant decreases in the amount of food intake in 0-1 hr, 1-3 hr and 3-6 hr were noted in the nesfatin-1-administration group relative to the control group ($p < 0.01$). In contrast, in 6-12 hr, an increase in the amount of food intake was noted in the nesfatin-1-administration group relative to the control group ($p < 0.01$). The above results demonstrated that the suppression of food intake by the intraventricular administration of nesfatin-1 is temporary, and the disappearance of the pharmacological effect of nesfatin with the passage of time and the resultant recovery of food intake can be noted.

Working Example 28

Study on the specificity of effect of intraventricular administration of anti-nesfatin-1 antibody on enhancing food intake

It was shown in Working Example 14 that the effect of enhancing food intake is noted when an antibody against nesfatin-1 is administered into the rat ventricle. In order to validate that the effect is due to the inhibition of the effect of nesfatin-1, a study was carried out to determine whether the suppression of food intake by the intraventricular administration of nesfatin-1 can be inhibited by the simultaneous administration of anti-nesfatin-1 antibody.

The experimental condition was similar to that in Working Example 12, and for a group that received 5 pmol of nesfatin-1 alone into the third ventricle of the rat brain immediately before the start of the dark period and a group that received 5 pmol of nesfatin-1 and 8 μ g of anti-nesfatin-1 antibody (nesfatin-1 IgG), the amount of food intake for 1 hour after administration was measured and compared to that for the control group (physiological saline alone was administered). Also, for a group that received only leptin (Rat leptin: R & D Systems, 598-LP-01M) (1 μ g) which is known to have an activity of suppressing food intake by intraventricular

administration and a group that received leptin and anti-nesfatin-1 antibody, its effect on food intake was also studied.

<Result>

5 Fig. 26 is a graph showing the amount of food intake
for 1 hour after administration for a group that received
nesfatin-1 alone (nesfatin-1 IgG/nesfatin-1/leptin: -/+/-
) , a group that received nesfatin-1 and anti-nesfatin-1
antibody (nesfatin-1 IgG/nesfatin-1/leptin: +/+/-) , a
10 group that received leptin (nesfatin-1 IgG/nesfatin-
1/leptin: -/-/+), and a group that received leptin and
anti-nesfatin-1 antibody (nesfatin-1 IgG/nesfatin-
1/leptin: +/-/+). The amount of food intake
significantly decreased in the group that received
15 nesfatin alone relative to the control group (nesfatin-1
IgG/nesfatin-1/leptin: -/-/-), whereas in the group that
received nesfatin-1 and anti-nesfatin-1 antibody, the
effect of enhancing the amount of food intake to about
the same degree as the control group was noted. In
20 contrast, in the group that received leptin, the amount
of food intake also decreased, whereas in the group that
received leptin and anti-nesfatin-1 antibody, no effect
of enhancing the suppression of the amount of food intake
by leptin was noted. This result suggests that the
25 effect of enhancing food intake by anti-nesfatin-1
antibody results from the specific suppression of the
effect of nesfatin-1.

Working Example 29

**Study on the binding specificity of anti-nesfatin-1
30 antibody**

It was shown in Working Example 28 that the
intraventricular administration of anti-nesfatin-1
antibody specifically inhibits the effect of nesfatin-1.
Its binding property with other factors known at present
35 to have an activity of controlling food intake was
further studied by the Western blotting method using
extracts of the rat brain.

Western blotting analysis was carried out by the method described in Working Example 3 with the primary antibody being changed from anti-NAP polyclonal antibody to anti-nesfatin-1 antibody. Before reacting anti-nesfatin-1 antibody to the membrane in Western blotting as an experiment for investigating the binding specificity of anti-nesfatin-1 antibody, 5 μ g of each peptide of NAP1-Ab (Working Example 10), and leptin (Rat leptin: R & D Systems, 598-LP-01M), α MSH (Melanocyte Stimulating Hormone; Peptide Institute, Inc., 4057-v), CART (Rat Cocaine- and Amphetamine-Regulated Transcript 55-102; Peptide Institute, Inc., 4351-s), NPY (Human, Rat Neuropeptide Y; Peptide Institute, Inc., 4158-v), MCH (Human Melanin-Concentrating Hormone; Peptide Institute, Inc., 4369-v) and Orexin-A (Human, Rat, Mouse, Bovine Orexin-A; Peptide Institute, Inc., 4346-s) per 1 μ g of anti-nesfatin-1 antibody was added, and reacted at room temperature for 1 hour, and then Western blotting was carried out in the method described above to examine whether or not the bands disappear.

<Result>

A of Fig. 27 shows an image of Western blotting carried out with anti-nesfatin-1 antibody using protein extracts from the rat brain. As a result, a band was noted at a position of molecular weight corresponding to 47.5 kd nesfatin polypeptide. B of Fig. 27 shows an image at about 47.5 kd of Western blotting carried out after anti-nesfatin-1 antibody and various peptides were previously reacted. When anti-nesfatin-1 antibody was reacted to NAP1-Ab, the 47.5 kd band disappeared, indicating that the previous binding of NAP-1 Ab to NAP1-Ab peptide blocked the binding site of nesfatin. In contrast, when it was reacted to leptin, α MSH, CART, NPY, MCH and Orexin-A, and when the peptide was not subjected to the anti-nesfatin-1 antibody reaction, the 47.5 kb band did not disappear. These results

demonstrated that anti-nesfatin-1 antibody specifically binds to nesfatin, but not to other food intake-related peptides such as letptin, α MSH, CART, NPY, MCH and Orexin-A.

5 **Working Example 30**

Study on the expression site of nesfatin in the rat medulla oblongata

10 In Working Example 4, expression in the vicinity of the hypothalamus of a rat brain was studied by immunohistochemical analysis. In order to further study the expression of nesfatin in the vicinity of the rat medulla oblongata, a similar immunohistochemical analysis was carried out.

15 Using eight week-old Wistar rats (purchased from Nippon SLC), brain tissue sections of the part containing the medulla oblongata were prepared from samples similarly to Working Example 4. The method of immunohistochemical stain was also similar to that in Working Example 4. Furthermore, the NAP peptide (Working
20 Example 3) was added to an antibody (1 μ g/ml) against the NAP peptide to 100 μ g/ml, reacted at room temperature for 1 hour, and then immunohistochemical stain was also carried out using the antibody.

<Result>

25 A of Fig. 28 shows an image of immunohistochemical stain in the brain tissue containing the medulla oblongata. In the immunohistochemical stain in the brain tissue containing the medulla oblongata, stain was noted at STN: the nucleus of solitary tract, and the expression
30 of the nesfatin polypeptide was noted. B of Fig. 28 shows an image of immunohistochemical stain carried out after an antibody against the NAP peptide and the NAP peptide were previously reacted. Since the stain
35 observed in A of Fig. 28 disappeared by reacting the anti-NAP antibody to the NAP peptide in advance, it was demonstrated, the nesfatin polypeptide has been

specifically stained in this immunohistochemical stain. The results revealed that nesfatin is also expressed in the nucleus of solitary tract which is a visceral sensory nerve nucleus and is thought to be involved in the mechanism of food intake control.

Working Example 31

Effect of troglitazone administration on blood leptin and the intracerebral expression of nesfatin in the normal and leptin-resistant obese rats

In Working Example 2, the induction of the nesfatin (NEFA) gene in cultured cells by troglitazone, a PPAR γ agonist that was used as an antidiabetic drug, was studied. In order to further study the induced expression of nesfatin by troglitazone, troglitazone was given to rats to study the induction of nesfatin in the brain. Similarly, blood levels of leptin which is known to be responsible for control of food intake were studied. In the study, normal Zucker rats (Zucker +/+; Lean) and Zucker fa/fa, an animal model of leptin-resistant obese rats, were used.

As animals, 8 week-old male Zucker fa/fa (Zucker) rats and Zucker +/+ (Lean) rats as the control animal were purchased from Nippon Charles River, and were housed in a cycle of 12 hours of the light period from 6 a.m. to 6 p.m. and 12 hours of the dark period from 6 p.m. to 6 a.m. the next morning and fed a powder food (Nippon Clea, CE-2), and kept at 22°C. After preliminary housing for over one week, individuals weighing 200-250 g were selected among 9 to 10 week-old individuals from the rats purchased and 6 animals per group were used in the experiment. Troglitazone (TGZ; Sankyo Co., Ltd.) was administered at a concentration of 0.2% blended in the powder food, to which the animals were allowed free access. As the control that did not receive troglitazone, rats that were housed on the normal powder food alone were used. After housing on the troglitazone-containing food or the normal food for 10 days on end,

the animals were weighed and sacrificed to collect whole blood. From the rat's blood collected, serum was separated, and leptin concentration in the serum was determined using a commercial ELISA kit (Yanaihara Institute Inc., YK050) according to the attached
5 protocol. Furthermore, the brain was harvested from the rats sacrificed, and was subjected to Western blotting in a similar manner to Working Example 3, the density of the bands was analyzed by an image analyzer (Imaging Research Inc., MCIDTM Basic), and the degree of color development
10 of the band was expressed as relative units.

<Result>

A of Fig. 29 is a graph showing the body weight of the group (TGZ: +) in which the troglitazone-containing food was given to the normal rats (Lean) and the Zucker
15 fa/fa rats (Zucker), and the group (TGZ: -) in which the troglitazone-free food was given thereto. In the normal rats and the Zucker fa/fa rats as well, no marked difference in body weight due to troglitazone
20 administration was noted. Also, irrespective of the administration of troglitazone, the Zucker fa/fa rats weighed significantly heavily ($p < 0.01$) relative to the normal rats.

B of Fig. 29 is a graph showing the concentration of leptin in the blood of the group (TGZ: +) in which the
25 troglitazone-containing food was given to the normal rats (Lean) and the Zucker fa/fa rats, and the group (TGZ: -) in which the troglitazone-free food was given thereto. In the normal rats and the Zucker fa/fa rats, the
30 concentration of leptin in the blood significantly decreased ($p < 0.05$) due to troglitazone administration. Also, irrespective of the administration of troglitazone, the concentration of leptin in the blood was
35 significantly high in the Zucker fa/fa rats ($p < 0.01$) relative to the normal rats.

C of Fig. 29 is a graph showing, as relative values, the density of bands obtained by Western blotting of

protein-extracted samples of the brains from the group (TGZ: +) in which the troglitazone-containing food was given to the normal rats (Lean) and the Zucker fa/fa rats and the group (TGZ: -) in which the troglitazone-free food was given thereto. In the normal rats, no difference in the amount expressed of nesfatin in the brain was noted between the group (TGZ: +) in which the troglitazone-containing food was given and the group (TGZ: -) in which the troglitazone-free food was given. In contrast, in the Zucker fa/fa rats, the amount expressed of nesfatin in the brain significantly increased ($p < 0.01$). Also, irrespective of the administration of troglitazone, the amount expressed of nesfatin in the brain was significantly high in the Zucker fa/fa rats ($p < 0.01$) relative to the normal rats.

The above results suggest that troglitazone that was used as an antidiabetic drug does not induce nesfatin in the brain of the normal animals, whereas it can induce the expression of nesfatin in the brain of the animal model that exhibits a leptin-resistant pathology. In contrast, it is suggested, the enhanced blood concentrations of leptin cannot be obtained by troglitazone in both the normal animals and the pathogenic animals, and conversely blood concentrations are decreased.

Working Example 32

Detection of the nesfatin-1 peptide in the HPLC fractions of the hypothalamus tissue extract

The peptide extracted from the hypothalamus tissue in Working Example 21 as the sample was subjected to HPLC fractionation, and the fractions were examined for the presence of nesfatin-1 by the competitive EIA assay system. Furthermore, in order to investigate the peptide detected in the fractions is a molecule corresponding to nesfatin-1, the HPLC fractions of the hypothalamus tissue extract were analyzed by the Western blotting method.

HPLC fractions of the hypothalamus tissue extract

was prepared in a similar manner to Working Example 21, and the fractions of No. (Fraction #) 43-47 were lyophilized. Samples after lyophilization were dissolved in 100 μ l of PBS, and 20 μ l of it was subjected to SDS-PAGE on a 12% polyacrylamide gel, and then to Western blotting with anti-nesfatin-1 antibody in a similar manner to Working Example 3.

<Result>

A of Fig. 30 shows an image of Western blotting of the fraction No. 45 obtained by fractionating a peptide extract from the rat hypothalamus by HPLC. The molecular weight of the band detected by Western blotting is about 9.7 kd, which almost agreed with that of the recombinantly prepared nesfatin-1 peptide (Working Example 10, Fig. 9C). B of Fig. 30 shows an image of the region at a molecular weight of about 9.7 kd in the image of Western blotting of the fraction Nos. 43-47 obtained by fractionating the peptide extract from the rat hypothalamus by HPLC. The 9.7 kd band is most strongly observed in fraction No. 45, which result agreed with the pattern obtained by determining by a competitive EIA method the fractions obtained by fractionating the peptide extract from the rat hypothalamus by HPLC (b-1 of Fig. 19B).

The above result indicated that a molecule corresponding to nesfatin-1 is present in the rat hypothalamus, and that a molecule corresponding to nesfatin-1 can be detected by fractionating it according to the method in Working Example 21 and then by detecting by a competitive EIA method and/or the Western blotting method.

Working Example 33

Study on the specificity of immunohistochemical stain of the regions of the rat hypothalamus

In Working Example 4, in order to analyze the NEFA expression site in the hypothalamus of the rat brain which is related to food intake the control, an

immunohistochemical analysis using rat brain sections was carried out. In order to study whether the stain is nesfatin-specific, an immunohistochemical analysis was carried out again taking into consideration the binding property of a peptide known to control food intake to an antibody.

In a method similar to that in Working Example 4, immunohistochemical stain was carried out on tissue sections of the rat brain each containing arcuate nucleus and paraventricular nucleus, in which anti-nesfatin-1 antibody (nesfatin-1 IgG; Working Example 10) was used instead of a anti-NAP polyclonal antibody as the primary antibody. Also, before reacting the primary antibody with the tissue sample, 5 μ g of each peptide of anti-nesfatin-1 antibody (Working Example 10), and leptin (Rat leptin: R & D Systems, 598-LP-01M), α MSH (Melanocyte Stimulating Hormone; Peptide Institute, Inc., 4057-v), CART (Rat Cocaine- and Amphetamine-Regulated Transcript 55-102; Peptide Institute, Inc., 4351-s) and NPY (Human, Rat Neuropeptide Y; Peptide Institute, Inc., 4158-v), known for the effect of controlling food intake, per 1 μ g of anti-nesfatin-1 antibody was added, and reacted at room temperature for 1 hour, and then used in the immunohistochemical stain to validate the reaction specificity of the antibody.

<Result>

A of Fig. 31 shows the result of immunohistochemical stain section using an anti-nesfatin-1 antibody in the rat brain tissue containing the arcuate nucleus, and the result of immunohistochemical stain carried out after the primary antibody and various peptides were previously reacted. In A of Fig. 31, since a-1 shows that the arcuate nucleus of the rat brain is immunologically stained with the anti-nesfatin-1 antibody and a-2 shows that when the anti-nesfatin-1 antibody is previously reacted with the nesfatin-1 peptide the stain disappears,

it was demonstrated that the anti-nesfatin-1 antibody detects nesfatin being expressed in the arcuate nucleus. In contrast, when the anti-nesfatin-1 antibody was previously reacted with leptin (a-3 in A of Fig. 31),
5 α MSH (a-4 in A of Fig. 31), CART (a-5 in A of Fig. 31) and NPY (a-6 in A of Fig. 31), the immunological stain in the arcuate nucleus does not disappear, indicating that these peptides do not bind to the anti-nesfatin-1 antibody, and thus it was demonstrated that in the
10 immunohistochemical stain said antibody does not react to these peptides.

B of Fig. 31 shows the result of immunohistochemical stain using an anti-nesfatin-1 antibody in the rat brain tissue section containing the paraventricular nucleus, and the result of immunohistochemical stain carried out
15 after the primary antibody and various peptides were previously reacted. In B of Fig. 31, since b-1 shows that the arcuate nucleus of the rat brain is immunologically stained with the anti-nesfatin-1 antibody and a-2 shows that when the anti-nesfatin-1 antibody is
20 previously reacted with the nesfatin-1 peptide the stain disappears, it was demonstrated that the anti-nesfatin-1 antibody detects nesfatin being expressed in the paraventricular nucleus. In contrast, when the anti-nesfatin-1 antibody was previously reacted with leptin
25 (b-3 in B of Fig. 31), α MSH (b-4 in B of Fig. 31), CART (b-5 in B of Fig. 31) and NPY (b-6 in B of Fig. 31), the immunological stain in the arcuate nucleus does not disappear, indicating that these peptides do not bind to
30 the anti-nesfatin-1 antibody, and thus it was demonstrated that in the immunohistochemical stain said antibody does not react to these peptides.

In the above result, the expression of nesfatin is specifically detected in the immunohistochemical stain
35 using the anti-nesfatin-1 antibody, indicating that in the rat brain nesfatin is being expressed in the arcuate nucleus and the paraventricular nucleus.

Working Example 34

Study on the effect of continuous administration of nesfatin-1 into the rat ventricle on the weight of the adipose tissue

5 In order to study the effect of nesfatin-1 on the amount of the adipose tissue, nesfatin-1 was continuously administered into the rat ventricle to analyze changes in the weight of the adipose tissue etc.

10 Nesfatin-1 (5 pmol per day) or physiological saline alone (the control group) was administered into the third ventricle of a rat using an osmotic pump for 10 consecutive days (5 and 4 rats were used per group). Intraventricular administration into rats by the osmotic pump was carried out in a manner similar to that in
15 Working Example 13.

 After the administration of nesfatin-1 or physiological saline alone for 10 days, each rat was sacrificed, dissected to excise all of the abdominal subcutaneous adipose tissue, the epididymal adipose
20 tissue (these are white adipose tissue) and the retroperitoneal brown adipose tissue, and the weight of them was measured. Also, the gastrocnemial muscles of the bilateral hind legs were harvested and the weight of them was measured. From the weight of the tissues
25 measured, their ratio to the body weight of each individual was determined (tissue weight/body weight, mg/g). For testing of significant difference, analysis of variance was used.

<Result>

30 Fig. 32 is a graph of the result showing the ratio of tissue weight of each adipose tissue (A-E) and the gastrocnemial muscle (F) obtained from the rats that were given nesfatin-1 or physiological saline alone for 10 days relative to the body weight. In the abdominal
35 subcutaneous adipose tissue (A of Fig. 32), the epididymal adipose tissue (B of Fig. 32) and the mesenteric adipose tissue (C of Fig. 32), significant

decreases in the ratio of tissue weight to body weight were noted in the nesfatin-1-administration group relative to the control group that received physiological saline alone. In the retroperitoneal adipose tissue (D of Fig. 32), the ratio of tissue weight to body weight tended to decrease but no significant difference was noted relative to the control group. Furthermore, in the brown adipose tissue (E of Fig. 32) and the gastrocnemial muscle (F of Fig. 32), no significant difference in the total tissue weight to the body weight was noted between the control group and the nesfatin-1-administration group.

The above result indicated that nesfatin-1 has an effect of decreasing the ratio of tissue weight of the white adipose tissue to body weight, i.e. body fat percentage. It was also shown that it does not affect the ratio of tissue weight of the brown adipose tissue and the muscle tissue to body weight.

Working Example 35

Study on the effect of intraperitoneal administration of nesfatin-1 into the rat on blood biochemical parameters

It was investigated whether the activities of suppressing food intake, suppressing body weight gain or reducing the amount of the adipose tissue are associated with changes in blood sugar (blood glucose values) and lipid-related parameters (cholesterol values, triglyceride values).

As experimental animals, 7 week-old male c57BL/6J mice were purchased from Nippon Clea, and were housed in a cycle of 12 hours of the light period from 6 a.m. to 6 p.m. and 12 hours of the dark period from 6 p.m. to 6 a.m. the next morning and were allowed free access to a pellet food (Nippon Clea, CE-2), and kept at 22°C.

Into the abdominal cavity of the c57BL/6J mice, 10 nmol of recombinant mouse nesfatin-1 prepared in Working Example 16 dissolved in 100 μ l of physiological saline

was administered, and as the control sample physiological saline alone was used. Using a tuberculin syringe equipped with a 26G needle, 100 μ l each of the sample was administered once into the abdominal cavity of each mouse (5 animals per group), and the time of administration was immediately before the start (6 p.m.) of the dark period.

Each mouse that received administration was placed in an individual cage, and during 0-3 hours after the administration the weight decreased of the pellet food was measured to determine the amount of food intake. Also, 3 hours after the administration each mouse was sacrificed by decapitation to collect whole blood, and serum was collected therefrom. For the serum, the contents of glucose, total cholesterol and triglyceride were measured using commercial reagents for determination (KYOWA MEDEX, Determiner L GLUII, Determiner L TCII, Determiner L TGII).

For testing of significant difference, analysis of variance was used.

<Result>

Fig. 33 is a graph of the assay result of the amount of food intake, glucose, total cholesterol and triglyceride in the blood when nesfatin-1 or physiological saline alone was intraperitoneally given to the mice.

Nesfatin-1 administration significantly reduced the amount of food intake as compared to the control group. In contrast, little difference was noted between the nesfatin-1-administration group and the control group in glucose content in the blood (glucose in the figure) which represents a blood sugar level, or lipid-related parameters such as total cholesterol content (cholesterol in the figure), triglyceride content (triglyceride in the figure).

The above result suggests that the effect of nesfatin-1 is to suppress food intake, suppress body weight gain and reduce the amount of the adipose tissue

without causing changes in blood sugar levels and blood levels of lipid-related parameters.

In accordance with the present invention, a factor involved in food intake control and/or body weight control can be obtained by using a PPAR γ agonist. Also by using nesfatin and/or nesfatin-1, diseases associated with metabolic and food intake disorders such as obesity or adiposis and nervous hyperphagia, and diseases associated with adiposis such as type 2 diabetes mellitus, impaired glucose tolerance, hypertension, hyperlipidemia, hyperuricemia, fatty liver, cardiac diseases, cerebral vascular diseases, sleep apnea syndrome, orthopedic diseases such as osteoarthritis, menstrual disorders and malignant tumors can be prevented or treated. Furthermore, by using a substance such as antibody that suppresses the activity of nesfatin or nesfatin-1, anorexia in post-surgery and/or cancer patients, and diseases associated with nutritional and feeding disorders such as cibophobia can be prevented or treated.

WHAT IS CLAIMED IS:

1. A polypeptide consisting of the amino acid sequence of SEQ ID NOs: 13 to 15, or a fragment thereof having an activity of suppressing food intake or suppressing body weight gain.
2. A polypeptide having an activity of suppressing food intake or suppressing body weight gain, wherein said polypeptide consists of an amino acid sequence at least 80% identical to SEQ ID NOs: 13 to 15.
3. A polypeptide having an activity of suppressing food intake or suppressing body weight gain, wherein said polypeptide comprises the amino acid sequence of SEQ ID NOs: 13 to 15, with the proviso that said polypeptide is not NUCB2.
4. A polypeptide having an activity of suppressing food intake or suppressing body weight gain, wherein said polypeptide comprises an amino acid sequence having at least 80% identity with the amino acid sequence of SEQ ID NOs: 13 to 15, with the proviso that said polypeptide is not NUCB2.
5. The polypeptide according to any one of claim 1 to 4, wherein at least one amino acid residue of the polypeptide has been chemically modified by a compound or a peptide.
6. A nucleic acid molecule encoding a polypeptide according to any one of claims 1 to 5.
7. A nucleic acid molecule consisting of a nucleotide sequence of SEQ ID NOs: 18 to 20.
8. A nucleic acid molecule that hybridizes to a nucleotide sequence of SEQ ID NOs: 18 to 20, or a partial sequence thereof under a highly stringent condition, and that encodes a polypeptide having an activity of suppressing food intake or suppressing body weight gain.

9. A vector comprising the nucleic acid molecule according to any one of claims 6 to 8.

10. A transformant comprising the nucleic acid molecule according to any one of claims 6 to 8.

11. A pharmaceutical composition comprising the polypeptide according to any one of claims 1 to 5, the vector according to claim 9, or the transformant according to claim 10 as an active ingredient, and pharmaceutically acceptable carrier.

12. The pharmaceutical composition according to claim 11 having an activity of suppressing food intake or suppressing body weight gain.

13. A transgenic non-human organism into which the nucleic acid molecule according to any one of claim 6 to 8 is introduced.

14. The transgenic non-human animal according to claim 13 wherein said transgenic non-human organism is a transgenic non-human animal that exhibits the state of suppressed food intake or the state of suppressed body weight gain.

15. A method producing the polypeptide according to any one of claims 1 to 5 comprising the following step (a) or (b):

(a) culturing the transformant according to claim 10 and recovering the polypeptide produced from the transformant; or

(b) conducting a peptide synthetic method.

16. An assay method of predicting or diagnosing the state of enhanced food intake or enhanced body weight gain comprising the step of:

detecting the amount contained of a nucleic acid molecule comprising a nucleotide sequence of SEQ ID NOs: 18 to 20, or of a polypeptide comprising an amino acid sequence of SEQ ID NOs: 13 to 15 in a biological sample from a mammal.

17. An assay kit for use in the assay method according to claim 16, said kit comprising:

at least one of a PCR primer, a probe or a DNA chip for detecting a nucleic acid molecule comprising a nucleotide sequence of SEQ ID NOs: 18 to 20; an antibody recognizing a polypeptide comprising an amino acid sequence of SEQ ID NOs: 13 to 15; a standard peptide, or a modified peptide for the binding competitive reaction.

18. A method of screening a therapeutic or preventive agent having an effect of suppressing food intake or suppressing body weight gain, said method comprising the steps of:

detecting the increased expression of a gene comprising a nucleotide sequence of SEQ ID NOs: 18 to 20, or the increased amount of a polypeptide comprising an amino acid sequence of SEQ ID NOs: 13 to 15 intracellularly contained in a mammalian cell to which a test substance has been contacted, or extracellularly secreted.

19. A method of screening a therapeutic or preventive agent having an effect of suppressing food intake or suppressing body weight gain, said method comprising the steps of:

detecting the enhanced expression of a gene comprising a nucleotide sequence of SEQ ID NOs: 18 to 20, or the enhanced production of a polypeptide comprising an amino acid sequence of SEQ ID NOs: 13 to 15 in a biological sample from a test animal into which a test substance has been administered.

20. A method of screening a therapeutic or preventive agent having an effect of suppressing food intake or suppressing body weight gain, said method comprising the steps of:

detecting the suppression of food intake or the suppression of body weight gain in a knock-out non-human animal in which the entire region or part thereof of a gene comprising a nucleotide sequence of SEQ ID NOs: 18 to 20 has been deleted, and into which a test substance has been administered.

21. A method of screening a therapeutic or preventive agent having an effect of enhancing food intake or enhancing body weight gain, said method comprising the steps of:

detecting the decreased expression of a gene comprising a nucleotide sequence of SEQ ID NOs: 18 to 20 in a mammalian cell to which a test substance has been contacted, or the decreased amount of a polypeptide comprising an amino acid sequence of SEQ ID NOs: 13 to 15 intracellularly contained in said cell or extracellularly secreted.

22. A method of screening a therapeutic or preventive agent having an effect of enhancing food intake or enhancing body weight gain, said method comprising the steps of:

detecting the suppressed expression of a gene comprising a nucleotide sequence of SEQ ID NOs: 18 to 20 or the suppressed production of a polypeptide comprising an amino acid sequence of SEQ ID NOs: 13 to 15 in a biological sample from a test animal to which a test substance has been administered.

23. A method of screening a therapeutic or preventive agent having an effect of enhancing food intake or enhancing body weight gain, said method comprising the steps of:

detecting the enhancement of food intake or the enhancement of body weight gain in the non-human organism according to claims 13 to 14.