

## DESCRIPTION

## RGMa BINDING PROTEIN AND USE THEREOF

## 5 TECHNICAL FIELD

[0001]

The present invention relates to an RGMa-binding protein and a use thereof.

## BACKGROUND ART

10 [0002]

RGM (repulsive guidance molecule), which is a GPI-anchored membrane protein with a molecular weight of about 33 kDa, has been initially identified as an axon guidance molecule in the visual system (see Non-Patent Document 1). The RGM family includes three members called RGMa, RGMb and RGMc. Among  
15 them, RGMa is re-expressed after central nervous system injury in adult humans and rats as well as at developmental stage, and RGMa inhibition in rat accelerates neurite outgrowth after spinal cord injury and promotes functional recovery (see Non-Patent Document 2). Thus, RGMa is thought to be a neurite inhibitor after central nervous system injury.

20 [0003]

RGMa also has been reported to have effects in the immune system. RGMa is expressed on dendritic cells and acts on T cells, thereby enhancing adhesion of T cells to ICAM-1 and fibronectin and inducing cytokine production (Patent Document 4). In a mouse model of multiple sclerosis, administration of anti-RGMa antibody  
25 suppresses symptoms due to encephalomyelitis and also shows effects of suppressing onset and relapse. It is thought that anti-RGMa antibody binds to RGMa expressed on dendritic cells to inhibit the activation of T cells, thereby exerting effects on multiple sclerosis.

[0004]

The signal transduction mechanism of RGMa is also being elucidated, and neogenin protein has been reported as an RGMa receptor (Patent Document 3).

Neogenin is a single-transmembrane protein expressed on neurons and T cells.

5 RGMa binds to neogenin on a cell membrane to induce intracellular RhoA activation and Ras inactivation, thereby providing a neurite outgrowth inhibitory effect. Meanwhile, neogenin is known to cause apoptosis in the absence of RGMa binding in a developing chicken brain (Matsunaga et al., Dev.Growth Differ.46, 481, 2004). Thus, the RGMa/neogenin pathway is thought to have two conflicting effects  
10 of promoting neuronal survival, which is a favorable effect for nerve regeneration, and of inhibiting neurite outgrowth, a negative effect.

[0005]

As a pharmaceutical agent targeting RGM, Patent Document 1 discloses an axon regeneration promoting agent containing an anti-RGM neutralizing antibody as  
15 an active ingredient. Patent Documents 2 and 3 disclose a therapeutic agent for mechanical damage to brain and spinal cord, an anti-RGM antibody that regulates the binding of RGM to its receptor neogenin. Patent Document 4 discloses medical uses of anti-RGM antibody such as for multiple sclerosis. Patent Document 5 discloses therapeutic uses of anti-RGM antibody for diseases including multiple sclerosis,  
20 mammalian brain injury, spinal cord injury, apoplexy, neurodegenerative disease and schizophrenia. Furthermore, Patent Document 6 discloses therapeutic uses of RGM modulators such as anti-RGM antibody for spinal cord injury and multiple sclerosis, and Non-Patent Document 3 discloses a therapeutic use for progressive multiple sclerosis.

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PRIOR ART DOCUMENTS

PATENT DOCUMENTS

[0006]

Patent Document 1: WO2005/087268

Patent Document 2: Japanese Translated PCT Patent Application Laid-open No.  
2010-537655

Patent Document 3: Japanese Translated PCT Patent Application Laid-open No.  
5 2009-510002

Patent Document 4: WO2011/071059

Patent Document 5: Japanese Translated PCT Patent Application Laid-open No.  
2011-512806

Patent Document 6: Japanese Translated PCT Patent Application Laid-open No.  
10 2004-525875

#### NON-PATENT DOCUMENTS

[0007]

Non-Patent Document 1: Neuron 5, 735-743 (1990)

15 Non-Patent Document 2: J. Cell Biol. 173, 47-58 (2006)

Non-Patent Document 3: Cell Reports 10, 1-12 (2015)

#### SUMMARY

[0007a]

In a first aspect of the present disclosure, there is provided an isolated anti-RGMA antibody, or an antigen-binding fragment thereof, wherein the amino acid sequence  
5 of each of the light chain complementarity determining region 1 (LCDR1), the light chain complementarity determining region 2 (LCDR2), the light chain complementarity determining region 3 (LCDR3), the heavy chain complementarity

determining region 1 (HCDR1), the heavy chain complementarity determining region 2 (HCDR2) and the heavy chain complementarity determining region 3 (HCDR3) comprises the following:

- LCDR1: RASQDISSYLN (SEQ ID NO: 30 in Sequence Listing),
- 5 LCDR2: YTSRLHS (SEQ ID NO: 31 in Sequence Listing),
- LCDR3: QQLNTLP (SEQ ID NO: 32 in Sequence Listing),
- HCDR1: DAWMD (SEQ ID NO: 33 in Sequence Listing),
- HCDR2: EIRSKANNHATYYAESVKG (SEQ ID NO: 34 in Sequence Listing) and
- HCDR3: RDGAY (SEQ ID NO: 35 in Sequence Listing);
- 10 or
- LCDR1: RSSQSLVHSNGNTYLH (SEQ ID NO: 36 in Sequence Listing)
- LCDR2: KVSNRFS (SEQ ID NO: 37 in Sequence Listing)
- LCDR3: SQSTHVP (SEQ ID NO: 38 in Sequence Listing)
- HCDR1: TSYAWN (SEQ ID NO: 39 in Sequence Listing)
- 15 HCDR2: YISYDGTNNYNPSLKN (SEQ ID NO: 40 in Sequence Listing) and
- HCDR3: SFG.

[0007b]

In a second aspect of the present disclosure, there is provided a nucleic acid molecule coding for the protein portion of the anti-RGMA antibody or an antigen-binding

fragment thereof according to the first aspect.

[0007c]

In a third aspect of the present disclosure, there is provided a recombinant vector comprising the nucleic acid molecule according to the second aspect.

5 [0007d]

In a fourth aspect of the present disclosure, there is provided a host cell containing the recombinant vector according to the third aspect.

[0007e]

In a fifth aspect of the present disclosure, there is provided a method for producing  
10 the anti-RGMA antibody or an antigen-binding fragment thereof according to the first aspect, the method comprising a step of culturing the host cell according to the fourth aspect.

[0007f]

In a sixth aspect of the present disclosure, there is provided a pharmaceutical  
15 composition comprising the anti-RGMA antibody or an antigen-binding fragment thereof according to the first aspect.

[0007g]

In a seventh aspect of the present disclosure, there is provided the pharmaceutical composition according to the sixth aspect for use in preventing, treating, or  
20 preventing the relapse of neurological or immunological diseases.

TECHNICAL PROBLEM

[0008]

Therapeutic uses of anti-RGMa antibody against neurological and immunological diseases are disclosed as described above, but conventional antibodies have problems such as insufficient activity, possibility of impairing intrinsic functions of RGMa, and side effects. In particular, conventional antibodies  
5 may inhibit binding between RGMa and neogenin, thereby also inhibiting favorable effects such as apoptosis suppression exerted by neogenin bound to RGMa.

Thus, an object of the present invention is to provide an RGMa-binding protein which does not inhibit RGMa/neogenin interaction but neutralizes the neurite

outgrowth inhibiting activity of RGMa.

## SOLUTION TO PROBLEM

[0009]

5           The inventors of the present invention intensively studied in order to solve the above problems. As a result, the present inventors have succeeded in obtaining an RGMa-binding protein which does not inhibit binding between RGMa and neogenin but neutralizes the neurite outgrowth inhibiting activity of RGMa, and have found that the RGMa-binding protein can be used as a medicine for neurological or  
10 immunological diseases, thereby completing the present invention.

[0010]

The present invention is as follows.

[1]       An isolated RGMa-binding protein, which does not inhibit binding between RGMa and neogenin but neutralizes the neurite outgrowth inhibiting activity of  
15 RGMa.

[2]       The RGMa-binding protein described in [1], which binds to human, rat and/or mouse RGMa.

[3]       The RGMa-binding protein described in [1] or [2], which binds to peptides of EEVVNAVEDWDSQG (SEQ ID NO: 26 in Sequence Listing),  
20 NQQIDFQAFHTNAE (SEQ ID NO: 27 in Sequence Listing), PTAPETFPYET (SEQ ID NO: 28 in Sequence Listing), and/or KLPVEDLYYQA (SEQ ID NO: 29 in Sequence Listing).

[4]       The RGMa-binding protein described in any one of [1] to [3], which binds to peptides of SEQ ID NOS: 26 and 27 in Sequence Listing.

25 [5]       The RGMa-binding protein described in any one of [1] to [4], which binds to peptides of SEQ ID NOS: 26, 27 and 28 in Sequence Listing.

[6]       The RGMa-binding protein described in any one of [1] to [4], which binds to peptides of SEQ ID NOS: 26, 27 and 29 in Sequence Listing.

- [7] The RGMa-binding protein described in any one of [1] to [6], wherein the RGMa-binding protein is a human antibody, a humanized antibody or a chimeric antibody, or an antigen-binding fragment thereof.
- [8] A nucleic acid molecule coding for the protein portion of the RGMa-binding  
 5 protein described in any one of [1] to [7].
- [9] A recombinant vector comprising the nucleic acid molecule described in [8].
- [10] A host cell containing the recombinant vector described in [9].
- [11] A method for producing the RGMa-binding protein described in [1] to [7], the method comprising a step of culturing the host cell described in [10].
- 10 [12] A pharmaceutical composition comprising the RGMa-binding protein described in any one of [1] to [7].
- [13] The pharmaceutical composition described in [12] for use in preventing, treating, or preventing the relapse of neurological or immunological diseases.
- [14] The pharmaceutical composition described in [13], wherein the neurological  
 15 diseases are selected from the group consisting of amyotrophic lateral sclerosis, brachial plexus injury, brain damage (including traumatic brain injury), cerebral palsy, Guillain-Barre syndrome, cerebral leukodystrophy, multiple sclerosis (including relapsing-remitting multiple sclerosis, primary progressive multiple sclerosis, secondary progressive multiple sclerosis), neuromyelitis optica, post-polio  
 20 syndrome, spina bifida, spinal cord injury, spinal muscular atrophy, spinal neoplasm, transverse myelitis, dementia (including senile dementia, mild cognitive impairment, Alzheimer's disease, dementia associated with Alzheimer's disease), Huntington's disease, tardive dyskinesia, mania, Parkinson's disease, Steele-Richardson syndrome, Down's syndrome, myasthenia gravis, neurotrauma (including optic nerve trauma),  
 25 vascular amyloidosis, cerebral hemorrhage associated with amyloidosis, brain infarction, cerebritis, acute confusional state, glaucoma, schizophrenia and retinal nerve fiber layer degeneration (including diabetic retinopathy, ischemic optic neuropathy, X-linked retinoschisis, drug-induced optic neuropathy, retinal dystrophy,

age-related macular degeneration, eye diseases characterized by optic disc drusen, eye diseases characterized by genetic determinant for photoreceptor degeneration, autosomal recessive cone-rod dystrophy, mitochondrial disorder associated with optic neuropathy).

- 5 [15] The pharmaceutical composition described in [13], wherein the immunological diseases are selected from the group consisting of multiple sclerosis (including relapsing-remitting multiple sclerosis, primary progressive multiple sclerosis, secondary progressive multiple sclerosis), neuromyelitis optica, psoriasis, arthritis (including rheumatoid arthritis, osteoarthritis, psoriatic arthritis), Guillain-  
10 Barre syndrome, neuro-Behcet disease, pernicious anemia, type I (insulin-dependent) diabetes mellitus, systemic lupus erythematosus (SLE), inflammatory bowel disease (IBD), Sjogren's syndrome, Goodpasture's syndrome, Graves' disease, autoimmune hemolytic anemia, autoimmune thrombocytopenic purpura, asthma, pollinosis, atopic dermatitis, glomerulonephritis, myasthenia gravis, Hashimoto's  
15 disease, and sarcoidosis.

- [16] The pharmaceutical composition described in [13], wherein the neurological or immunological diseases are selected from the group consisting of spinal cord injury, neurotrauma (including optic nerve trauma) and multiple sclerosis (including relapsing-remitting multiple sclerosis, primary progressive multiple sclerosis,  
20 secondary progressive multiple sclerosis).

- [17] An isolated anti-RGMA antibody, or an antigen-binding fragment thereof, wherein the amino acid sequence of each of the light chain complementarity determining region 1 (LCDR1), the light chain complementarity determining region 2 (LCDR2), the light chain complementarity determining region 3 (LCDR3), the  
25 heavy chain complementarity determining region 1 (HCDR1), the heavy chain complementarity determining region 2 (HCDR2) and the heavy chain complementarity determining region 3 (HCDR3) comprises the following:  
LCDR1:RASQDISSYLN (SEQ ID NO: 30 in Sequence Listing)

LCDR2:YTSRLHS (SEQ ID NO: 31 in Sequence Listing)

LCDR3:QQLNTLP (SEQ ID NO: 32 in Sequence Listing)

HCDR1:DAWMD (SEQ ID NO: 33 in Sequence Listing)

HCDR2:EIRSKANNHATYYAESVKG (SEQ ID NO: 34 in Sequence Listing) and

5 HCDR3:RDGAY (SEQ ID NO: 35 in Sequence Listing);

or

LCDR1:RSSQSLVHSNGNTYLH (SEQ ID NO: 36 in Sequence Listing)

LCDR2:KVSNRFS (SEQ ID NO: 37 in Sequence Listing)

LCDR3:SQSTHVP (SEQ ID NO: 38 in Sequence Listing)

10 HCDR1:TSYYWN (SEQ ID NO: 39 in Sequence Listing)

HCDR2:YISYDGTNNYNPSLKN (SEQ ID NO: 40 in Sequence Listing) and

HCDR3:SFG, and

wherein in each of the CDR sequences one or several amino acids may be substituted, deleted, and/or added.

15 [18] The anti-RGMa antibody or an antigen-binding fragment thereof described in [17],

wherein the heavy chain variable region (VH) comprises the following:

VH:

EVQLVESGGGLVQPGRSLRLSCTASGFTFSDAWMDWVRQAPGKGLEWVAE

20 IRSKANNHATYYAESVKG RFTISRDDSKSIVY LQMNSLRTE DTALYYCTRRD

GAYWGKGTTVTVSS (SEQ ID NO: 41 in Sequence Listing) or an amino acid sequence having an identity of at least 90% with said amino acid sequence; and

wherein the light chain variable region (VL) comprises the following:

VL:

25 DIQMTQSPSSVSASVGDRV TITCRASQDISSYLNWYQQKPGKAPKLLIYYTSR

LHSGVPSRFSGSGSGTDFTLTIS SLQPEDFASYFCQQLNTLPWTFGGG TKVEM

E (SEQ ID NO: 42 in Sequence Listing) or an amino acid sequence having an identity of at least 90% with said amino acid sequence.

[19] The anti-RGMa antibody or an antigen-binding fragment thereof described in [17] or [18], wherein the anti-RGMa antibody is a humanized antibody.

[20] The anti-RGMa antibody or an antigen-binding fragment thereof described in any one of [17] to [19], wherein the anti-RGMa antibody comprises a constant

5 region of human IgG.

[21] An RGMa-binding protein, which competes with the anti-RGMa antibody described in [17] or [18] for binding to RGMa.

[22] A nucleic acid molecule coding for the protein portion of the anti-RGMa antibody or an antigen-binding fragment thereof described in any one of [17] to [20].

10 [23] The nucleic acid molecule described in [22], wherein the nucleotide sequences coding for the VH and VL amino acid sequences each is a nucleotide sequence comprising:

VH:

gaagtgcagctgggtggaatctggcggcgactgggtgcagcctggcagatccctgagactgtcctgtaccgcctccggct  
 15 tcaccttctccgacgcctggatggattgggtgcgacaggctcctggcaagggcctggaatgggtggccgagatccggtc  
 caaggccaacaaccacgccactactacgccgagctgtgaagggccgggtcaccatctcccgaggacgactccaagtcc  
 atcgtgtacctgcagatgaactccctgcggaccgaggacaccgcctgtactactgcaccagaagggacggcgctact  
 ggggcaagggcaccacagtgcagtgctctcc (SEQ ID NO: 43 in Sequence Listing), and

VL:

20 gacatccagatgaccagtcctccctcctcctgtgtctgcttccgtggcgacagagtgaccatcacctgtcgggcctccca  
 ggacatctcctctactgaactgggtatcagcagaagcccggaaggccccaagctgtgatctactacacctccggc  
 tgcactccggcgtgcctctagatttccggctctggctccggcaccgactttaccctgaccatctccagcctgcagcccg  
 aggacttcgcctcctacttctgtcagcagctgaacaccctgcctggaccttggcggaggcaccaaggtggaaatggaa  
 (SEQ ID NO: 44 in Sequence Listing).

25 [24] A recombinant vector comprising the nucleic acid molecule described in [22] or [23].

[25] A host cell containing the recombinant vector described in [24].

[26] A method for producing the anti-RGMa antibody or an antigen-binding

fragment thereof described in any one of [17] to [20], the method comprising a step of culturing the host cell described in [25].

[27] A pharmaceutical composition comprising the anti-RGMA antibody or an antigen-binding fragment thereof described in any one of [17] to [20].

5 [28] The pharmaceutical composition described in [27] for use in preventing, treating, or preventing the relapse of neurological or immunological diseases.

[29] The pharmaceutical composition described in [28], wherein the neurological diseases are selected from the group consisting of amyotrophic lateral sclerosis, brachial plexus injury, brain damage (including traumatic brain injury), cerebral  
10 palsy, Guillain-Barre syndrome, cerebral leukodystrophy, multiple sclerosis (including relapsing-remitting multiple sclerosis, primary progressive multiple sclerosis, secondary progressive multiple sclerosis), neuromyelitis optica, post-polio syndrome, spina bifida, spinal cord injury, spinal muscular atrophy, spinal neoplasm, transverse myelitis, dementia (including senile dementia, mild cognitive impairment,  
15 Alzheimer's disease, dementia associated with Alzheimer's disease), Huntington's disease, tardive dyskinesia, mania, Parkinson's disease, Steele-Richardson syndrome, Down's syndrome, myasthenia gravis, neurotrauma (including optic nerve trauma), vascular amyloidosis, cerebral hemorrhage associated with amyloidosis, brain infarction, cerebritis, acute confusional state, glaucoma, schizophrenia and retinal  
20 nerve fiber layer degeneration (including diabetic retinopathy, ischemic optic neuropathy, X-linked retinoschisis, drug-induced optic neuropathy, retinal dystrophy, age-related macular degeneration, eye diseases characterized by optic disc drusen, eye diseases characterized by genetic determinant for photoreceptor degeneration, autosomal recessive cone-rod dystrophy, mitochondrial disorder associated with  
25 optic neuropathy).

[30] The pharmaceutical composition described in [28], wherein the immunological diseases are selected from the group consisting of multiple sclerosis (including relapsing-remitting multiple sclerosis, primary progressive multiple

sclerosis, secondary progressive multiple sclerosis), neuromyelitis optica, psoriasis, arthritis (including rheumatoid arthritis, osteoarthritis, psoriatic arthritis), Guillain-Barre syndrome, neuro-Behcet disease, pernicious anemia, type I (insulin-dependent) diabetes mellitus, systemic lupus erythematosus (SLE), inflammatory  
 5 bowel disease (IBD), Sjogren's syndrome, Goodpasture's syndrome, Graves' disease, autoimmune hemolytic anemia, autoimmune thrombocytopenic purpura, asthma, pollinosis, atopic dermatitis, glomerulonephritis, myasthenia gravis, Hashimoto's disease, and sarcoidosis.

[31] The pharmaceutical composition described in [28], wherein the neurological  
 10 or immunological diseases are selected from the group consisting of spinal cord injury, neurotrauma (including optic nerve trauma) and multiple sclerosis (including relapsing-remitting multiple sclerosis, primary progressive multiple sclerosis, secondary progressive multiple sclerosis).

[32] A method for preventing, treating, or preventing the relapse of neurological  
 15 or immunological diseases, the method comprising a step of administering a effective dose of the RGMa-binding protein described in any one of [1] to [7] to a subject in need thereof.

[33] A method for preventing, treating, or preventing the relapse of neurological  
 20 or immunological diseases, the method comprising a step of administering an effective dose of the anti-RGMa antibody or an antigen-binding fragment thereof described in any one of [17] to [20] to a subject in need thereof.

## ADVANTAGEOUS EFFECT OF THE INVENTION

[0011]

25 The RGMa-binding protein of the present invention does not inhibit the interaction between RGMa and neogenin, thereby being able to maintain effects such as apoptosis inhibition on neurons and the like exerted by neogenin bound to RGMa, thus having a strong protective effect on neurons and little concern for side effects

associated with neuron depletion. In addition, the humanized anti-RGMa antibody of the present invention is superior to conventional antibodies in properties such as binding to human RGMa and thermal stability. Therefore, the antibody can be used as a medicine for neurological or immunological diseases which has an excellent efficacy and few side effects.

## BRIEF DESCRIPTION OF THE DRAWINGS

[0012]

Fig. 1 shows a result of an RGMa-neogenin binding inhibition assay using an anti-RGMa polyclonal antibody (AF2459), an antibody of comparative example (r5F9), and antibodies of the present invention (r70E4 and 116A3).

Fig. 2 shows a result of an RGMa-BMP2 binding inhibition assay using a control mouse-IgG and antibodies of the present invention (B5.70E4 and B5.116A3).

Fig. 3 shows a result of a thermal stability test for antibodies using an antibody of comparative example (rH5F9), a chimeric antibody of the present invention (r116A3C) and humanized antibodies of the present invention (HE/KA and HA/KC).

Fig. 4 shows a result of a neurite outgrowth assay using the antibodies of the present invention (B5.70E4 (left) and B5.116A3 (right)).

Fig. 5 shows a result of an efficacy test using a control mouse-IgG (mouse IgG2bk), and the antibodies of the present invention (r70E4 and r116A3) and using a rat model of spinal cord injury. Results of efficacy tests in (A) a spinal cord crush model and (B) a spinal cord hemisection model are shown.

Fig. 6 shows a result of an efficacy test for the antibody of the present invention (B5.116A3) using a mouse model of multiple sclerosis induced by a PLP<sub>139-151</sub> peptide. The left side shows EAE scores, the right side shows changes in body weight, and the top and bottom parts show results when the test antibodies were administered after 7 and 10 days and after 18 and 21 days, respectively.

## DESCRIPTION OF THE EMBODIMENTS

[0013]

In order to facilitate understanding of the present invention, the terms used  
5 in the present invention will be explained below.

[0014]

*RGMa*

RGMa is a neurite outgrowth inhibitory protein in the central nervous  
system and human RGMa protein is biosynthesized as a precursor protein comprising  
10 450 amino acids as shown in SEQ ID NO: 1 in Sequence Listing. The signal peptide  
Met 1 to Pro 47 present at the N terminus (which refers to the peptide from the first  
methionine residue to the 47th proline residue from the N-terminal side, similarly  
described hereafter) is removed, the peptide bond between Asp 168 and Pro 169 is  
cleaved, and the C-terminal peptide Arg 423 to Cys 450 is removed and  
15 simultaneously a GPI anchor is added to the C-terminal carboxyl group of Gly 422  
which has become the C-terminus. A human RGMa protein is expressed on a cell  
membrane via the GPI anchor as a mature protein in which the N-terminal domain  
(Cys 48 to Asp 168) and the C-terminal domain (Pro 169 to Ala 424) are joined  
together by a disulfide bond. A precursor protein of mouse RGMa comprises the  
20 amino acid sequence shown in SEQ ID NO: 2 in Sequence Listing and a precursor  
protein of rat RGMa comprises the amino acid sequence shown in SEQ ID NO: 3 in  
Sequence Listing. Since their C terminal peptides are removed, mature proteins  
therefrom have the same amino acid sequences. In the present invention, RGMa may  
refer to either a precursor protein, a mature protein or an active fragment thereof, or  
25 may be a derivative or variant thereof, as long as it acts via binding to neogenin as  
described later. RGMa may be human RGMa or RGMa derived from other  
organisms, but human RGMa is preferred.

[0015]

*Neogenin*

Neogenin is expressed in, for example, central nervous neurons and functions as one of RGMa receptors. As shown in SEQ ID NO: 10 in Sequence Listing, the human neogenin protein comprises 1461 amino acids and is expressed as

5 a mature membrane protein after removal of the signal peptide Met 1 to Ala 33. In the present invention, neogenin may refer to either a precursor protein, a mature protein or an RGMa-binding fragment, or may be a derivative or variant thereof, as long as it binds to RGMa. Neogenin may be human neogenin or neogenin derived from other organisms, but human neogenin is preferred.

10 [0016]

*Neutralization*

The term "neutralization" as used herein refers to an action through which binding to a target of interest and inhibition of any function of the target can occur. In other words, the phrase "neutralizing the neurite outgrowth inhibitory activity of

15 RGMa" means that an RGMa binding protein binds to RGMa, thereby inhibiting the neurite outgrowth inhibitory activity of RGMa. Neurite outgrowth inhibitory activity can be assessed by one or more of several in vitro or in vivo assays known in the art, and can be assessed by, for example, the neurite outgrowth inhibition assay described herein.

20 [0017]

*Isolated*

The term "isolated" such as in isolated RGMa-binding protein means being identified, and separated and/or recovered from components in its natural state. Impurities in the natural state are substances that can interfere with the diagnostic or

25 therapeutic use of the antibody, including enzymes, hormones and other proteinous or nonproteinous solutes. Generally, isolation of RGMa-binding protein or the like can be achieved by at least one purification step. The RGMa binding protein purified by at least one purification step can be referred to as "isolated RGMa-binding

protein".

[0018]

### *RGMa-Binding Protein*

As used herein, the term "RGMa-binding protein" refers to a molecule  
 5 comprising a protein that binds to RGMa. Examples of the RGMa-binding proteins  
 include anti-RGMa antibodies and antigen-binding fragments thereof; RGMa-  
 binding scaffold proteins; soluble RGMa receptor proteins such as extracellular  
 domain of Neogenin; and fusion proteins thereof. The term "RGMa-binding scaffold  
 protein" refers to a protein that realizes the function of binding to RGMa by  
 10 introduction of mutations into the Kunitz domain of a serine protease inhibitor, the  
 extracellular domain of human fibronectin, ankyrin, lipocalin or the like. The term  
 "fusion protein" refers to RGMa-binding proteins bound chemically or genetically to  
 function molecules other than the RGMa-binding protein of the present application  
 such as nonpeptidic polymers such as polyethylene glycol (PEG), radioactive  
 15 substances, toxins, low molecular weight compounds, cytokines, growth factors (e.g.,  
 TGF- $\beta$ , NGF, neurotrophin), albumin, enzymes, and other antibodies.

[0019]

### *Human Antibody*

The term "human antibody" refers to an antibody in which light and heavy  
 20 chains are both derived from human immunoglobulin. Depending on the difference  
 in constant regions of heavy chains, human antibodies include IgG having  $\gamma$ -heavy  
 chains (including IgG1, IgG2, IgG3 and IgG4), IgM having  $\mu$ -heavy chains, IgA  
 having  $\alpha$ -heavy chains (including IgA1 and IgA2), IgD having  $\delta$ -heavy chains, or  
 IgE having  $\epsilon$ -heavy chains. Light chains, in principle, comprise either  $\kappa$  chains or  $\lambda$   
 25 chains.

[0020]

### *Humanized Antibody*

The term "humanized antibody" refers to an antibody comprising variable

regions comprising complementarity determining regions of an antibody derived from a nonhuman animal and framework regions derived from a human antibody, and constant regions derived from a human antibody.

[0021]

## 5 *Chimeric Antibody*

The term "chimeric antibody" refers to an antibody in which the light chain, the heavy chain, or both comprises a non-human derived variable region and a human derived constant region.

[0022]

## 10 *Anti-RGMa antibody*

As used herein, the term "anti-RGMa antibody" refers to immunoglobulin molecules which bind to RGMa, or modified molecules thereof. Modified molecules include multispecific antibodies, chimeric antibodies, humanized antibodies, functionally modified antibodies, and conjugated antibodies.

15 [0023]

## *Multispecific Antibody*

The term "multispecific antibody" refers to an asymmetric antibody comprising two or more independent antigen recognition sites having two or more different antigen specificities, including bispecific antibody having two antigen specificities and trispecific antibody having three antigen specificities.

20

[0024]

## *Functionally Modified Antibody*

As used herein, the term "functionally modified antibody" refers to an antibody in which functions other than the antigen binding function of the antibody, including cell killing function, complement activating function and serum half-life extending function, are modified by mainly modifying the amino acid or sugar chain of the Fc region of the antibody.

25

[0025]

### *Conjugated Antibody*

As used herein, the term "conjugated antibody" refer to an antibody bound chemically or genetically to function molecules other than antibody such as nonpeptidic polymers such as polyethylene glycol (PEG), radioactive substances, toxins, low molecular weight compounds, cytokines, growth factors (e.g., TGF- $\beta$ , NGF, neurotrophin), albumin, and enzymes.

[0026]

### *Antigen-Binding Fragment*

As used herein, the term "antigen-binding fragment" refers to a protein which comprises a part of an antibody and can bind to an antigen. Examples of the antigen-binding fragment include F(ab')<sub>2</sub>, Fab', Fab, Fv (variable fragment of antibody), disulfide-linked Fv, single-chain antibody (scFv), and polymers thereof. In addition, the antigen-binding fragment includes conjugated antigen-binding fragments bound chemically or genetically to function molecules other than the anti-RGMa antibody in the present application such as nonpeptidic polymers such as polyethylene glycol (PEG), radioactive substances, toxins, low molecular weight compounds, cytokines, growth factors (e.g., TGF- $\beta$ , NGF, neurotrophin), albumin, enzymes, and other antibodies.

[0027]

### *20 Complementarity Determining Region*

The term "complementarity determining region (CDR)" refers to a region forming an antigen binding site in a variable region of an immunoglobulin molecule, which is also called a hypervariable region, and particularly refers to a portion in which the amino acid sequence changes greatly for each immunoglobulin molecule. As CDR, light and heavy chains each have three CDRs (LCDR1, LCDR2, LCDR3, and HCDR1, HCDR2, HCDR3). In the present application, CDRs of an immunoglobulin molecule are determined according to the Kabat numbering system (Kabat et al., 1987, Sequences of Proteins of Immunological Interest, US Department

of Health and Human Services, NIH, USA).

[0028]

*Percent (%) Identity of Amino Acid Sequence*

"Percent (%) identity" with respect to the identified reference polypeptide  
5 sequence, such as variable region, is defined as the percentage of amino acid residues  
in a candidate sequence that are identical to the amino acid residues of a particular  
reference polypeptide sequence, after arranging the sequences and introducing gaps  
as necessary in order to obtain maximal % identity and assuming that no  
conservative substitutions are considered part of the sequence identity. Alignment  
10 for purposes of determining % identity can be accomplished by using various  
methods within the skill of the art, for example, using publicly available computer  
software such as BLAST, BLAST-2, ALIGN, or Megalign (DNASTAR) software.  
Those skilled in the art can determine appropriate parameters for aligning sequences,  
including any algorithms necessary to achieve maximal alignment over the full  
15 length of the sequences being compared. However, for purposes herein, % identity  
values are obtained by using the sequence comparison computer program BLAST in  
pairwise alignments.

In situations where BLAST is used for amino acid sequence comparisons,  
the % identity of a given amino acid sequence A to a given amino acid sequence B is  
20 calculated as follows:

100 times the fraction  $X/Y$

where X is the number of amino acid residues scored as identical matches  
by the sequence alignment program Blast in that program's alignment of A and B,  
and where Y is the total number of amino acid residues in B. It will be appreciated  
25 that if the length of amino acid sequence A is different from the length of amino acid  
sequence B, the % identity of A to B will be different from the % identity of B to A.  
Unless stated otherwise, all % identity values herein are obtained using the BLAST  
computer program as shown in the immediately preceding paragraph.

[0029]

*Competing*

As used herein, the term "competing" with the anti-RGMa antibody of the present invention means that, as measured by the surface plasmon resonance (SPR) described herein, the binding between the anti-RGMa antibody of the present invention and RGMa is decreased with a significant difference due to the presence of said anti-RGMa antibody or an antigen-binding fragment thereof.

[0030]

The present invention will be described in detail below.

10 *RGMa-Binding Protein*

The RGMa-binding protein of the present invention is an isolated RGMa-binding protein, which does not inhibit binding between RGMa and neogenin but neutralizes neurite outgrowth inhibiting activity of RGMa.

[0031]

15 Preferably, RGMa proteins are RGMa proteins derived from mammals. For example, human RGMa proteins include a protein having the amino acid sequence of SEQ ID NO: 1 in Sequence Listing, mouse RGMa proteins include a protein having the amino acid sequence of SEQ ID NO: 2 in Sequence Listing, and rat RGMa proteins include a protein having the amino acid sequence of SEQ ID NO: 20 3 in Sequence Listing. A polypeptide comprising an amino acid sequence wherein one or several (preferably 1 to 20, more preferably 1 to 10, still more preferably 1 to 5) amino acids in these sequences are substituted, deleted, inserted and/or added and having substantially the same activity as RGMa protein; or a polypeptide comprising an amino acid sequence having 90% or more (preferably 95% or more) identity to 25 the amino acid sequence may also be preferred.

[0032]

As used herein, a polypeptide "having substantially the same activity as RGMa protein" includes any polypeptide as long as the polypeptide has neurite

outgrowth inhibitory action.

[0033]

The amino acid substitution is preferably conservative substitution. As used herein, "conservative substitution" means replacing an amino acid residue with  
5 another chemically similar amino acid residue so as not to substantially alter the activity of the peptide. For example, substitution of one hydrophobic residue by another hydrophobic residue, substitution of one polar residue by another polar residue having the same electric charge, and the like are included. Examples of functionally similar amino acids with which such substitution is possible include, as  
10 nonpolar (hydrophobic) amino acids, alanine, valine, isoleucine, leucine, proline, tryptophan, phenylalanine and methionine. Examples of polar (neutral) amino acids include glycine, serine, threonine, tyrosine, glutamine, asparagine, and cysteine. Examples of positively charged (basic) amino acids include arginine, histidine and lysine. Examples of negatively charged (acidic) amino acids include aspartic acid  
15 and glutamic acid.

[0034]

Binding of the RGMa binding protein of the present invention to RGMa means RGMa specific binding. More preferred is a RGMa-binding protein having a low dissociation constant ( $K_d$ ) for human RGMa, for example, of  $10^{-8}$  M or less,  
20 more preferably  $10^{-9}$  M or less, still more preferably  $10^{-10}$  M or less as the upper limit value, and for example without limitation, of  $10^{-14}$  M or more, more preferably  $10^{-13}$  or more as the lower limit value.

[0035]

The RGMa protein comprises an N-terminal domain and a C-terminal  
25 domain as a mature protein, but has neurite outgrowth inhibitory activity with the C-terminal domain alone. The RGMa-binding protein of the present invention preferably binds only to the C-terminal domain of RGMa to neutralize neurite outgrowth inhibitory activity. More preferred is a RGMa-binding protein having a

low dissociation constant (Kd) for the C-terminal domain of human RGMa, for example, of  $10^{-8}$  M or less, more preferably  $10^{-9}$  M or less, still more preferably  $10^{-10}$  M or less as the upper limit value, and for example without limitation, of  $10^{-14}$  M or more, more preferably  $10^{-13}$  or more as the lower limit value.

5 [0036]

The RGMa-binding protein of the present invention does not inhibit binding between RGMa and neogenin. Here, the phrase "does not inhibit binding between RGMa and neogenin" means that, in the binding system of RGMa and neogenin shown in Examples described later, even when the concentration of RGMa-binding  
10 protein is increased, the binding between RGMa and neogenin is not substantially decreased. For example, in the case where RGMa-binding protein is added to the binding system of RGMa and neogenin and its concentration is increased, if the concentration of the RGMa-binding protein showing IC50 is not less than 10  $\mu\text{g/mL}$ , more preferably not less than 50  $\mu\text{g/mL}$ , most preferably not less than 100  $\mu\text{g/mL}$ , it  
15 can be said that the RGMa-binding protein does not inhibit the binding between RGMa and neogenin.

[0037]

Neogenin used for the binding assay with RGMa is preferably neogenin of the same type as RGMa. In other words, it is preferred that mouse or human  
20 neogenin is used for mouse or human RGMa, respectively. An example of human neogenin includes a protein having the amino acid sequence of SEQ ID NO: 10 in Sequence Listing. However, as long as it is capable of binding to RGMa, a protein having an amino acid sequence with 90% or more (preferably 95% or more) identity with SEQ ID NO: 10 in Sequence Listing may be preferred.

25 [0038]

The RGMa-binding protein of the present invention neutralizes the neurite outgrowth inhibiting activity of RGMa. The neurite outgrowth inhibitory activity can be evaluated by a neurite outgrowth assay shown in Examples described later.

Addition of RGMa inhibits neurite outgrowth, but addition of RGMa-binding protein prevents neurite outgrowth inhibition by RGMa. The RGMa-binding protein of the present invention can neutralize the neurite outgrowth inhibition by addition of RGMa by 50% or more, more preferably by 80% or more, and most preferably by 90% or more.

[0039]

Since amino acid sequences of RGMa proteins vary depending on the animal species, there are also differences in amino acid sequence between the human RGMa represented by SEQ ID NO: 1 in Sequence Listing, the mouse RGMa represented by SEQ ID NO: 2 in Sequence Listing, and the rat RGMa represented by SEQ ID NO: 3 in Sequence Listing. Since rodents such as mice and rats are generally used as experimental materials in pharmacological and safety tests of protein preparations such as antibody preparations, the RGMa-binding protein of the present invention preferably binds to mouse and/or rat RGMa, and more preferred are those having a low  $K_d$  for mouse and/or rat RGMa. RGMa-binding proteins whose  $K_d$  has the upper limit of, for example,  $5 \times 10^{-7}$  M or less, more preferably  $10^{-8}$  M or less, still more preferably  $10^{-9}$  M or less, and the lower limit value of, for example without limitation,  $10^{-12}$  M or more, more preferably  $10^{-11}$  or more are included.

[0040]

The RGMa binding protein of the present invention is preferably excellent in thermal stability. The thermal stability can be evaluated by a decrease in the binding with RGMa by heat treatment, and the RGMa binding protein of the present invention is preferably stable even by heat treatment at 60°C or higher, more preferably even by heat treatment at 65°C or higher, most preferably even by heat treatment at 70°C or higher.

[0041]

The binding site when the RGMa binding protein of the present invention

binds to RGMa is not particularly limited. For example, in human RGMa, binding to one or more of the following peptides is preferred: EEVVNAVEDWDSQG (SEQ ID NO: 26 in Sequence Listing) (amino acid numbers 298-311 of SEQ ID NO: 1 in Sequence Listing), NQQIDFQAFHTNAE (SEQ ID NO: 27 in Sequence Listing)  
 5 (amino acid numbers 322-335 of SEQ ID NO: 1 in Sequence Listing), PTAPETFPYET (SEQ ID NO: 28 in Sequence Listing) (amino acid numbers 349-359 of SEQ ID NO: 1 in Sequence Listing), KLPVEDLYYQA (SEQ ID NO: 29 in Sequence Listing) (amino acid numbers 367-377 of SEQ ID NO: 1 in Sequence Listing). The RGMa binding protein of the present invention binds more preferably  
 10 to SEQ ID NOS: 26 and 27 of the sequence listing, more preferably to SEQ ID NOS: 26 and 27 of the sequence listing and to SEQ ID NO: 28 or 29 of the sequence listing.

[0042]

Specific examples of RGMa-binding protein include anti-RGMa antibody,  
 15 RGMa-binding scaffold protein, and fusion proteins thereof.

[0043]

#### *Anti-RGMa Antibody*

The anti-RGMa antibody of the present invention includes polyclonal or monoclonal antibodies obtained by using the RGMa protein or a partial fragment  
 20 thereof (for example, a fragment containing one or more of SEQ ID NOS: 26 to 29 in the above sequence listing) as an antigen and immunizing mammals such as mice with the antigens; chimeric antibodies and humanized antibodies produced using gene recombination technology; human antibodies produced using, for example, human antibody-producing transgenic animals; and the like. When the antibody of  
 25 the present invention is administered as a pharmaceutical to humans, a humanized antibody or a human antibody is preferable from the viewpoint of side effects.

[0044]

Antigens may be directly used for immunization or may be used as a

complex with a carrier protein. For preparing a complex of an antigen and a carrier protein, condensing agents such as glutaraldehyde, carbodiimide and maleimide active ester can be used. Examples of the carrier protein include bovine serum albumin, thyroglobulin, hemocyanin, and KLH.

5 [0045]

Examples of mammals to be immunized include mice, rats, hamsters, guinea pigs, rabbits, cats, dogs, pigs, goats, horses and cattle, and inoculation methods include subcutaneous, intramuscular or intraperitoneal administration. Upon administration, antigens may be administered in admixture with complete Freund's adjuvant or incomplete Freund's adjuvant, and administration is usually carried out  
10 once every 2 to 5 weeks. Antibody-producing cells obtained from the spleen or lymph nodes of the immunized animals are fused with myeloma cells and isolated as hybridomas. As the myeloma cells, those derived from a mammal such as mouse, rat, or human are used.

15 [0046]

#### *Polyclonal Antibody*

Polyclonal antibodies can be obtained by existing common production methods. That is, polyclonal antibodies can be obtained from serum obtained from animals subjected to immunization, for example, by immunizing a mammal as  
20 described above with an antigen as described above together with Freund's adjuvant as necessary.

[0047]

#### *Monoclonal Antibody*

Specifically, monoclonal antibodies can be obtained as follows. That is, an  
25 antigen as described above is used as an immunogen, and the immunogen is injected or transplanted one or several times in combination with Freund's adjuvant, if necessary, to a mammal as described above subcutaneously, intramuscularly, intravenously, into a footpad or intraperitoneally for immunization. Generally,

immunization is performed 1 to 4 times every 1 to 14 days from the initial immunization, and antibody-producing cells are obtained from the mammal immunized from about 1 to 5 days after the final immunization.

[0048]

- 5            Monoclonal antibodies can be obtained using methods well known to those skilled in the art (for example, "Current Protocols in Molecular Biology" (John Wiley & Sons (1987)), Antibodies: A Laboratory Manual, Ed. Harlow and David Lane, Cold Spring Harbor Laboratory (1988))).

[0049]

- 10           Preparation of "hybridomas" secreting monoclonal antibodies can be carried out according to the method of Köhler and Milstein et al. (Nature, 256, 495, 1975) and methods similar thereto. That is, Hybridomas are prepared by fusing an antibody-producing cell contained in a spleen or the like obtained from an immunized mammal with a myeloma cell having no autoantibody producing ability
- 15           derived from a mammal, preferably mouse, rat or human.

[0050]

- Examples of myeloma cells which can be used for cell fusion include mouse-derived myeloma P3/X63-Ag8.653 (653), P3/NSI/1-Ag4-1 (NS-1), P3/X63-Ag8.U1 (P3U1), SP2/0-Ag14 (Sp2/O, Sp2), PAI, F0 and BW5147, rat-derived
- 20           myeloma 210RCY3-Ag.2.3., and human-derived myeloma U-266AR1, GM1500-6TG-A1-2, UC729-6, CEM-AGR, D1R11 and CEM-T15.

[0051]

- Examples of fusion accelerators include polyethylene glycol and the like. In general, antibody-producing cells and myeloma cells at a number ratio of usually
- 25           about 1: 1 to 10: 1 are allowed to react, using polyethylene glycol (average molecular weight: 1000 to 4000) at a concentration of about 20 to 50%, at a temperature of 20 to 40°C, preferably 30 to 37°C for about 1 to 10 minutes, whereby cell fusion can be carried out.

[0052]

Screening of hybridoma clones producing monoclonal antibodies can be carried out by culturing the hybridomas, for example, in microtiter plates and measuring the reactivity of the culture supernatants in the wells to the immunogen by immunochemical methods such as ELISA.

[0053]

In the screening of the antibody-producing hybridomas, in addition to the binding assay with RGMa protein, whether the antibody does not inhibit the binding between RGMa protein and neogenin and whether the antibody neutralizes the function of RGMa protein (neurite outgrowth inhibitory activity) are also evaluated. These screening methods allow selection of the anti-RGMa antibody of the present invention.

[0054]

Clones can be further obtained from the wells containing hybridomas producing the desired antibodies by cloning using limiting dilution. Selection and breeding of hybridomas are usually carried out in an animal cell culture medium containing 10 to 20% fetal bovine serum supplemented with HAT (hypoxanthine, aminopterin and thymidine).

[0055]

Monoclonal antibodies from hybridomas can be produced by culturing the hybridomas in vitro or growing them in vivo, for example, in ascitic fluid of mammals such as mice and rats and isolating monoclonal antibodies from the resulting culture supernatant or the ascitic fluid of the mammal.

[0056]

When cultured in vitro, hybridomas are grown, maintained, and stored in accordance with various conditions such as the characteristics of and the culture method for the cells species to be cultured, and a nutrient medium suitable for producing monoclonal antibodies in the culture supernatant can be used.

[0057]

Examples of basic media include a low-calcium medium such as Ham's F12 medium, MCDB 153 medium or low-calcium MEM medium and a high-calcium medium such as MCDB 104 medium, MEM medium, D-MEM medium, RPMI 1640 medium, ASF 104 medium or RD medium. The basic media can contain, for example, serum, hormones, cytokines and/or various inorganic or organic substances according to the purpose.

[0058]

Monoclonal antibodies can be isolated and purified by, for example, subjecting the above-mentioned culture supernatant or ascitic fluid to saturated ammonium sulfate, euglobulin precipitation method, caproic acid method, caprylic acid method, ion exchange chromatography (such as DEAE or DE52) or affinity column chromatography such as anti-immunoglobulin column or protein A column chromatography. Specifically, purification of the monoclonal antibody can be carried out by any methods known as immunoglobulin purification method, and can be easily achieved by means such as ammonium sulfate fractionation, PEG fractionation, ethanol fractionation, and affinity chromatography utilizing an anion exchanger and further using RGMA proteins.

[0059]

Monoclonal antibodies can also be obtained by a phage display method. In the phage display method, phages selected from an optional phage antibody library are screened using the desired immunogen and phages having desired binding capacity to the immunogen are selected. Next, the antibody-corresponding sequence contained in the phage is isolated or sequenced and an expression vector comprising a nucleic acid molecule encoding an antibody or an antigen binding domain is constructed based on the isolated sequence or the determined sequence information. Finally, monoclonal antibodies can be produced by culturing cell lines transfected with such expression vectors. A human antibody library can be used as a phage

antibody library to generate human antibodies having desired binding properties.

[0060]

As a scaffold protein, for example, a Kunitz domain of human serine protease inhibitor and an extracellular domain of human fibronectin are utilized, and  
 5 the sequence of a target binding site on the scaffold can be modified to generate a scaffold protein which binds to RGMa (Clifford Mintz et.al BioProcess International, 2013, Vol.11(2), pp40-48).

[0061]

Fusion proteins include RGMa-binding proteins bound chemically or  
 10 genetically to function molecules other than the RGMa-binding protein of the present application such as nonpeptidic polymers such as polyethylene glycol (PEG), radioactive substances, toxins, low molecular weight compounds, cytokines, growth factors (e.g., TGF- $\beta$ , NGF, neurotrophin), albumin, enzymes, and other antibodies.

[0062]

15 When PEG is bound as a functional molecule, PEG can be used having a molecular weight of, without limitation, 2,000 to 100,000 Da, more preferably 10,000 to 50,000 Da, which may be linear or branched. By using, for example, an NHS active group, PEG can be bound, for example, to N-terminal amino groups of amino acids of the RGMa-binding protein.

20 [0063]

In the case of using a radioactive substance as a functional molecule, for example,  $^{131}\text{I}$ ,  $^{125}\text{I}$ ,  $^{90}\text{Y}$ ,  $^{64}\text{Cu}$ ,  $^{99}\text{Tc}$ ,  $^{77}\text{Lu}$  or  $^{211}\text{At}$  is used. Radioactive substances can be directly bound to the RGMa-binding protein by, for example, chloramine-T method.

25 [0064]

When using a toxin as a functional molecule, for example, bacterial toxins (e.g., diphtheria toxin), phytotoxins (e.g., ricin), small toxins (e.g., geldanamycin), maytansinoids and calicheamicin are used.

[0065]

When using a low molecular weight compound as a functional molecule, for example, daunomycin, doxorubicin, methotrexate, mitomycin, neocarzinostatin, vindesine and fluorescent dyes such as FITC are used.

5 [0066]

When using an enzyme as a functional molecule, for example, luciferase (e.g., firefly luciferase and bacterial luciferase; US Pat. No. 4,737,456), malate dehydrogenase, urease, peroxidase (e.g., horseradish peroxidase (HRPO)), alkaline phosphatase,  $\beta$ -galactosidase, glucoamylase, lysozyme, saccharide oxidase (e.g.,  
 10 glucose oxidase, galactose oxidase and glucose-6-phosphate dehydrogenase), heterocyclic oxidase (e.g., uricase and xanthine oxidase), lactoperoxidase, and microperoxidase are used.

[0067]

Examples of linkers used for chemically bonding a toxin, a low molecular  
 15 weight compound or an enzyme include divalent radicals (e.g., alkylene, arylene, heteroarylene), a repeating unit of a linker or alkoxy represented by  $-(CR_2)_nO(CR_2)_n-$  (wherein R is an optional substituent, and n is a positive integer) (e.g., polyethyleneoxy, PEG and polymethyleneoxy) and alkylamino (e.g., polyethyleneamino, Jeffamine<sup>TM</sup>) and a diacid ester and amide (e.g., succinate,  
 20 succinamide, diglycolate, malonate, and caproamide). Chemical modification methods for binding functional molecules have already been established in this field (D.J.King., Applications and Engineering of Monoclonal antibodies., 1998, T.J. International Ltd, Monoclonal Antibody-Based Therapy of Cancer., 1998, Marcel Dekker Inc; Chari et al., Cancer Res., 1992 Vol 152:127; Liu et al., Proc Natl Acad  
 25 Sci USA., 1996 Vol 93:8681).

[0068]

A preferred embodiment of the RGMa-binding protein of the present invention is a chimeric antibody. As the "chimeric antibody", a chimeric antibody in

which the variable region is a variable region derived from an immunoglobulin of a non-human animal (such as mouse, rat, hamster or chicken) and the constant region is a constant region of human immunoglobulin is exemplified. A chimeric antibody can be prepared, for example, by immunizing a mouse with an antigen, cutting out a  
5 variable region that binds to the antigen from the gene encoding the mouse monoclonal antibody, and combining the variable region with a constant region of an antibody derived from human bone marrow. The constant region derived from human immunoglobulin has a unique amino acid sequence depending on the isotype such as IgG (IgG1, IgG2, IgG3 and IgG4), IgM, IgA (IgA1 and IgA2), IgD and IgE,  
10 but the constant region of the recombinant chimeric antibody according to the present invention may be a constant region of human immunoglobulin belonging to any isotype. The constant region is preferably a constant region of human IgG. An expression vector can be prepared using the gene of the chimeric antibody thus prepared. Host cells are transformed with the expression vector to obtain chimeric  
15 antibody-producing transformant cells, and then the transformed cells are cultured to obtain the desired chimeric antibody from the culture supernatant.

[0069]

Another preferred embodiment of the RGMa-binding protein of the present invention is a humanized antibody. The "humanized antibody" in the present  
20 invention is an antibody obtained by grafting only the DNA sequence of the antigen binding site (CDR; complementarity determining region) of a nonhuman animal antibody such as mouse to a human antibody gene (CDR grafting). Humanized antibodies can be prepared by referring to the methods described in, for example, Japanese Translated PCT Patent Application Laid-open No. Hei 4-506458 and  
25 Japanese Patent No. 2912618. Specifically, a humanized antibody is contemplated characterized in that part or all of the CDRs are CDRs derived from monoclonal antibodies of non-human mammals (such as mouse, rat and hamster), that the framework regions of the variable region are framework regions of variable regions

derived from human immunoglobulin, and that the constant regions are constant regions derived from human immunoglobulin.

[0070]

The humanized antibody of the present invention can be produced, for  
5 example, as follows, but it goes without saying that the production method is not limited thereto.

[0071]

For example, a recombinant humanized antibody derived from a mouse  
monoclonal antibody can be produced by genetic engineering with reference to  
10 Japanese Translated PCT Patent Application Laid-open Nos. Hei 4-506458 and Sho 62-296890. That is, DNA coding for mouse heavy chain CDR portion and DNA coding for mouse light chain CDR portion are isolated from hybridomas producing a mouse monoclonal antibody, and a human heavy chain gene coding for the whole region other than CDR of human heavy chain and a human light chain gene coding  
15 for the whole region other than CDR of human light chain are isolated from a human immunoglobulin gene.

[0072]

The human heavy chain gene to which the isolated DNA coding for mouse heavy chain CDR portion is grafted is introduced into an appropriate expression  
20 vector so that expression thereof is possible. Similarly, the human light chain gene to which the DNA coding for mouse light chain CDR portion is grafted is introduced into another appropriate expression vector so that expression thereof is possible. Alternatively, human heavy and light chain genes to which mouse CDR is grafted may be introduced into the same expression vector so that expression thereof is  
25 possible. Host cells are transformed with the expression vector thus prepared to obtain humanized antibody-producing transformant cells, and then the transformed cells are cultured to obtain the desired humanized antibody from the culture supernatant.

[0073]

Another preferred embodiment of the RGMa-binding protein of the present invention is a human antibody. A human antibody refers to an antibody in which all regions including heavy chain variable regions and heavy chain constant regions and  
5 light chain variable regions and light chain constant regions constituting the immunoglobulin are derived from genes encoding human immunoglobulin. Human antibodies can be prepared by introducing human antibody genes into mice. Human antibodies can be produced in the same manner as the above-mentioned method for preparing polyclonal antibodies or monoclonal antibodies, specifically, for example,  
10 by immunizing a transgenic animal prepared by integrating at least human immunoglobulin genes into a gene locus of a mammal other than a human such as a mouse.

[0074]

For example, transgenic mice that produce human antibodies can be  
15 prepared according to the methods described, for example, in Nature Genetics, Vol.7, p.13-21, 1994; Nature Genetics, Vol.15, p.146-156, 1997; Japanese Translated PCT Patent Application Laid-open Nos. Hei 4-504365 and Hei 7-509137; WO 94/25585; Nature, Vol.368, p.856-859, 1994; and Japanese Translated PCT Patent Application Laid-open No. Hei 6-500233. Specific examples of the transgenic mice include  
20 HuMab<sup>TM</sup> mouse (Medarex, Princeton NJ), KMTM mouse (Kirin Pharma Company, Japan), and KM (FC $\gamma$ RIIb-KO) mouse.

[0075]

Specific examples of the monoclonal antibody of the present invention include those in which CDRs in the heavy chain variable region comprise the amino  
25 acid sequences of SEQ ID NOS: 33 (HCDR 1), 34 (HCDR 2) and 35 (HCDR 3) in Sequence Listing and in which CDRs in the light chain variable region comprise the amino acid sequences of SEQ ID NOS: 30 (LCDR 1), 31 (LCDR 2) and 32 (LCDR 3) in Sequence Listing. One to several amino acids in one or more of the CDRs may

be substituted as long as the properties of the antibody of the present invention of having the ability to bind to RGMa, not inhibiting the binding between RGMa and neogenin, and neutralizing the neurite outgrowth inhibitory activity of RGMa are maintained. One to several means, for example, one or two. The amino acid  
5 substitution is preferably conservative substitution in order to maintain the properties of the present invention. Maintaining the properties of the antibody means that these properties are maintained to the same degree, for example, 80% or more, preferably 90% or more, more preferably 95% or more as compared with the properties before the CDR amino acid sequence modifications. Maintenance also includes  
10 improvement.

[0076]

The region other than CDRs is not particularly limited as long as it is a sequence that can maintain the structure as an antibody and exert its function, and may be any of the sequences derived from mouse, human, and other mammals,  
15 chimeric sequences thereof, and artificial sequences. In the case of comprising a constant region, the amino acid sequences of the constant regions in heavy chain and light chain are exemplified by those described in Nucleic Acids Research vol.14, p1779, 1986, The Journal of Biological Chemistry vol.257, p1516, 1982 and Cell vol.22, p197, 1980.

20 [0077]

Examples of mouse antibodies having these CDRs include antibodies in which the light chain has the amino acid sequence of SEQ ID NO: 4 in Sequence Listing and in which the heavy chain has the amino acid sequence of SEQ ID NO: 5 in Sequence Listing. In these amino acid sequences, there may be substitution,  
25 deletion, addition or insertion of one or several (1 to 20, 1 to 10 or 1 to 5) amino acids as long as the properties of the antibody of having the ability to bind to RGMa, not inhibiting the binding between RGMa and neogenin, and neutralizing the neurite outgrowth inhibitory activity of RGMa are maintained. Such substitution, deletion or

addition may be introduced into CDRs, but it is preferably introduced into a region other than the CDRs. The amino acid substitution is preferably conservative substitution in order to maintain the properties of the present invention.

[0078]

5           Mouse/human chimeric antibodies in which constant regions in the above-mentioned mouse antibody are derived from human are also included. An example of such mouse/human chimeric antibodies is an antibody in which the light chain has the amino acid sequence of SEQ ID NO: 8 in Sequence Listing (the variable region extends from 1 to 107) and in which the heavy chain has the amino acid sequence of  
10   SEQ ID NO: 9 in Sequence Listing (the variable region extends from 1 to 116). In these amino acid sequences, there may be substitution, deletion, addition or insertion of one or several (1 to 20, 1 to 10 or 1 to 5) amino acids as long as the properties of the antibody of having the ability to bind to RGMa, not inhibiting the binding between RGMa and neogenin, and neutralizing the neurite outgrowth inhibitory  
15   activity of RGMa are maintained. Such substitution, deletion or addition may be introduced into CDRs, but it is preferably introduced into a region other than the CDRs. The amino acid substitution is preferably conservative substitution in order to maintain the properties of the present invention.

[0079]

20           Further, humanized antibodies in which the region other than CDRs is derived from human are exemplified. An example of such humanize antibodies is an antibody in which the heavy chain has an amino acid sequence of any one of SEQ ID NOS: 11 to 18 (the variable region is up to the 116th residue on the N terminal side) and in which the light chain has an amino acid sequence of any one of SEQ ID NOS:  
25   19-25 in Sequence Listing (the variable region extends from the 1st to 107th residues on the N-terminal side).

In the amino acid sequences of the humanized antibody (heavy chain: SEQ ID NOS: 11-18 in Sequence Listing, light chain: SEQ ID NOS: 19 to 25 in Sequence

Listing), there may be substitution, deletion, addition or insertion of one or several (1 to 20, 1 to 10 or 1 to 5) amino acids as long as the properties of the antibody of having the ability to bind to RGMa, not inhibiting the binding between RGMa and neogenin, and neutralizing the neurite outgrowth inhibitory activity of RGMa are maintained. Such substitution, deletion or addition may be introduced into CDRs, but it is preferably introduced into a region other than the CDRs. The amino acid substitution is preferably conservative substitution in order to maintain the properties of the present invention.

[0080]

10           The heavy chain amino acid sequence and the light chain amino acid sequence may be any combination thereof, but particularly preferred is an antibody having heavy chains comprising the amino acid sequence of SEQ ID NO: 15 in Sequence Listing and light chains comprising the amino acid sequence of SEQ ID NO: 19 in Sequence Listing. In the amino acid sequence of SEQ ID NO: 15 in  
15           Sequence Listing, the amino acid sequence corresponding to the heavy chain variable region is shown in SEQ ID NO: 41 in Sequence Listing and the amino acid sequence corresponding to the light chain variable region is shown in SEQ ID NO: 42 in Sequence Listing. Thus, the particularly preferred antibody of the present invention is an antibody in which the heavy chain variable region has the amino acid sequence  
20           of SEQ ID NO: 41 in Sequence Listing and in which the light chain variable region has the amino acid sequence of SEQ ID NO: 42 in Sequence Listing.

          In these amino acid sequences, there may be substitution, deletion, addition or insertion of one or several (1 to 20, 1 to 10 or 1 to 5) amino acids as long as the properties of the antibody of having the ability to bind to RGMa, not inhibiting the  
25           binding between RGMa and neogenin, and neutralizing the neurite outgrowth inhibitory activity of RGMa are maintained. Such substitution, deletion or addition may be introduced into CDRs, but it is preferably introduced into a region other than the CDRs. The amino acid substitution is preferably conservative substitution in

order to maintain the properties of the present invention.

In the amino acid sequence of the antibody of the present invention comprising substitution, deletion or the like in the amino acid sequence of SEQ ID NO: 41 and/or 42 in Sequence Listing as described above, the heavy chain variable region is an amino acid sequence having 90% or more (more preferably 95%, 96%, 97%, 98%, 99% or more) identity with SEQ ID NO: 41 of the sequence listing, and the light chain variable region is an amino acid sequence having 90% or more (more preferably 95%, 96%, 97%, 98%, 99% or more) identity with SEQ ID NO: 42 of the sequence listing.

10 [0081]

Other specific examples of the monoclonal antibody of the present invention include those in which CDRs in the heavy chain variable region comprise the amino acid sequences of SEQ ID NOS: 39 (HCDR 1), 40 (HCDR 2) and SFG (HCDR 3) in Sequence Listing and in which CDRs in the light chain variable region comprise the amino acid sequences of SEQ ID NOS: 36 (LCDR 1), 37 (LCDR 2) and 38 (LCDR 3) in Sequence Listing. One to several amino acids in one or more of the CDRs may be substituted as long as the properties of the antibody of the present invention of having the ability to bind to RGMa, not inhibiting the binding between RGMa and neogenin, and neutralizing the neurite outgrowth inhibitory activity of RGMa are maintained.

One to several means, for example, one or two. The amino acid substitution is preferably conservative substitution in order to maintain the properties of the present invention.

The region other than CDRs is not particularly limited as long as it is a sequence that can maintain the structure as an antibody and exert its function, and may be any of the sequences derived from mouse, human, and other mammals, chimeric sequences thereof, and artificial sequences. In the case of comprising a constant region, the amino acid sequences of the constant regions in heavy chain and

light chain are exemplified by those described in Nucleic Acids Research vol.14, p1779, 1986, The Journal of Biological Chemistry vol.257, p1516, 1982 and Cell vol.22, p197, 1980.

[0082]

5           Examples of mouse antibodies having these CDRs include antibodies in which the light chain has the amino acid sequence of SEQ ID NO: 6 in Sequence Listing and in which the heavy chain has the amino acid sequence of SEQ ID NO: 7 in Sequence Listing. In these amino acid sequences, there may be substitution, deletion, addition or insertion of one or several (1 to 20, 1 to 10 or 1 to 5) amino  
10   acids as long as the properties of the antibody of having the ability to bind to RGMa, not inhibiting the binding between RGMa and neogenin, and neutralizing the neurite outgrowth inhibitory activity of RGMa are maintained. Such substitution, deletion or addition may be introduced into CDRs, but it is preferably introduced into a region other than the CDRs. The amino acid substitution is preferably conservative  
15   substitution in order to maintain the properties of the present invention.

[0083]

As the above-mentioned mouse antibody, chimeric antibodies whose constant regions are derived from human are also included. Humanized antibodies in which the region other than CDRs is derived from human are further included.

20   [0084]

The anti-RGMa antibody of the present invention includes multispecific antibodies, functionally modified antibodies, and conjugated antibodies having CDRs comprising specific amino acid sequences (for example, amino acid sequences of SEQ ID NO: 30 in Sequence Listing for LCDR1, SEQ ID NO: 31 in Sequence  
25   Listing for LCDR2, SEQ ID NO: 32 in Sequence Listing for LCDR3, SEQ ID NO: 33 in Sequence Listing for HCDR1, SEQ ID NO: 34 in Sequence Listing for HCDR2, and SEQ ID NO: 35 in Sequence Listing for HCDR3), or having variable regions comprising specific amino acid sequences (for example, amino acid

sequences of SEQ ID NO: 41 in Sequence Listing for heavy chain variable region, and SEQ ID NO: 42 for light chain variable region).

[0085]

The anti-RGMA antibody of the present invention itself can be bound to an antibody having another antigen binding specificity other than anti-RGMA specificity by genetic engineering techniques to prepare multispecific antibodies such as bispecific antibodies. The genetic engineering techniques have already been established in this field. For example, by using a technique for DVD-Ig in which variable regions are connected in series (Wu et al., Nature Biotechnology 25(11), 1290 (2007)) or a technique for ART-Ig in which heavy chains of two types of antibodies binding to different antigens are combined by modifying the Fc region of an antibody (Kitazawa et al., Nature Medicine 18(10), 1570(2012), desired bispecific antibodies can be obtained. Other antigens than RGMA include, but not limited to, factors inhibiting neurite outgrowth such as Nogo, MAG, Omgp, CSPG, Sema 3A and Lingo-1, and immune-related molecules such as TNF- $\alpha$ , IL-6 receptor, CD3, CD20,  $\alpha$ 4 integrin, BLys, Thymic Stromal Lymphopoietin, IgE, IL-1, IL-2, IL-4, IL-5, IL-6, IL-13, IL-17, IL-23 and IL-25.

[0086]

Functionally modified antibodies are exemplified as modified molecules of the anti-RGMA antibody of the present invention. Functionally modified antibody means an antibody in which functions such as cell killing function, complement activating function and function to extend blood half-life are modified mainly by modifying the Fc region or the like (Shitara, Journal of the Pharmaceutical Society of Japan, 2009, Vol. 129(1), p3; Ishii et al., Nippon Yakubutsugaku Zasshi (Folia Pharmacologica Japonica), 2010, Vol.136(5), p280; Hashiguchi et al., The Journal of Japanese Biochemical Society, 2010, Vol.82(8),p710).

[0087]

Functionally modified antibodies of the anti-RGMA antibody are prepared

by the following method. For example, when the anti-RGMa antibody of the present application is produced using, as host cells, CHO cells whose  $\alpha$ 1,6-fucosyltransferase (FUT 8) gene has been disrupted, antibodies with reduced content of sugar chain fucose and increased cell killing function are obtained, and when the anti-RGMa antibody of the present application is produced using, as a host cell, CHO cells into which FUT8 gene has been introduced, antibodies with low cell killing function are obtained (WO 2005/035586, WO 2002/31140 and WO 00/61739). The complement activation function can be regulated by modifying amino acid residues in the Fc region (U.S. Patent Nos: 6737056, 7297775 and 7317091). The blood half-life extension can be achieved by using Fc region variants with increased binding to FcRn which is one of Fc receptors (Hashiguchi et al., The Journal of Japanese Biochemical Society, 2010, Vol.82 (8), p710). These functionally modified antibodies can be produced by genetic engineering techniques.

[0088]

Conjugated antibodies are exemplified as modified molecules of the anti-RGMa antibody of the present invention. Examples of the conjugated antibody include conjugated antibodies in which anti-RGMa antibodies are bound chemically or genetically to function molecules other than the anti-RGMa antibody in the present application such as nonpeptidic polymers such as polyethylene glycol (PEG), radioactive substances, toxins, low molecular weight compounds, cytokines, growth factors (e.g., TGF- $\beta$ , NGF, neurotrophin), albumin, enzymes, and other antibodies.

[0089]

When PEG is bound as a functional molecule, PEG can be used having a molecular weight of, without limitation, 2,000 to 100,000 Da, more preferably 10,000 to 50,000 Da, which may be linear or branched. By using, for example, an NHS active group, PEG can be bound, for example, to N-terminal amino groups of amino acids of antibodies.

[0090]

In the case of using a radioactive substance as a functional molecule, for example,  $^{131}\text{I}$ ,  $^{125}\text{I}$ ,  $^{90}\text{Y}$ ,  $^{64}\text{Cu}$ ,  $^{99}\text{Tc}$ ,  $^{77}\text{Lu}$  or  $^{211}\text{At}$  is used. Radioactive substances can be directly bound to antibodies by, for example, chloramine-T method.

[0091]

- 5           When using a toxin as a functional molecule, for example, bacterial toxins (e.g., diphtheria toxin), phytotoxins (e.g., ricin), small toxins (e.g., geldanamycin), maytansinoids and calicheamicin are used.

[0092]

- 10           When using a low molecular weight compound as a functional molecule, for example, daunomycin, doxorubicin, methotrexate, mitomycin, neocarzinostatin, vindesine and fluorescent dyes such as FITC are used.

[0093]

- 15           When using an enzyme as a functional molecule, for example, luciferase (e.g., firefly luciferase and bacterial luciferase; US Pat. No. 4,737,456), malate dehydrogenase, urease, peroxidase (e.g., horseradish peroxidase (HRPO)), alkaline phosphatase,  $\beta$ -galactosidase, glucoamylase, lysozyme, saccharide oxidase (e.g., glucose oxidase, galactose oxidase and glucose-6-phosphate dehydrogenase), heterocyclic oxidase (e.g., uricase and xanthine oxidase), lactoperoxidase, and microperoxidase are used.

- 20           [0094]

- Examples of linkers used for chemically bonding a toxin, a low molecular weight compound or an enzyme include divalent radicals (e.g., alkylene, arylene, heteroarylene), a repeating unit of a linker or alkoxy represented by  $-(\text{CR}_2)_n\text{O}(\text{CR}_2)_n-$  (wherein R is an optional substituent, and n is a positive integer) (e.g., polyethyleneoxy, PEG and polymethyleneoxy) and alkylamino (e.g., polyethyleneamino, Jeffamine<sup>TM</sup>) and a diacid ester and amide (e.g., succinate, succinamide, diglycolate, malonate, and caproamide). Chemical modification methods for binding functional molecules have already been established in this field

(D.J.King., Applications and Engineering of Monoclonal antibodies., 1998 T.J. International Ltd, Monoclonal Antibody-Based Therapy of Cancer., 1998 Marcel Dekker Inc; Chari et al., Cancer Res., 1992 Vol152:127; Liu et al., Proc Natl Acad Sci USA., 1996 Vol 93:8681).

5 [0095]

"Antigen binding fragments" of antibodies in the present invention means a partial region having antigen binding property, of the above-described antibodies, including, in particular, F(ab')<sub>2</sub>, Fab', Fab, Fv (variable fragment of antibody), disulfide-linked Fv, single-chain antibody (scFv), and polymers thereof. Antigen-  
10 binding fragments further include conjugated antigen-binding fragments bound chemically or genetically to function molecules other than the anti-RGMa antibody in the present application such as nonpeptidic polymers such as polyethylene glycol (PEG), radioactive substances, toxins, low molecular weight compounds, cytokines, growth factors (e.g., TGF- $\beta$ , NGF, neurotrophin), albumin, enzymes, and other  
15 antibodies.

[0096]

As used herein, "F (ab')<sub>2</sub>" and "Fab" means antibody fragments which are produced by treating immunoglobulins with a protease pepsin or papain and are generated by digestion upstream and downstream of the disulfide bond existing  
20 between two heavy chains in the hinge region. For example, papain treatment of IgG can cause cleavage upstream of the disulfide bond existing between two heavy chains in the hinge region to produce of two homologous antibody fragments each comprising a light chain comprising a VL (light chain variable region) and a CL (light chain constant region) and a heavy chain fragment comprising a VH (heavy  
25 chain variable region) and a CH $\gamma$ 1 ( $\gamma$ 1 region within heavy chain constant region) in which the light chain and the heavy chain fragment are linked to each other via a disulfide bond at C-terminal domain. Each of the two homologous antibody fragments is referred to as Fab. Pepsin treatment of IgG can cause cleavage

downstream of the disulfide bond existing between two heavy chains in the hinge region to produce an antibody fragment slightly larger than one in which the two Fabs are linked to each other at the hinge region. This antibody fragment is referred to as F(ab')<sub>2</sub>.

5 [0097]

Conjugated antigen-binding fragments are exemplified as modified molecules of the antigen-binding fragment of the anti-RGMa antibody of the present invention. Examples of the conjugated antigen-binding fragment include those in which a partial region having the antigen-binding property of the anti-RGMa  
10 antibody is bound chemically or genetically to a function molecule other than the anti-RGMa antibody in the present application such as nonpeptidic polymers such as polyethylene glycol (PEG), radioactive substances, toxins, low molecular weight compounds, cytokines, growth factors (e.g., TGF- $\beta$ , NGF, neurotrophin), albumin, enzymes, and other antibodies.

15 [0098]

When PEG is bound as a functional molecule, PEG can be used having a molecular weight of, without limitation 2,000 to 100,000 Da, more preferably 10,000 to 50,000 Da, which may be linear or branched. PEG can be bound, for example, to the N-terminal amino group of a partial region having the antigen-binding property  
20 of the anti-RGMa antibody by using, for example, an NHS active group.

[0099]

In the case of using a radioactive substance as a functional molecule, for example, <sup>131</sup>I, <sup>125</sup>I, <sup>90</sup>Y, <sup>64</sup>Cu, <sup>99</sup>Tc, <sup>77</sup>Lu or <sup>211</sup>At is used. Radioactive substances can be directly bound to a partial region having the antigen-binding property of the anti-  
25 RGMa antibody by, for example, chloramine-T method.

[0100]

When using a toxin as a functional molecule, for example, bacterial toxins (e.g., diphtheria toxin), phytotoxins (e.g., ricin), small toxins (e.g., geldanamycin),

maytansinoids and calicheamicin are used.

[0101]

When using a low molecular weight compound as a functional molecule, for example, daunomycin, doxorubicin, methotrexate, mitomycin, neocarzinostatin,  
 5 videsine and fluorescent dyes such as FITC are used.

[0102]

When using an enzyme as a functional molecule, for example, luciferase (e.g., firefly luciferase and bacterial luciferase; US Pat. No. 4,737,456), malate dehydrogenase, urease, peroxidase (e.g., horseradish peroxidase (HRPO)), alkaline  
 10 phosphatase,  $\beta$ -galactosidase, glucoamylase, lysozyme, saccharide oxidase (e.g., glucose oxidase, galactose oxidase and glucose-6-phosphate dehydrogenase), heterocyclic oxidase (e.g., uricase and xanthine oxidase), lactoperoxidase, and microperoxidase are used.

[0103]

15 Examples of linkers used for chemically bonding a toxin, a low molecular weight compound or an enzyme include divalent radicals (e.g., alkylene, arylene, heteroarylene), a repeating unit of a linker or alkoxy represented by  $-(CR_2)_nO(CR_2)_n-$  (wherein R is an optional substituent, and n is a positive integer) (e.g., polyethyleneoxy, PEG and polymethyleneoxy) and alkylamino (e.g.,  
 20 polyethyleneamino, Jeffamine<sup>TM</sup>) and a diacid ester and amide (e.g., succinate, succinamide, diglycolate, malonate, and caproamide). Chemical modification methods for binding functional molecules have already been established in this field (D.J.King., Applications and Engineering of Monoclonal antibodies., 1998 T.J. International Ltd, Monoclonal Antibody-Based Therapy of Cancer., 1998 Marcel  
 25 Dekker Inc; Chari et al., Cancer Res., 1992 Vol152:127; Liu et al., Proc Natl Acad Sci USA., 1996 Vol 93:8681).

[0104]

In the anti-RGMA antibody comprising CDRs or variable regions having the

specific amino acid sequences of the present invention, the constant region preferably is a constant region of human IgG (IgG1, IgG2, IgG3 or IgG4) for maintenance with a long half-life in blood.

[0105]

5           The present invention also includes an anti-RGMa antibody which competes with the antibody having the amino acid sequences of the specific CDRs as described above for binding to RGMa proteins, and antigen binding fragments thereof. The antibody which competes with the antibody having the amino acid sequences of the specific CDRs as described above for binding to RGMa proteins include epitopes in  
10 the regions selected from Glu 298 to Gly 311, Asn 322 to Glu 335, Lys 367 to Ala 377, and Pro 349 to Thr 359.

          The antibody can be obtained (screened) or evaluated by allowing the antibody to coexist in the binding system of the antibody having the CDR sequences as described above and RGMa proteins. For example, the antibody can be obtained  
15 by screening using the following surface plasmon resonance (SPR) method.

[0106]

          Biotinylated human RGMa protein (4  $\mu\text{g/mL}$ ) as a ligand is loaded on an avidin-immobilized sensor chip to immobilize human RGMa proteins equivalent to 1300 to 1600 RU. Next, an optional anti-RGMa antibody (15  $\mu\text{g/mL}$ ) is loaded as an  
20 analyte and bound to the human RGMa protein immobilized on the sensor chip. By repeating this a plurality of times, a state in which the optional anti-RGMa antibody is bound to all of the human RGMa protein molecules on the sensor chip is created (saturated state) and the amount of binding in the saturated state (saturated binding amount 1) is determined.

25           A similar experiment is carried out with the anti-RGMa antibody comprising the amino acid sequences of the specific CDRs of the present invention to determine the amount of binding in the saturated state (saturated binding amount 2).

Next, the human RGMa proteins on the sensor chip are saturated with the anti-RGMa antibody comprising the amino acid sequences of the specific CDRs of the present invention, and then an optional anti-RGMa antibody (15 µg/mL) is loaded as an analyte to investigate whether the optional anti-RGMa antibody binds  
5 additionally to the human RGMa protein saturated with the anti-RGMa antibody comprising the amino acid sequences of the specific CDRs of the invention.

If the optional anti-RGMa antibody can bind additionally to the human RGMa protein saturated with the anti-RGMa antibody comprising the amino acid sequences of the specific CDRs of the invention while showing the saturated binding  
10 amount 1 of an optional anti-RGMa antibody calculated above, then the antibody is judged as "not competing". On the other hand, if the optional anti-RGMa antibody can not bind additionally to the human RGMa protein saturated with the anti-RGMa antibody comprising the amino acid sequences of the specific CDRs of the invention, then the antibody is judged as "competing". Even if the optional anti-RGMa  
15 antibody can bind additionally to the human RGMa protein saturated with the anti-RGMa antibody comprising the amino acid sequences of the specific CDRs of the invention, when the added binding amount does not reach the saturated binding amount 1 with a significant difference, then the antibody is judged as "competing". Significant differences are examined by general statistical methods (for example,  
20 Student's t-test), and the significance level is set to 5% or less.

[0107]

The anti-RGMa antibody which competes with an anti-RGMa antibody comprising the specific CDR amino acid sequence as described above for binding with RGMa may be an antibody derived from any animal such as mouse, human, rat,  
25 rabbit, goat, or camel antibody, or may be a chimeric or humanized antibody which is a combination of these antibodies, but preferably is a chimeric, humanized, or human antibody.

[0108]

*Nucleic Acid Molecules of the Present Invention*

Examples of the nucleic acid molecules of the present invention include polynucleotides in which the region encoding a heavy chain variable region comprises a base sequence encoding the amino acid sequence of SEQ ID NOS: 33, 34, and 35 in Sequence Listing, respectively (in which one or several amino acids may be substituted, deleted, inserted or added) and the region encoding a light chain variable region comprises a base sequence encoding the amino acid sequence of SEQ ID NOS: 30, 31, and 32 in Sequence Listing, respectively (in which one or several amino acids may be substituted, deleted, inserted or added); and polynucleotides in which the region encoding a heavy chain variable region comprises a base sequence encoding the amino acid sequence of SEQ ID NOS: 39, 40, and SFG in Sequence Listing, respectively (in which one or several amino acids may be substituted, deleted, inserted or added) and the region encoding a light chain variable region comprises a base sequence encoding the amino acid sequence of SEQ ID NOS: 36, 37, and 38 in Sequence Listing, respectively (in which one or several amino acids may be substituted, deleted, inserted or added).

[0109]

Other examples of the nucleic acid molecule of the present invention include polynucleotides in which the region encoding a heavy chain comprises a base sequence encoding the amino acid sequence of SEQ ID NO: 5 in Sequence Listing and the region encoding a light chain comprises a base sequence encoding the amino acid sequence of SEQ ID NO: 4 in Sequence Listing; and polynucleotides in which the region encoding a heavy chain comprises a base sequence encoding the amino acid sequence of SEQ ID NO: 7 in Sequence Listing and the region encoding a light chain comprises a base sequence encoding the amino acid sequence of SEQ ID NO: 6 in Sequence Listing.

[0110]

Other examples of the nucleic acid molecule of the present invention include

polynucleotides in which the region encoding a heavy chain comprises a base sequence encoding the amino acid sequence of SEQ ID NO: 9 in Sequence Listing and the region encoding a light chain comprises a base sequence encoding the amino acid sequence of SEQ ID NO: 8 in Sequence Listing.

5 [0111]

Other examples of the nucleic acid molecule of the present invention include polynucleotides in which the region encoding a heavy chain comprises a base sequence encoding the amino acid sequence of any one of SEQ ID NOS: 11 to 18 in Sequence Listing and the region encoding a light chain comprises a base sequence  
10 encoding the amino acid sequence of any one of SEQ ID NOS: 19 to 25 in Sequence Listing.

[0112]

Particularly preferred examples of the nucleic acid molecule of the present invention include polynucleotides in which the region encoding a heavy chain  
15 comprises a base sequence encoding the amino acid sequence of SEQ ID NO: 15 in Sequence Listing and the region encoding a light chain comprises a base sequence encoding the amino acid sequence of SEQ ID NO: 19 in Sequence Listing.

[0113]

Other examples of the nucleic acid molecule of the present invention include  
20 polynucleotides in which the region encoding a heavy chain variable region comprises a base sequence encoding the amino acid sequence of SEQ ID NO: 41 in Sequence Listing and the region encoding a light chain variable region comprises a base sequence encoding the amino acid sequence of SEQ ID NO: 42 in Sequence Listing.

25 [0114]

Specific examples of the nucleic acid molecule of the present invention include polynucleotides in which the region encoding a heavy chain variable region comprises the base sequence of SEQ ID NO: 43 in Sequence Listing and the region

encoding a light chain variable region comprises the base sequence of SEQ ID NO: 44 in Sequence Listing.

[0115]

5 The nucleic acid molecule of the present invention may be polynucleotides which hybridizes under stringent conditions to a complementary strand DNA having the base sequence of SEQ ID NO: 43 in Sequence Listing and polynucleotides which hybridizes under stringent conditions to a complementary strand DNA having the base sequence of SEQ ID NO: 44 in Sequence Listing as long as the nucleic acid molecule of the present invention encodes a monoclonal antibody which has ability  
10 to bind to RGMa, does not inhibit the binding between RGMa and neogenin, and neutralizes the neurite outgrowth inhibitory activity of RGMa. Examples of the stringent conditions include a condition of conducting southern hybridization and washing at a salt concentration corresponding to 0.1 x SSC, 0.1% SDS at 68°C.

[0116]

15 The nucleic acid molecule of the present invention may encode all of the constant regions and the variable regions of heavy and light chains, and may encode only the variable regions of heavy and light chains. The base sequence of the constant region of heavy and light chains in the case of encoding all of the constant region and the variable region is preferably those described in Nucleic Acids  
20 Research vol.14, p1779, 1986, The Journal of Biological Chemistry vol.257, p1516, 1982 and Cell vol.22, p197, 1980.

[0117]

The nucleic acid molecule of the present invention can be obtained, for example, by the following method. First, total RNA is prepared from cells such as  
25 hybridomas using a commercial RNA extraction kit, and cDNA is synthesized with reverse transcriptase using random primers or the like. Next, cDNAs encoding the antibody are amplified by a PCR method using, for primers, oligonucleotides having sequences conserved in the variable regions of known human antibody heavy and

light chain genes, respectively. For the sequence encoding the constant region, it can be obtained by amplifying the known sequence by PCR method. The base sequence of the DNA can be determined by a conventional method, for example by incorporating it into a plasmid for sequencing.

5           Alternatively, a DNA encoding the monoclonal antibody of the present invention can also be obtained by chemically synthesizing a sequence of the variable region or a part thereof and binding it to a sequence comprising the constant region.  
[0118]

          The present invention also provides a recombinant vector comprising the  
10   nucleic acid molecule of the present invention and a transformant (host cell) comprising the recombinant vector. The recombinant vector may be vectors which can be expressed in prokaryotic cells such as *E. coli* (*Escherichia coli*) (e.g., pBR322, pUC119 or a derivative thereof), and preferably are vectors which can be expressed in eukaryotic cells, and more preferably are vectors which can be expressed in  
15   mammalian-derived cells. Examples of the vectors which can be expressed in mammalian-derived cells include plasmid vectors such as pcDNA 3.1 (Invitrogen), pConPlus, pcDM8, pcDNA I/Amp, pcDNA 3.1, pREP4; and viral vectors such as pDON-AI DNA (Takara Bio). The vector may be one vector comprising a heavy chain coding sequence and a light chain coding sequence or may be two vectors of a  
20   vector comprising a heavy chain coding sequence and a vector comprising a light chain coding sequence.

[0119]

          The transformant into which the recombinant vector of the present invention is introduced may be a prokaryotic cell such as *Escherichia coli* or *Bacillus subtilis*,  
25   preferably a eukaryotic cell, more preferably a mammalian cell. Examples of the mammalian cell include Chinese hamster ovary cells (CHO cells), COS, myeloma, BHK, HeLa, Vero, 293, NS0, Namalwa and YB2/0.

[0120]

The anti-RGMA antibody or antigen-binding fragment thereof obtained can be purified to homogeneity. Separation and purification methods used for ordinary proteins may be used for separation and purification of antibodies and the like. Separation and purification of antibodies can be achieved by appropriately selecting and combining, for example, but not limited to, chromatography columns such as affinity chromatography, filters, ultrafiltration, salting out, dialysis, SDS polyacrylamide gel electrophoresis or isoelectric focusing (Antibodies: A Laboratory Manual. Ed Harlow and David Lane, Cold Spring Harbor Laboratory, 1988). Examples of columns used for affinity chromatography include protein A column, protein G column, anti-immunoglobulin antibody-conjugated column and antigen-conjugated column. Examples of the protein A column include Hyper D, POROS and Sepharose F. F. (Amersham Biosciences).

[0121]

*Agents for Preventing or Treating Immunological and Neurological Diseases*

The RGMA-binding protein, particularly the anti-RGMA antibody or antigen-binding fragment thereof of the present invention neutralizes the neurite outgrowth inhibitory activity of RGMA to promote the repair of neuronal function and thus can be used as an agent for preventing, treating, or preventing the relapse of neurological diseases.

The RGMA-binding protein, particularly the anti-RGMA antibody or antigen-binding fragment thereof of the present invention also neutralizes T-cell activation by RGMA and thus can be used as an agent for preventing, treating, or preventing the relapse of immunological diseases.

[0122]

Examples of the neurological disease include amyotrophic lateral sclerosis, brachial plexus injury, brain damage (including traumatic brain injury), cerebral palsy, Guillain-Barre syndrome, cerebral leukodystrophy, multiple sclerosis (including relapsing-remitting multiple sclerosis, primary progressive multiple

sclerosis, secondary progressive multiple sclerosis), neuromyelitis optica, post-polio syndrome, spina bifida, spinal cord injury, spinal muscular atrophy, spinal neoplasm, transverse myelitis, dementia (including senile dementia, mild cognitive impairment, Alzheimer's disease, dementia associated with Alzheimer's disease), Huntington's disease, tardive dyskinesia, mania, Parkinson's disease, Steele-Richardson syndrome, Down's syndrome, myasthenia gravis, neurotrauma (including optic nerve trauma), vascular amyloidosis, cerebral hemorrhage associated with amyloidosis, brain infarction, cerebritis, acute confusional state, glaucoma, schizophrenia and retinal nerve fiber layer degeneration (including diabetic retinopathy, ischemic optic neuropathy, X-linked retinoschisis, drug-induced optic neuropathy, retinal dystrophy, age-related macular degeneration, eye diseases characterized by optic disc drusen, eye diseases characterized by genetic determinant for photoreceptor degeneration, autosomal recessive cone-rod dystrophy, mitochondrial disorder associated with optic neuropathy). Spinal cord injury and neurotrauma (including optic nerve trauma) are preferred.

[0123]

Examples of the immunological disease include multiple sclerosis (including relapsing-remitting multiple sclerosis, primary progressive multiple sclerosis, secondary progressive multiple sclerosis), neuromyelitis optica, psoriasis, arthritis (including rheumatoid arthritis, osteoarthritis, psoriatic arthritis), Guillain-Barre syndrome, neuro-Behcet disease, pernicious anemia, type I (insulin-dependent) diabetes mellitus, systemic lupus erythematosus (SLE), inflammatory bowel disease (IBD), Sjogren's syndrome, Goodpasture's syndrome, Graves' disease, autoimmune hemolytic anemia, autoimmune thrombocytopenic purpura, asthma, pollinosis, atopic dermatitis, glomerulonephritis, myasthenia gravis, Hashimoto's disease, and sarcoidosis. Multiple sclerosis is preferred.

[0124]

The RGMa-binding protein, particularly the anti-RGMa antibody or antigen-

binding fragment thereof of the present invention can be used as an agent for preventing, treating, or preventing the relapse of neurological/immunological diseases, which preferably include spinal cord injury, neurotrauma (including optic nerve trauma) and multiple sclerosis (including relapsing-remitting multiple sclerosis, primary progressive multiple sclerosis, secondary progressive multiple sclerosis).

[0125]

As used herein, the term "treating" includes any treatment of diseases in a mammal, particularly a human, and includes inhibiting disease symptoms, that is, inhibiting their progression or eliminating the diseases or symptoms, and alleviating disease symptoms, that is, causing regression of the diseases or symptoms or a delay in the development of the symptoms.

[0126]

"Preventing" includes prevention of onset of the above-described diseases in a mammal, particularly a human.

[0127]

"Preventing the relapse" includes prevention of relapse of the above-described diseases repeating remission and relapse in a mammal, particularly a human.

[0128]

The RGMa-binding protein (anti-RGMa antibody or antigen-binding fragment thereof) of the present invention can be used as a pharmaceutical composition for preventing or treating neurological or immunological diseases.

[0129]

The administration form of the RGMa-binding protein (anti-RGMa antibody or antigen-binding fragment thereof) of the present invention is not particularly limited and can be administered to mammals including humans by any route of oral or parenteral administration (e.g., intravenous, intramuscular, subcutaneous, rectal,

transcutaneous, intracerebral, intraspinal or other topical administration).

[0130]

Dosage forms for oral and parenteral administration and preparation methods thereof are well known to those skilled in the art, and a pharmaceutical  
5 composition can be prepared by combining the antibody according to the invention with a pharmaceutically acceptable carrier or the like.

Dosage forms for parenteral administration include injectable preparations (e.g., drip injections, intravenous injections, intramuscular injections, subcutaneous injections, intradermal injections, intracerebral administration preparations, and  
10 intraspinal administration preparations), external preparations (e.g., ointments, poultices, and lotions), suppository inhalants, eye drops, ophthalmic ointments, nasal drops, ear drops, and liposomes. In particular, when the antibody according to the present invention is to be directly acted on a central nervous tissue, it can be infused continuously using a medical micropump which is an osmotic pump, or mixed with  
15 fibrin glue or the like to prepare a sustained-release preparation and then placed in the affected tissue.

[0131]

For example, injectable preparations are usually prepared by dissolving the antibody in injectable distilled water and, if necessary, a solubilizing agent, a buffer,  
20 a pH adjusting agent, an isotonicizing agent, a soothing agent, a preservative, and a stabilizing agent may be added. The injectable preparations may also be lyophilized preparations prepared before use.

[0132]

Dosage forms for oral administration include solid or liquid dosage forms,  
25 in particular, tablets, coated tablets, pills, fine granules, granules, powders, capsules, syrups, emulsions, suspensions, injections and troches.

[0133]

The pharmaceutical composition of the present invention may further

contain other therapeutically effective drugs, and if necessary, components such as microbicides, antiphlogistics, vitamins and amino acids may be blended.

[0134]

Pharmacologically acceptable carriers include, for example, excipients,  
5 lubricants, binders and disintegrants for solid preparations; and solvents, solubilizing agents, suspending agents, isotonizing agents, buffers and soothing agents for liquid preparations. If necessary, additives such as usual antiseptics, antioxidants, colorants, sweeteners, adsorbents, and wetting agents may be appropriately used in appropriate amounts.

10 [0135]

The dosage of the antibody according to the present invention can be determined based on various factors such as the route of administration, the type of disease, the degree of symptoms, age, sex, body weight, severity of disease of patients, pharmacological findings such as pharmacokinetic and toxicological  
15 characteristics, whether or not a drug delivery system is used, and whether or not it is administered as a part of a combination with other drugs. Usually, 1 to 5,000  $\mu\text{g/day}$ , preferably 10 to 2,000  $\mu\text{g/day}$ , more preferably 50 to 2,000  $\mu\text{g/day}$  for oral administration or 1 to 5,000  $\mu\text{g/day}$ , preferably 5 to 2,000  $\mu\text{g/day}$ , more preferably 50 to 2,000  $\mu\text{g/day}$  for injection can be administered per adult (60 kg body weight) in  
20 one or several doses. For parenteral administration to the whole body, 10 to 100,000  $\mu\text{g/kg}$ , more preferably 100 to 50,000  $\mu\text{g/kg}$ , and even more preferably 500 to 20,000  $\mu\text{g/kg}$  per body weight can be administered at an interval of once a day, once a week, once a month, or 1 to 7 times a year. For topical administration using an osmotic pump or the like, in usual, continuous infusion at a rate of 10 to 100,000  $\mu\text{g/day}$ ,  
25 more preferably 100 to 10,000  $\mu\text{g/day}$ , still more preferably 500 to 5,000  $\mu\text{g/day}$  per adult (60 kg body weight) is possible.

## EXAMPLES

[0136]

The present invention will be described in detail below with reference to Examples, but the present invention is not limited to the aspects of the following Examples.

5 [0137]

Example 1: Preparation of Human RGMa Protein (C-terminal Domain)

A CHO cell expressing a recombinant human RGMa protein in which a histidine tag was fused to the C terminus of Pro 169 to Gly 422 (which refers to the proline residue at position 169 to the glycine residue at position 422 from the N-  
10 terminal side, hereinafter similarly described) of the human RGMa protein (SEQ ID NO: 1 in Sequence Listing) was established.

The C-terminal domain of the human RGMa protein contained in the culture supernatant of CHO cells was adsorbed on a nickel column (GE Healthcare, 17-5247-01), and eluted with a 100 mM imidazole solution. By dialysis, the imidazole  
15 elution fraction was replaced with Phosphate buffered Saline (PBS) and used as an immunogen.

[0138]

Example 2: Preparation of Mouse Anti-human RGMa Monoclonal Antibody

Ten microgram of the recombinant human RGMa protein prepared in  
20 Example 1 was mixed with complete Freund's adjuvant (Sigma) to prepare an emulsion, and a BALB/c mouse (Charles River Japan) was immunized therewith at several subcutaneous sites on the back. Thereafter, immunization was carried out similarly at 1 to 2 week intervals with 10 µg of recombinant human RGMa protein prepared into an emulsion with incomplete Freund's adjuvant (Sigma), and blood was  
25 collected after several immunizations. Antibody titer was measured by ELISA described below in which human or mouse RGMa proteins were immobilized. Into individuals which showed an increased antibody titer, 10 µg of human RGMa protein was intravenously administered for boosting, and splenocytes were recovered 2 or 3

days later.

For cell fusion, the spleen cells and mouse myeloma cells (SP2/0, Dainippon Sumitomo Pharma) with half the number of the spleen cells were mixed and centrifuged, and polyethylene glycol (Roche Diagnostics) was added to the resulting precipitate fraction to obtain cell fusions. The cells were then centrifuged and washed twice with D-MEM (Invitrogen). The cells were resuspended in GIT medium (Nippon Pharmaceutical) containing 10% fetal bovine serum (Invitrogen), 1% BM condimed (Roche Diagnostics) and HAT (Sigma-Aldrich) and seeded in each well of a 96-well plate at  $5 \times 10^4$  myeloma cells/well. The culture supernatant was recovered and screened for antibody-producing cells by the human RGMa protein-immobilized ELISA of Example 3.

Antibody-producing cells obtained by the screening were cloned by the limiting dilution method, and hybridoma cells producing two kinds of monoclonal antibodies (B5.116A3 and B5.70E4) were selected.

Isotypes of both of the monoclonal antibodies determined by using an isotyping kit (Mouse MonoAB ID/SP KIT, ZYMED, 93-6550) were mouse IgG2b for heavy chain and kappa for light chain.

The culture supernatants of the hybridomas were subjected to affinity chromatography using agarose on which anti-mouse IgG antibodies were immobilized (Anti-Mouse IgG-Agarose manufactured by Sigma) to purify the monoclonal antibodies. After the antibody was bound to the column, the column was washed with PBS. Then the antibody was eluted with 10 mM glycine hydrochloride (pH 2.7) and the eluate was neutralized immediately. Thereafter, the neutralized elute was replaced with PBS through an ultrafilter.

[0139]

#### Example 3: ELISA on Which Human or Mouse RGMa Proteins are Immobilized

Human RGMa protein (R&D systems, 2459-RM) or mouse RGMa protein (R&D systems, 2458-RG) prepared at 2  $\mu\text{g/mL}$  with PBS was dispensed in a 96-well

plate at 50  $\mu$ L/well each, and the plate was allowed to stand at room temperature for 1 hour. After removal of the solution, ApplieBlock (Seikagaku Bio-Business, 200150) diluted 5-fold with PBS was dispensed at 200  $\mu$ L/well each and the plate was allowed to stand at room temperature for 1 hour to block nonspecific binding.

5 After washing three times with PBST (PBS containing 0.05% Tween 20), samples (e.g., mouse serum, hybridoma culture supernatant, recombinant antibody-expressed culture supernatant described later, or purified antibody) serially diluted with PBS were added at 50  $\mu$ L/well each, and the plate was allowed to stand at room temperature for 1 hour. Thereafter, the plate was washed three times with PBST, and

10 then peroxidase-labeled sheep anti-mouse IgG antibody (GE Healthcare, NA9310V) diluted with PBS was dispensed at 50  $\mu$ L/well each, and the plate was allowed to stand at room temperature for 1 hour. After washing three times, a peroxidase-coloring kit (Sumitomo Bakelite, ML-1130O) was added and allowed to color develop for a certain period of time, and the absorbance at 492 nm was measured

15 with a plate reader.

[0140]

#### Example 4: Antibody Epitope Analysis

Epitopes to which the antibodies bind were determined by the peptide scanning method. A total of 83 kinds of peptides were synthesized by fusing the N-

20 terminal side of the amino acid sequences consisting of 11 consecutive residues shifted by 3 residues contained in Arg 172 to Ala 424 of the human RGMa protein (SEQ ID NO: 1 in Sequence Listing) with a spacer sequence (SGSG) with biotinylated N terminus (SEQ ID NO: 46 in Sequence Listing). After immobilizing the peptides on avidin-coated plates, the test antibodies (B5.116A3 and B5.70E4)

25 were allowed to react. Subsequently, a peroxidase-labeled rabbit anti-mouse Ig antibody (Dako, P026002) was allowed to react. After a substrate solution was added and color developed for a certain time, the absorbance was measured with a plate reader.

As a result, B5.116A3 bound to peptides derived from human RGMa of Glu 298 to Gly 311 (two types of peptides: Glu 298 to Asp 308 and Val 301 to Gly 311), Asn 322 to Glu 335 (two types of peptides: Asn 322 to Thr 332 and Ile 325 to Glu 335), and Lys 367 to Ala 377; and B5.70E4 bound to peptides derived from human RGMa of Glu 298 to Gly 311 (two types of peptides: Glu 298 to Asp 308 and Val 301 to Gly 311), Asn 322 to Glu 335 (two types of peptides: Asn 322 to Thr 332 and Ile 325 to Glu 335), and Pro 349 to Thr 359.

[0141]

#### Example 5: Sequence Analysis and Cloning of Mouse Antibody Gene

Total RNA was extracted from hybridoma cells producing a mouse monoclonal antibody (B5.116A3 or B5.70E4). Using the total RNA as a template, cDNA was synthesized by reverse transcription reaction. Using the cDNA as a template, genes of light chain variable and constant regions, and heavy chain variable and constant regions were amplified by PCR and the DNA sequences were determined. Next, based on the determined sequences of the variable and constant regions, full-length antibody genes were amplified by PCR and cloned. The amino acid sequences encoded by these antibody genes were as follows.

[0142]

(1) B5.116A3 light chain amino acid sequence (SEQ ID NO: 4 in Sequence Listing)

DIQMTQTTSSLSASLGDRVTISCRASQDISSYLNWYQQKPDGTVKLLIYYTSR  
LHSGVPSRFSGSGSGTDYSLTISNLEQEDIATYFCQQLNTLPWTFGGGKLEI  
KRADAAPTVSIFPPSSEQLTSGGASVVCFLNNFYPKDINVKWKIDGSRQNG  
VLNSWTDQDSKDYMSSTLTITKDEYERHNSYTCEATHKTSTSPIVKSFN  
RNEC

[0143]

(2) B5.116A3 heavy chain amino acid sequence (SEQ ID NO: 5 in Sequence Listing)

EVKLEESGGGLVQPGGSMKLSCAASGFTFSDAWMDWVRQSPEKGLEWVAE  
IRSKANNHATYYAESVKGRFTISRDDSKRSVYLMNNLRAEDTGIYYCTRRD

GAYWGGTLTVSAAKTTPPSVYPLAPGCGDTTGSSVTLGCLVKGYFPESV  
 TVTWNSGSLSSSVHTFPALLQSGLYTMSSSVTVPSSTWPSQTVTCSVAHPASS  
 TTVDDKKLEPSGPISTINPCPPCKECHKCPAPNLEGGPSVFIFPPNIKDVLMISLT  
 PKVTCVVDVSEDDPDVQISWVFNNEVHTAQQTQTHREDYNSTIRVVSTLPI  
 5 QHQDWMSGKEFKCKVNNKDLPSPIERTISKIKGLVRAPQVYILPPPAEQLSRK  
 DVSLTCLVVGFNPGDISVEWTSNGHTEENYKDTAPVLDSGSGSYFIYSKLNMK  
 TSKWEKTDSFSCNVRHEGLKNYYLKKTISRSPGK

[0144]

(3) B5.70E4 light chain amino acid sequence (SEQ ID NO: 6 in Sequence Listing)

10 DVVMTQTPLSLPVSLGDQASISCRSSQSLVHSNGNTYLHWYLQRPQGSPKLL  
 IYKVSNRFSGVDPDRFSGSGSGTDFTLKISRVEAEDLGLYFCSQSTHVPYTFGG  
 GTKLEIKRADAAPTVSIFPPSSEQLTSGGASVVCFLNNFYPKDINVKWKIDGS  
 ERQNGVLNSWTDQDSKDYMSSTLTTLTKDEYERHNSYTCEATHKTSTSPI  
 VKSFNRNEC

15 [0145]

(4) B5.70E4 heavy chain amino acid sequence (SEQ ID NO: 7 in Sequence Listing)

DVKLQESGPGLVKPSQSLSLTCSVTGYSITTSYYWNWIRQFPGNKLEWMGYI  
 SYDGTNNYNPSLKNRISITRDTSKNQFFLRNLNSVTTEDTATYYCAGSFGYSQG  
 TLTVTSAAKTTPPSVYPLAPGCGDTTGSSVTLGCLVKGYFPESVTVTWNSGS  
 20 LSSSVHTFPALLQSGLYTMSSSVTVPSSTWPSQTVTCSVAHPASSTTVDKKLE  
 PSGPISTINPCPPCKECHKCPAPNLEGGPSVFIFPPNIKDVLMISLTPKVTCVVV  
 DVSEDDPDVQISWVFNNEVHTAQQTQTHREDYNSTIRVVSTLPIQHQDWMS  
 GKEFKCKVNNKDLPSPIERTISKIKGLVRAPQVYILPPPAEQLSRKDVSLTCLV  
 VGFNPGDISVEWTSNGHTEENYKDTAPVLDSGSGSYFIYSKLNMKTSKWEKT  
 25 DSFSCNVRHEGLKNYYLKKTISRSPGK

[0146]

Example 6: Preparation of Recombinant Mouse Antibody and Recombinant Rat  
 Mouse Chimeric Antibody

Recombinant mouse antibodies of two types of anti-RGMa antibodies B5.116A3 and B5.70E4 derived from hybridomas were prepared (hereinafter referred to as "r116A3" and "r70E4", respectively).

In addition, as a comparative example, recombinant rat mouse chimeric antibody (hereinafter referred to as "r5F9") was prepared by fusing the variable regions of rat antibody 5F9 with the constant regions of mouse antibody (IgG2b $\kappa$ ) (the light chain constant region is Arg 108 to Cys 214 of SEQ ID NO: 4 in Sequence Listing, and the heavy chain constant region is Ala 117 to Lys 452 of SEQ ID NO: 5 of the sequence listing) according to Patent Document 1 (WO2009/106356).

DNA encoding the light and heavy chains of each of the antibodies was inserted into pcDNA 3.3 (Life Technologies) to prepare an expression vector. The expression vector was introduced into HEK293F cells (Life Technologies) using Neofection 293 (ASTEC). The cells were cultured at 37°C under 8% carbon dioxide gas atmosphere for 6 days, and then culture supernatant was recovered. For the purification of the recombinant antibody, the culture supernatant was applied to an affinity column on which Protein A or Protein G was immobilized (GE Healthcare), and antibodies bound to the column were eluted with 10 mM glycine hydrochloride (pH2.8). The eluate was neutralized immediately, and then replaced with PBS.

In the case where refined purity needs to be increased according to purpose of use, the antibodies purified with the Protein A column were purified with a Ceramic Hydroxyapatite Type 1 (CHT) column (BIORAD). Antibodies bound to the CHT column were washed with 10 mM KH<sub>2</sub>PO<sub>4</sub> (pH6.5) and then eluted with 20 mM KH<sub>2</sub>PO<sub>4</sub> (pH6.5), 0.5 M NaCl. Eluted fractions were collected and then replaced with PBS.

[0147]

#### Example 7: Binding Assay on RGMa Protein Expressing Cells

A vector expressing full-length human RGMa protein (Met 1 to Cys 450 of SEQ ID NO: 1 in Sequence Listing), human RGMa protein C-terminal domain (Pro

169 to Cys 450 of SEQ ID NO: 1 in Sequence Listing), full-length mouse RGMa protein (Met 1 to Trp 454 of SEQ ID NO: 2 in Sequence Listing), or rat RGMa protein C-terminal domain (Pro 170 to Trp 449 of SEQ ID NO: 3 in Sequence Listing) was introduced into CHO or HEK 293 cells to prepare antigen-expressing  
 5 cells.

In RGMa proteins, the C-terminal peptide is processed during GPI anchor addition reaction. Mouse and rat RGMa proteins are both cleaved at Ala 427 and the C-terminal peptide is removed. Therefore, the amino acid sequences of the full-length protein and the C-terminal domain expressed on a cell via a GPI anchor are  
 10 the same in mouse and rat.

Test antibodies (r116A3 and r70E4) and r5F9 (Comparative Example) with a final concentration of 10 µg/mL were reacted with the above-described antigen-expressing cells and then the cells were washed with PBS containing 0.1% bovine serum albumin and 0.05% NaN<sub>3</sub>. FITC-labeled anti-mouse immunoglobulin  
 15 antibody (DAKO) was allowed to react and the cells were washed. Fluorescence was measured with flow cytometry (FACSCalibur manufactured by Beckton Dickinson), and the binding properties of the test antibodies to the antigen-expressing cells were evaluated (Table 1).

As a result, r116A3 and r70E4 were found to bind to the C-terminal domain  
 20 of human and rat RGMa proteins, unlike r5F9. RGMa protein has neurite outgrowth inhibitory effect even in the C-terminal domain alone, and r116A3 and r70E4 inhibit both the full-length RGMa protein and the C-terminal domain.

[0148]

Table 1: Evaluation of binding of various recombinant anti-RGMa antibodies to  
 25 antigen-expressing cells\*

|        | Full-length human RGMa | Human RGMa C-terminal domain | Full-length mouse RGMa | Rat RGMa C-terminal domain |
|--------|------------------------|------------------------------|------------------------|----------------------------|
| r116A3 | ++                     | ++                           | ++                     | ++                         |
| r70E4  | ++                     | ++                           | +                      | ++                         |

|      |    |   |    |   |
|------|----|---|----|---|
| r5F9 | ++ | - | ++ | - |
|------|----|---|----|---|

\*; ++ Strongly bind

+ Weakly bind

- Not bind

5 [0149]

#### Example 8: Determination of Dissociation Constant for RGMa Protein

The affinity of the test antibodies (r116A3 and r70E4) and r5F9 (Comparative Example) for RGMa protein were measured by the surface plasmon resonance (SPR) method using Proteon XPR36 (Bio-Rad).

10 Human RGMa protein (R&D Systems, 2459-RM), the C-terminal domain of human RGMa protein (prepared in Example 1) or mouse RGMa protein (R&D Systems, 2458-RG) diluted to 10 µg/mL with 10 mM acetate buffer (pH4.5) was immobilized on a GLC sensor chip by amine coupling method. The serially diluted test antibodies were applied as analytes at a flow rate of 100 µL/min for 60 seconds  
15 to determine the dissociation constant (Kd value).

As shown in Table 2, r116A3 and r70E4 also bound to the C-terminal domain of human RGMa protein, but r5F9 did not. R116A3 bound to human RGMa protein 32 times stronger than r5F9 and to mouse RGMa protein 44 times stronger than r5F9.

20 [0150]

Table 2: Affinity of anti-RGMa monoclonal antibody

| Antibody                      | Dissociation Constant (Kd value, nM) |                              |            |
|-------------------------------|--------------------------------------|------------------------------|------------|
|                               | Human RGMa                           | Human RGMa C-terminal domain | Mouse RGMa |
| r116A3                        | 0.0487                               | 0.0568                       | 0.201      |
| r70E4                         | 2.43                                 | 1.12                         | 174        |
| r5F9<br>(Comparative Example) | 1.59                                 | not bind                     | 8.98       |

[0151]

Example 9: RGMa-Neogenin Binding Inhibition Assay

The extracellular domain (Ala 34 to Leu 1105) of the recombinant human neogenin protein (SEQ ID NO: 10 in Sequence Listing) was purified. A CHO cell  
5 line expressing human Neogenin protein extracellular domain was established. A histidine tag was fused to the C-terminus. From the culture supernatant of CHO cells, the extracellular domain was adsorbed on a nickel column (GE Healthcare, 17-5247-01), and then eluted with a 100 mM imidazole solution. By dialysis, the imidazole elution fraction was replaced with PBS.

10 Human RGMa protein (R&D systems, 2459-RM) was labeled with biotin using ChromaLink Biotin Labeling Kit (Solulink). Equal amounts of the biotin-labeled human RGMa protein adjusted to 2 µg/mL and the test antibodies (r116A3 and r70E4) subjected to 2-fold serial dilution were mixed and allowed to react at room temperature for 2 hours to prepare a mixed solution.

15 At the same time, 50 µL/well of the human neogenin protein extracellular domain adjusted to 2 µg/mL with PBS was added to a 96-well plate, and the plate was allowed to stand at room temperature for 1 hour to prepare a neogenin-immobilized plate. After removal of the solution, 2.5% bovine serum albumin solution was added and the plate was left to stand for 1 hour to block nonspecific  
20 binding. To the neogenin-immobilized plate, the above-described mixed solution was added at 50 µL/well, and the plate was allowed to stand at room temperature for 1 hour. Then, a washing operation was performed, and peroxidase-labeled Avidin (VECTASTAIN ABC system, manufactured by Vector Laboratories) was added, and the plate was allowed to stand at room temperature for 1 hour. A washing operation  
25 was then performed, a substrate solution was added, color development was carried out for a certain period of time, and the absorbance was measured with a plate reader. The absorbance ratio in the absence of the antibody was plotted as 1, and the concentration-dependent RGMa-neogenin binding inhibition by the antibody was

evaluated (Fig. 1).

As a result, unlike anti-human RGMa polyclonal antibody (R&D Systems, AF2459) and r5F9, r116A3 and r70E4 did not inhibit RGMa-neogenin binding. [0152]

#### 5 Example 10: RGMa-BMP2 Binding Inhibition Assay

Human RGMa protein (R&D systems, 2459-RM) adjusted to 2 µg/mL with PBS was added to a 96-well plate at 50 µL/well each, and the plate was allowed to stand at room temperature for 1 hour. A 2.5% bovine serum albumin solution was added and the mixture was allowed to stand for 1 hour to block nonspecific binding, thereby preparing RGMa protein-immobilized plate. The test antibodies (B5.116A3 and B5.70E4) serially diluted to 0.01 to 10 µg/mL were added to the RGMa protein-immobilized plate and the plate was allowed to stand at room temperature for 1 hour. Then, a washing operation was performed, and human BMP2 protein (R&D systems, 355-BM) diluted to 0.5 µg/mL was added and the plate was left to stand at room temperature for 1 hour. 15

A biotin-labeled anti-BMP2 antibody was allowed to react, and further added with peroxidase-labeled Avidin (VECTASTAIN ABC system, manufactured by Vector Laboratories) and a substrate solution were added, color development was carried out for a certain period of time, and the absorbance was measured with a plate reader (Fig. 2). 20

As a result, in particular, the anti-RGMa antibody (B5.116A3) weakly inhibited the binding of RGMa-BMP2 in a concentration-dependent manner (Absorbance: 0.45 at 0.01 µg/mL, 0.4 at 0.1 µg/mL, 0.32 at 1 µg/mL, and 0.1 at 10 µg/mL). 25 [0153]

#### Example 11: Design of Humanized Antibody

Humanization of the mouse monoclonal antibody B5.116A3 was carried out by complementarity determining region (CDR) grafting according to the method by

Winter et al. described in Patent No. 2912618.

First, 3D homology models of the light and heavy chain variable regions of the mouse monoclonal antibody B5.116A3 were prepared and amino acid residues within the framework (FW) region, located near CDR were identified. FWs of human antibodies in which these amino acids are maintained as many as possible were selected, CDRs of the mouse antibody were grafted thereto, and thus designing a humanized antibody. In the designed humanized antibody sequence, the heavy chain is described as HA (SEQ ID NO: 11 in Sequence Listing) and the light chain is described as KA (SEQ ID NO: 19 in Sequence Listing). In addition, a plurality of humanized antibody sequences were designed by introducing additional mutations into amino acids within FWs involved in structural stability of the variable region (A total of 8 heavy chains ranging from HB to HH including HA and a total of 7 light chains ranging from KB to KG including KA).

[0154]

Table 3: Designed humanized antibody sequences

| Heavy Chain |                                                                                        | SEQ ID NO in the sequence listing |
|-------------|----------------------------------------------------------------------------------------|-----------------------------------|
| HA          | Sequence in which CDRs of mouse antibody B5.116A3 are grafted to FWs of human antibody | 11                                |
| HB          | HA sequence with substitution of Ala 81 by Val and Lys 89 by Arg                       | 12                                |
| HC          | HA sequence with substitution of Ala 81 by Val                                         | 13                                |
| HD          | HA sequence with substitution of Lys 89 by Arg                                         | 14                                |
| HE          | HA sequence with substitution of Ala 81 by Val, Lys 89 by Arg, and Phe 37 by Val       | 15                                |
| HF          | HA sequence with substitution of Leu 95 by Val                                         | 16                                |
| HG          | HA sequence with substitution of Phe 37 by Val                                         | 17                                |
| HH          | HA sequence with substitution of Phe 37 by Val, and Leu 95 by Val                      | 18                                |
| Light Chain |                                                                                        |                                   |
| KA          | Sequence in which CDRs of mouse antibody B5.116A3 are grafted to FWs of human antibody | 19                                |
| KB          | KA sequence with substitution of Phe 71 by Tyr                                         | 20                                |
| KC          | KA sequence with substitution of Phe 71 by Tyr, and Phe 44 by Val                      | 21                                |

|    |                                                                                  |    |
|----|----------------------------------------------------------------------------------|----|
| KD | KA sequence with substitution of Ser 85 by Thr                                   | 22 |
| KE | KA sequence with substitution of Pro 44 by Val                                   | 23 |
| KF | KA sequence with substitution of Pro 44 by Val, and Ser 85 by Thr                | 24 |
| KG | KA sequence with substitution of Phe 71 by Tyr, Pro 44 by Val, and Ser 85 by Thr | 25 |

[0155]

Example 12: Preparation of Recombinant Mouse Human Chimeric Antibody and Recombinant Humanized Antibody

- 5 (1) Recombinant mouse human chimeric anti-RGMA antibody 116A3 (r116A3C) having the following amino acid sequences was prepared according to Example 6.

[0156]

Light chain (SEQ ID NO: 8 in Sequence Listing)

10 DIQMTQTTSSLSASLGDRVTISCRASQDISSYLNWYQQKPDGTVKLLIYYTSR  
LHSGVPSRFSGSGSGTDYSLTISNLEQEDIATYFCQQLNTPWTFGGGKLEI  
KRTVAAPSVFIFPPSDEQLKSGTASVCLLNNFYPREAKVQWKVDNALQSG  
NSQESVTEQDSKDSSTYSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSF  
NRGEC

15 [0157]

Heavy chain (SEQ ID NO: 9 in Sequence Listing)

EVKLEESGGGLVQPGGSMKLSCAASGFTFSDAWMDWVRQSPEKGLEWVAE  
IRSKANNHATYYAESVKGRFTISRDDSKRSVYLQMNNLRAEDTGIYYCTRDR  
GAYWGQGTLLTVSAASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVT  
20 VSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPS  
NTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVT  
CVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLH  
QDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQ  
VSLTCLVKGFYPSDIAVEWESNGQPENNYKTPPVLDSDGSFFLYSKLTVDK

SRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

[0158]

(2) A total of 20 humanized anti-RGMA antibodies comprising the following combinations of heavy and light chains were prepared according to Example 6.

5 [0159]

Heavy chain/light chain combinations;

HA/KA, HA/KB, HA/KC, HA/KG, HB/KC, HC/KA, HC/KB, HD/KA, HD/KB,  
HD/KC, HD/KD, HE/KA, HF/KA, HF/KF, HF/KG, HG/KD, HG/KH, HH/KA,  
HH/KD, HH/KF

10 [0160]

(3) A recombinant humanized anti-RGMA antibody (hereinafter referred to as rH5F9) was prepared according to Example 6 (Comparative Example).

The variable region of the humanized anti-RGMA monoclonal antibody h5F9 described in Patent Document 1 (WO2009/106356) (the light chain is seq  
15 ID\_53 of Patent Document 1, and the heavy chain is seq ID\_50 of Patent Document 1) was ligated with the human antibody constant region (the light chain is Arg 108 to Cys 214 of SEQ ID NO: 26 in Sequence Listing, and heavy chain is Ala 117 to Lys 446 of SEQ ID NO: 27 in Sequence Listing).

[0161]

#### 20 Example 13: Thermal Stability Test for Antibody

Twenty  $\mu$ L aliquots of the culture supernatant in which the recombinant antibody described in Example 12 was expressed were collected and heat-treated for 10 minutes at 8 temperature points of 40, 45, 50, 55, 60, 65, 70 and 75°C, respectively, using a thermal cycler (Takara bio, TP 600). The culture supernatant  
25 was diluted with PBS so that the final concentration of the antibody was 125 ng/mL. Thereafter, the culture supernatant was subjected to ELISA on which human RGMA proteins are immobilized described in Example 3 to evaluate the antigen binding property of the antibody (Fig. 3).

As a result, a humanized antibody comprising a combination of heavy chain HE and light chain KA (hereinafter referred to as "rH116A3"), and a mouse human chimeric antibody (r116A3C) showed better thermal stability than the humanized antibody (rH5F9). Hereinafter, the humanized antibody comprising this combination  
 5 of HE/KA is described as rH116A3.

When the heat treatment was not performed, the mouse human chimeric antibody (r116A3C) and the humanized antibody (rH116A3) showed equivalent antigen binding properties, and there was no decrease in antigen binding property associated with humanization.

10 [0162]

Example 14: Establishment of a CHO Stable Cell Line Producing Humanized Anti-RGMA Antibody (rH116A3)

A CHO stable cell line producing humanized anti-RGMA antibody (rH116A3) was established using Lonza GS Xceed system (Lonza). A pXC double  
 15 gene vector comprising the light chain coding sequence (SEQ ID NO: 44 in Sequence Listing) and the heavy chain coding sequence (SEQ ID NO: 43 in Sequence Listing) of the humanized anti-RGMA antibody (rH116A3) was introduced into a CHOK1SV GS knock out parent cell line, and a pool of transformed cells was obtained under methionine sulfoximine (MSX) selection. After separating into  
 20 single cells by flow cytometry, antibody production amount in culture supernatant, cell proliferation and the like were evaluated, and a CHO stable cell line was obtained.

[0163]

Example 15: Neurite Outgrowth Assay

25 A cerebellum was excised from a neonatal rat (P7), suspended in a trypsin solution (0.25% trypsin solution in PBS containing 0.2% DNase) and digested at 37 °C for 10 to 15 minutes. Next, DMEM medium containing 10% fetal bovine serum was added and the mixture was centrifuged. The cells were resuspended in the same

medium and centrifuged, and the same procedure was repeated twice to wash the cells. Further, this cell suspension was filtered through a 70  $\mu\text{m}$  cell strainer and centrifuged, and the precipitated fraction was resuspended in the same medium. B27 supplement (GIBCO) was added to the cell suspension to prepare neonatal rat cerebellar granule cells.

Next, the neonatal rat cerebellar granule cells were seeded on a cell culture plate and cultured at 37 °C for 1 day.

Recombinant RGMa protein (R&D systems, 2459-RM) with a final concentration of 2  $\mu\text{g/mL}$  was added and the cells were cultured at 37 °C for 2 days.

The neurite length was measured by microscopy. As shown in Fig. 4, the addition of RGMa resulted in neurite length changes from 37  $\mu\text{m}$  to 26  $\mu\text{m}$  in the experiment on the left figure and from 38  $\mu\text{m}$  to 27  $\mu\text{m}$  in the right figure, thus inhibiting neurite outgrowth. Although addition of only the test antibody (B5.116A3 or B5.70E4) at a final concentration of 10  $\mu\text{g/mL}$  did not change the neurite length, simultaneous addition of recombinant RGMa protein and the test antibody induced neurite outgrowth to the same extent as a control (no RGMa added), which indicate neutralizing effect of the antibody against RGMa protein.

[0164]

#### Example 16: Efficacy Test Using a Rat Spinal Cord Injury Model.

A Wistar rat (female, 8 weeks old, weighing about 200 g) anesthetized by aspiration of halothane (Takeda Pharmaceutical) was subjected to a laminectomy of the anterior and posterior vertebrae around the spinal level T9 (T8 to T10), and the spinal cord was exposed. In the case of evaluation using spinal cord crush model, pressure of 200 kdyn was applied to the exposed spinal cord using IH impactor (Precision System).

Immediately after damaging the spinal cord as described above, an osmotic minipump filled with 400  $\mu\text{g/mL}$  of the test antibody (r116A3 or r70E4) or a control mouse antibody (mo-IgG2b $\kappa$ ) (200  $\mu\text{L}$  volume, 0.5  $\mu\text{L}/\text{hour}$ , 14 Day delivery) (Alzet,

model 2002) was placed under the skin of the back of the rat. The tip of a silicon tube connected to the outlet of the osmotic mini pump was placed under the dura at the site of spinal cord injury. The tube was sewn and fixed to the spinous processes on the right lower limb side of the laminectomized site, the muscle and skin layers were sutured, and the rat was raised.

The motor function of spinal cord injury model rat was evaluated using Basso-Beattie-Bresnahan (BBB) score (Basso, D.M., Beattie, M.S., & Bresnahan, J.C., A sensitive and reliable locomotor rating scale for open field testing in rats. *J Neurotrauma* 12, 1-21 (1995)) at 0, 1, 3 and 7 days after injury, and every week thereafter for up to 8 weeks. As a result, as shown in Fig. 5(A), r116A3 or r70E4 significantly improved the BBB score 4 or 3 weeks after administration, respectively, compared to the control antibody (mo-IgG2bκ) ( $p < 0.05$ , Student's t-test).

In the case of evaluation using a spinal cord hemisection model, the dorsal side of the exposed spinal cord was cut to a depth of 1.8 mm to 2.0 mm. In the same manner as above, the test antibody (B5.116A3) or the control mouse antibody (mo-IgG2bκ) was administered using osmotic minipump and evaluated using BBB score at 0, 1, 3 and 7 days after injury, and every week thereafter for up to 10 weeks. As shown in Fig. 5(B), B5.116A3 significantly improved the BBB score 4 weeks after administration compared to the control antibody (mo-IgG2bκ) ( $p < 0.01$ , Student's t-test).

[0165]

#### Example 17: Efficacy Test Using Mouse Model with Multiple Sclerosis

PLP<sub>139-151</sub> peptide (HSLGKWLGHDPKF: SEQ ID NO: 45 in Sequence Listing, Peptide Institute) was dissolved in physiological saline (Otsuka Pharmaceutical Factory) and mixed with incomplete Freund's adjuvant (Sigma) to which dead tubercle bacillus H37 Ra (Difco Laboratories) was added to prepare an emulsion. SJL/JorllcoCrj (SJL/J) mouse (Charles River Japan) was immunized subcutaneously in the back with the PLP<sub>139-151</sub> peptide at 50 µg/head, and EAE score

and body weight change were evaluated (H. Kataoka, K Sugahara, K. Shimano, K. Teshima, M. Koyama, A. Fukunari and K. Chiba. FTY720, sphingosine 1-phosphate receptor modulator, ameliorates experimental autoimmune encephalomyelitis by inhibition of T cell infiltration. Cellular & Molecular Immunology 6, 439-448, 2005.)

5 (Fig. 6).

Test antibody (B5.116A3) diluted in physiological saline was intraperitoneally administered at 20 mg/kg each at 7 and 10 days or 18 and 21 days after the immunization with PLP<sub>139-151</sub> peptide.

As a result, as shown in Fig. 6, compared with the control antibody (mo-  
10 IgG2bk), the anti-RGMA mouse monoclonal antibody (B5.116A3) inhibited the deterioration of EAE score by administration before onset (upper part of Fig. 6) and showed relapse prevention effect by administration after onset (lower part of Fig. 6).  
[0166]

#### Example 18: Immunogenicity Test of Antibody

15 Undifferentiated dendritic cells contained in peripheral blood derived from 51 healthy donors were matured by granulocyte-monocyte colony-stimulating factor (GM-CSF) and Interleukin-4 stimulations. The test antibody (rH116A3) at a final concentration of 50 µg/mL was added to the mature dendritic cells and the cells were cultured for 4, 5 days to allow the antibody to be taken up into the dendritic cells.  
20 Peripheral blood CD4<sup>+</sup> T cells (helper T cells) derived from the same donor were mixed with the dendritic cells, and co-cultured for another week, and then the proliferation of T cells was measured by flow cytometry. Using the T cell proliferation activity of the test antibody as an index, the immunogenicity risk in human was evaluated. As a result, T cell proliferation was observed in 4 (7.8%) out  
25 of 51 donors, and thus the immunogenicity risk was low.

## CLAIMS

1. An isolated anti-RGMA antibody, or an antigen-binding fragment thereof,  
 wherein the amino acid sequence of each of the light chain complementarity  
 5 determining region 1 (LCDR1), the light chain complementarity determining region  
 2 (LCDR2), the light chain complementarity determining region 3 (LCDR3), the  
 heavy chain complementarity determining region 1 (HCDR1), the heavy chain  
 complementarity determining region 2 (HCDR2) and the heavy chain  
 complementarity determining region 3 (HCDR3) comprises the following:  
 10 LCDR1: RASQDISSYLN (SEQ ID NO: 30 in Sequence Listing),  
 LCDR2: YTSRLHS (SEQ ID NO: 31 in Sequence Listing),  
 LCDR3: QQLNTLP (SEQ ID NO: 32 in Sequence Listing),  
 HCDR1: DAWMD (SEQ ID NO: 33 in Sequence Listing),  
 HCDR2: EIRSKANNHATYYAESVKG (SEQ ID NO: 34 in Sequence Listing) and  
 15 HCDR3: RDGAY (SEQ ID NO: 35 in Sequence Listing);  
 or  
 LCDR1: RSSQSLVHSNGNTYLH (SEQ ID NO: 36 in Sequence Listing)  
 LCDR2: KVSNRFS (SEQ ID NO: 37 in Sequence Listing)  
 LCDR3: SQSTHVP (SEQ ID NO: 38 in Sequence Listing)  
 20 HCDR1: TSYAWN (SEQ ID NO: 39 in Sequence Listing)  
 HCDR2: YISYDGTNNYNPSLKN (SEQ ID NO: 40 in Sequence Listing) and  
 HCDR3: SFG.

2. The anti-RGMa antibody or an antigen-binding fragment thereof according to claim 1,

wherein the heavy chain variable region (VH) comprises the following:

VH:

5 EVQLVESGGGLVQPGRSLRLSCTASGFTFSDAWMDWVRQAPGKGLEWVAE  
IRSKANNHATYYAESVKGRFTISRDDSKSIVYLQMNSLRTEDTALYYCTRRD  
GAYWGKGTTVTVSS (SEQ ID NO: 41 in Sequence Listing) or an amino acid  
sequence having one or two amino acids may be substituted in said amino acid  
sequence by conservative substitution; and

10 wherein the light chainvariable region (VL) comprises the following:

VL:

DIQMTQSPSSVSASVGDRVTITCRASQDISSYLNWYQQKPGKAPKLLIYYTSR  
LHSGVPSRFSGSGSGTDFTLTISLQPEDFASYFCQQLNTLPWTFGGGGTKVEM  
E (SEQ ID NO: 42 in Sequence Listing) or an amino acid sequence having one or  
15 two amino acids may be substituted in said amino acid sequence by conservative  
substitution.

3. The anti-RGMa antibody or an antigen-binding fragment thereof according to claim 1 or 2, wherein the anti-RGMa antibody is a humanized antibody.

20

4. The anti-RGMa antibody or an antigen-binding fragment thereof according to any one of claims 1 to 3, wherein the anti-RGMa antibody comprises constant regions of human IgG.

25 5. A nucleic acid molecule coding for the protein portion of the anti-RGMa antibody or an antigen-binding fragment thereof according to any one of claims 1 to 4.

6. The nucleic acid molecule according to claim 5, wherein the nucleotide sequences coding for the VH and VL amino acid sequences each is a nucleotide sequence comprising:

VH:

5 gaagtgcagctggtggaatctggcggcggactggtgcagcctggcagatccctgagactgtcctgtaccgcctccggct  
tcaccttctccgacgcctggatggattgggtgcgacaggctcctggcaagggcctggaatgggtggccgagatccggctc  
caaggccaacaaccacgccacctactacgccgagctctgtgaagggccggtcaccatctccgggacgactccaagtcc  
atcgtgtacctgcagatgaactccctgcggaccgaggacaccgccctgtactactgcaccagaagggacggcgctact  
ggggcaagggcaccacagtgcagtgctctcc (SEQ ID NO: 43 in Sequence Listing), and

10 VL:

gacatccagatgaccagtcctccctcctcgtgtctgcttccgtgggcgacagagtgaccatcacctgtcgggcctccca  
ggacatctcctctacctgaactggtatcagcagaagcccggcaagggccccaagctgctgatctactacacctccggc  
tgactccggcgtgcctctagattttcggctctggctccggcaccgactttaccctgaccatctccagcctgcagccg  
aggacttcgcctctacttctgtcagcagctgaacacctgccttgacctttggcggaggcaccaaggtggaaatggaa

15 (SEQ ID NO: 44 in Sequence Listing).

7. A recombinant vector comprising the nucleic acid molecule according to claim 5 or 6.

20 8. A host cell containing the recombinant vector according to claim 7.

9. A method for producing the anti-RGMa antibody or an antigen-binding fragment thereof according to any one of claims 1 to 4, the method comprising a step of culturing the host cell according to claim 8.

25

10. A pharmaceutical composition comprising the anti-RGMa antibody or an antigen-binding fragment thereof according to any one of claims 1 to 4.

11. The pharmaceutical composition according to claim 10 for use in preventing, treating, or preventing the relapse of neurological or immunological diseases.
- 5 12. The pharmaceutical composition according to claim 11, wherein the neurological diseases are selected from the group consisting of amyotrophic lateral sclerosis, brachial plexus injury, brain damage (including traumatic brain injury), cerebral palsy, Guillain-Barre syndrome, cerebral leukodystrophy, multiple sclerosis (including relapsing-remitting multiple sclerosis, primary progressive multiple
- 10 sclerosis, secondary progressive multiple sclerosis), neuromyelitis optica, post-polio syndrome, spina bifida, spinal cord injury, spinal muscular atrophy, spinal neoplasm, transverse myelitis, dementia (including senile dementia, mild cognitive impairment, Alzheimer's disease, dementia associated with Alzheimer's disease), Huntington's disease, tardive dyskinesia, mania, Parkinson's disease, Steele-Richardson syndrome,
- 15 Down's syndrome, myasthenia gravis, neurotrauma (including optic nerve trauma), vascular amyloidosis, cerebral hemorrhage associated with amyloidosis, brain infarction, cerebritis, acute confusional state, glaucoma, schizophrenia and retinal nerve fiber layer degeneration (including diabetic retinopathy, ischemic optic neuropathy, X-linked retinoschisis, drug-induced optic neuropathy, retinal dystrophy,
- 20 age-related macular degeneration, eye diseases characterized by optic disc drusen, eye diseases characterized by genetic determinant for photoreceptor degeneration, autosomal recessive cone-rod dystrophy, mitochondrial disorder associated with optic neuropathy).
- 25 13. The pharmaceutical composition according to claim 11, wherein the immunological diseases are selected from the group consisting of multiple sclerosis (including relapsing-remitting multiple sclerosis, primary progressive multiple sclerosis, secondary progressive multiple sclerosis), neuromyelitis optica, psoriasis,

arthritis (including rheumatoid arthritis, osteoarthritis, psoriatic arthritis), Guillain-Barre syndrome, neuro-Behcet disease, pernicious anemia, type I (insulin-dependent) diabetes mellitus, systemic lupus erythematosus (SLE), inflammatory bowel disease (IBD), Sjogren's syndrome, Goodpasture's syndrome, Graves' disease, 5 autoimmune hemolytic anemia, autoimmune thrombocytopenic purpura, asthma, pollinosis, atopic dermatitis, glomerulonephritis, myasthenia gravis, Hashimoto's disease, and sarcoidosis.

14. The pharmaceutical composition according to claim 11, wherein the 10 neurological or immunological diseases are selected from the group consisting of spinal cord injury, neurotrauma (including optic nerve trauma), multiple sclerosis (including relapsing-remitting multiple sclerosis, primary progressive multiple sclerosis, secondary progressive multiple sclerosis).