Nogo A Binding Molecules And Pharmaceutical Use Thereof

This invention relates to NogoA binding molecules, such as for example monoclonal antibodies or Fab fragments thereof.

Neuronal regeneration following injury in the adult central nervous system (CNS) is limited due to the presence of the inhibitory myelin environment that ensheaths axons and formation of scar tissue. In the last few years important insights have been gained into the molecular understanding why the CNS is unable to spontaneously repair itself following injury. Inhibitory molecules in the myelin are the major impediment for the axonal regeneration, particularly immediately after the injury. So far NogoA, Myelin-Associated Glycoprotein (MAG) and myelin-oligodendrocyte glycoprotein (OMgp) have been characterised as potent inhibitors of neurite outgrowth. In addition, myelin also contains other inhibitory components, such as, chondroitin sulphate proteoglycans. Nogo-A is a member of the reticulon protein family and it has at least two biologically active and pharmacologically distinct domains termed Amino-Nogo and Nogo-66. While the receptor site for the former is not known so far, Nogo-66 inhibits neuronal growth in vitro and in vivo via the neuronal receptor NgR. In addition to Nogo-66, MAG and OMgp also bind to the NgR with high affinity and inhibit neurite outgrowth.

Potential new research approaches currently pursued for enhancement of nerve repair include digestion of scar tissue using an enzyme chondroitinase ABC, bridging techniques using Olfactory ensheathing cells and stem cells and protein growth factors to boost neuronal growth. Blocking actions of neurite outgrowth inhibitors by modulation of intracellular signalling mediators such as Rho , a membrane-bound guanosine trisphosphatase (GTPase), which appears to be a key link in the inhibition of axonal growth. Cyclic adenosine monophosphate (cAMP) which can overcome myelin associated inhibition in vitro and induce regeneration in vivo. Use of peptide inhibitor of the NgR receptor (NEP 1-40) to induce neuronal regrowth and functional recovery in rats following spinal injury.

In addition to the use of the approaches described above, attention has also focused upon the use of certain monoclonal antibodies to neutralize neurite growth inhibitory molecules of the central and peripheral nervous system, in particular to neutralize the neurite growth inhibitory activity of NogoA. Thus it has been shown that the monoclonal antibody IN-1 or the

IN-1 Fab fragment thereof induce neurite outgrowth in vitro and enhance sprouting and regeneration in vivo (Schwab ME et al. (1996) Physiol. Rev. 76, 319-370). Testing different domains of the NogoA for neurite growth inhibitory acitvity have delineated several inhibitory domains in the molecule (Chen et al. (2000) Nature 403, 434-439; GrandPre eta l. (2000) Nature 403, 439-444; Prinjha et al. (2000) Nature 403, 383-384; see also detailed analysis in Example 1).

Natural immunoglobulins or antibodies comprise a generally Y- shaped multimeric molecule having an antigen-binding site at the end of each upper arm. The remainder of the structure, in particular the stem of the Y mediates effector functions associated with the immunoglobulins. Antibodies consists of a 2 heavy and 2 light chains. Both heavy and light chains comprise a variable domain and a constant part. An antigen binding site consists of the variable domain of a heavy chain associated with the variable domain of a light chain. The variable domains of the heavy and light chains have the same general structure. More particularly, the antigen binding characteristics of an antibody are essentially determined by 3 specific regions in the variable domain of the heavy and light chains which are called hypervariable regions or complementarity determining regions (CDRs). These 3 hypervariable regions alternate with 4 framework regions (FRs) whose sequences are relatively conserved and which are not directly involved in binding. The CDRs form loops and are held in close proximity by the framework regions which largely adopt a β-sheet conformation. The CDRs of a heavy chain together with the CDRs of the associated light chain essentially constitute the antigen binding site of the antibody molecule. The determination as to what constitutes an FR or a CDR region is usually made by comparing the amino acid sequence of a number of antibodies raised in the same species. The general rules for identifying the CDR and FR regions are general knowledge of a man skilled in the art and can for example be found in the webside (http://www.bioinf.org.uk/abs/).

It has now surprisingly been found that a novel monoclonal mouse antibody (hereinafter called "11C7") raised against a polypeptide fragment of rat NogoA (SEQ ID NO: 1) and of the IgG1 type has better properties than the NogoA antibodies of the prior art especially with regard to the binding affinity to NogoA of different species including the homo sapiens and with regard to its higher NogoA neurite outgrowth neutralizing activity at a given antibody concentration. Moreover it is now possible to construct other NogoA binding molecules having the same hypervariable regions as the said antibody.

Accordingly, the invention provides binding molecules to a particular region or epitope of NogoA (hereinafter referred to as "the Binding Molecules of the invention" or simply "Binding Molecules"). Preferably the Binding Molecules of the invention bind to human NogoA_623-640 (orthologous fragment against which 11C7 was raised; = SEQ ID NO: 6), human Nig-D20 (orthologous to the smallest fragment of NogoA with neurite outgrowth inhibitory activity, SEQ ID NO: 24), human NogoA (SEQ ID NO: 5) or human NiG (which is the most potent neurite outgrowth inhibitory fragment of NogoA and starts at amino acid No. 186 and ends at amino acid No. 1004 of human NogoA, = SEQ ID NO: 5) with a dissociation constant (Kd) < 1000nM, more preferably with a Kd < 100 nM, most preferably with a Kd < 10 nM. The binding reaction may be shown by standard methods (qualitative assays) including, for example, the ELISA method described in Example 6 and the biosensor affinity method described in the example 7. In addition, the binding to human NogoA and almost more importantly the efficiency may be shown in a neurite outgrowth assay, e.g. as described below.

Thus, in a further preferred embodiment the Binding Molecules (at a concentration of 1 mg/ml, more preferably at 0.1 mg/ml even more preferably at 0.01 mg/ml culture medium) enhance the number of neurites of rat cerebellar granule cells on a substrate of rat spinal cord protein extract by at least 20%, preferably 50%, most preferred 100% compared to the number of neurites of rat cerebellar granule cells which are treated with a control antibody that does not bind to the human NogoA, human NiG, human Nig-D20 or NogoA_623-640 polypeptide (i.e. that has a dissociation constant > 1000 nM).

In a further preferred embodiment the Binding Molecules of the invention comprises at least one antigen binding site, said antigen binding site comprising in sequence, the hypervariable regions CDR1-11C7, CDR2-11C7 and CDR3-11C7; said CDR1-11C7 having the amino acid sequence SEQ ID NO: 8, said CDR2-11C7 having the amino acid sequence SEQ ID NO: 9, and said CDR3-11C7 having the amino acid sequence SEQ ID NO: 10; and direct equivalents thereof.

In a further aspect of the invention, the Binding Molecule of the invention comprises at least one antigen binding site, said antigen binding site comprising either

a) in sequence the hypervariable regions CDR1-11C7, CDR2-11C7 and CDR3-11C7; said CDR1-11C7 having the amino acid sequence of SEQ ID NO: 8, said CDR2-11C7 having the amino acid sequence of SEQ ID NO: 9, and said CDR3-11C7 having the amino acid sequence SEQ ID NO: 10; or
b) in sequence the hypervariable regions CDR1'-11C7, CDR2'-11C7 and CDR3'-11C7, said CDR1'-11C7 having the amino acid sequence of SEQ ID NO: 11, said CDR2'-11C7 having the amino acid sequence of SEQ ID NO: 12, and said CDR3'-11C7 having the amino acid sequence of SEQ ID NO: 13; or
c) direct equivalents thereof.

In a further aspect of the invention, the Binding Molecule of the invention comprises at least
a) a first domain comprising in sequence the hypervariable regions CDR1-11C7, CDR2-11C7 and CDR3-11C7; said CDR1-11C7 having the amino acid sequence of SEQ ID NO: 8, said CDR2-11C7 having the amino acid sequence of SEQ ID NO: 9, and said CDR3-11C7 having the amino acid sequence SEQ ID NO: 10; and
b) a second domain comprising in sequence the hypervariable regions CDR1'-11C7, CDR2'-11C7 and CDR3'-11C7, said CDR1'-11C7 having the amino acid sequence of SEQ ID NO: 11, said CDR2'-11C7 having the amino acid sequence of SEQ ID NO: 12, and said CDR3'-11C7 having the amino acid sequence of SEQ ID NO: 13; or
c) direct equivalents thereof.

Moreover, the invention also provides the following Binding Molecule of the invention, which comprises at least one antigen binding site comprising
a) either the variable part of the heavy chain of 11C7 (SEQ ID NO: 2); or
b) the variable part of the light chain of 11C7 (SEQ ID NO: 3), or direct equivalents thereof.

When the antigen binding site comprises both the first and second domains, these may be located on the same polypeptide molecule or, preferably, each domain may be on a different chain, the first domain being part of an immunoglobulin heavy chain or fragment thereof and the second domain being part of an immunoglobulin light chain or fragment thereof.

Examples of Binding Molecules of the invention include antibodies as produced by B-cells or hybridomas and chimeric or humanized antibodies or any fragment thereof, e.g. $F(ab')_2$; and Fab fragments, as well as single chain or single domain antibodies.

A single chain antibody consists of the variable domains of an antibody heavy and light chains covalently bound by a peptide linker usually consisting of from 10 to 30 amino acids, preferably from 15 to 25 amino acids. Therefore, such a structure does not include the constant part of the heavy and light chains and it is believed that the small peptide spacer should be less antigenic than a whole constant part. By "chimeric antibody" is meant an antibody in which the constant regions of heavy or light chains or both are of human origin while the variable domains of both heavy and light chains are of non- human (e.g. murine) origin. By "humanized antibody" is meant an antibody in which the hypervariable regions (CDRs) are of non-human (e.g. murine) origin, while all or substantially all the other parts of the immunoglobulin e.g. the constant regions and the highly conserved parts of the variable domains, i.e. the framework regions, are of human origin. A humanized antibody may however retain a few amino acids of the murine sequence in the parts of the framework regions adjacent to the hypervariable regions.

Hypervariable regions may be associated with any kind of framework regions, preferably of murine or human origin. Suitable framework regions are described in "Sequences of proteins of immunological interest", Kabat E.A. et al, US department of health and human services, Public health service, National Institute of Health. Preferably the constant part of a human heavy chain of the Binding Molecules may be of the IgG4 type, including subtypes, preferably the constant part of a human light chain may be of the κ or λ type, more preferably of the κ type.

Monoclonal antibodies raised against a protein naturally found in all humans may be developed in a non-human system e. g. in mice. As a direct consequence of this, a xenogenic antibody as produced by a hybridoma, when administered to humans, elicits an undesirable immune response, which is predominantly mediated by the constant part of the xenogenic immunoglobulin. This clearly limits the use of such antibodies as they cannot be administered over a prolonged period of time. Therefore it is particularly preferred to use single chain, single domain, chimeric or humanized antibodies which are not likely to elicit a substantial allogenic response when administered to humans.

In view of the foregoing, a more preferred Binding Molecule of the invention is selected from a chimeric antibody, which comprises at least

a) one immunoglobulin heavy chain or fragment thereof which comprises (i) a variable domain comprising in sequence the hypervariable regions CDR1-11C7, CDR2-11C7 and CDR3-11C7 and (ii) the constant part or fragment thereof of a human heavy chain; said CDR1-11C7 having the amino acid sequence (SEQ ID NO: 8), said CDR2-11C7 having the amino acid sequence (SEQ ID NO: 9), and said CDR3-11C7 having the amino acid sequence (SEQ ID NO: 10), and

b) one immunoglobulin light chain or fragment thereof which comprises (i) a variable domain comprising in sequence the hypervariable regions CDR1'-11C7, CDR2'-11C7 and CDR3'-11C7 and (ii) the constant part or fragment thereof of a human light chain; said CDR1'-11C7 having the amino acid sequence (SEQ ID NO: 11), said CDR2'-11C7 having the amino acid sequence (SEQ ID NO: 12), and said CDR3'-11C7 having the amino acid sequence (SEQ ID NO: 13); or

direct equivalents thereof.

Alternatively, a Binding Molecule of the invention may be selected from a single chain binding molecule which comprises an antigen binding site comprising

a) a first domain comprising in sequence the hypervariable CDR1-11C7, CDR2-11C7 and CDR3-11C7; said CDR1-11C7 having the amino acid sequence (SEQ ID NO: 8), said CDR2-11C7 having the amino acid sequence (SEQ ID NO: 9), and said CDR3-11C7 having the amino acid sequence (SEQ ID NO: 10); and

b) a second domain comprising in sequence the hypervariable CDR1'-11C7, CDR2'-11C7 and CDR3'-11C7; said CDR1'-11C7 having the amino acid sequence (SEQ ID NO: 11), said CDR2'-11C7 having the amino acid sequence (SEQ ID NO: 12), and said CDR3'-11C7 having the amino acid sequence (SEQ ID NO: 13); and

c) a peptide linker which is bound either to the N- terminal extremity of the first domain and to the C-terminal extremity of the second domain or to the C-terminal extremity of the first domain and to the N-terminal extremity of second domain;

or direct equivalents thereof.

As it is well known, minor changes in an amino acid sequence such as deletion, addition or substitution of one or several amino acids may lead to an allelic form of the original protein which has substantially identical properties. Thus, by the term "direct equivalents thereof" is meant either any single domain Binding Molecule of the invention (molecule X)

(i) in which each of the hypervariable regions CDR1, CDR2, and CDR3 of the Binding Molecule is at least 50 or 80% homologous, preferably at least 90% homologous, more preferably at least 95, 96, 97, 98, 99% homologous to the equivalent hypervariable regions of CDR1-11C7 (SEQ ID NO: 8), CDR2-11C7 (SEQ ID NO: 9) and CDR3-11C7 (SEQ ID NO: 10), whereas CDR1 is equivalent to CDR1-11C7, CDR2 is equivalent to CDR2-11C7, CDR3 is equivalent to CDR3-11C7; and

(ii) which is capable of binding to the human NogoA, human NiG, human NiG-D20, or human NogoA_623-640, preferably with a dissociation constant (Kd) < 1000nM, more preferably with a Kd < 100 nM, most preferably with a Kd < 10 nM, or

any binding molecule of the invention having at least two domains per binding site (molecule X')

(iii) in which each of the hypervariable regions CDR1, CDR2, CDR3, CDR1', CDR2' and CDR3' is at least 50 or 80% homologous, preferably at least 90% homologous, more preferably at least 95, 96, 97, 98, 99% identical to the equivalent hypervariable regions of CDR1-11C7 (SEQ ID NO: 8), CDR2-11C7 (SEQ ID NO: 9), CDR3-11C7 (SEQ ID NO: 10), CDR1'-11C7 (SEQ ID NO: 11), CDR2'-11C7 (SEQ ID NO: 12), and CDR3'-11C7 (SEQ ID NO: 13), whereas CDR1 is equivalent to CDR1-11C7, CDR2 is equivalent to CDR2-11C7, CDR3 is equivalent to CDR3-11C7, CDR1' is equivalent to CDR1'-11C7, CDR2' is equivalent to CDR2'-11C7, CDR3' is equivalent to CDR3'-11C7; and

(iv) which is capable of binding the human NogoA, human NiG, human NiG-D20, or human NogoA_623-640, preferably with a dissociation constant (Kd) < 1000nM, more preferably with a Kd < 100 nM, most preferably with a Kd < 10 nM.

Thus further embodiments of the inventions are for example a Binding Molecule which is capable of binding to the human NogoA, human NiG, human NiG-D20, or human NogoA_623-640 with a dissociation constant < 1000nM and comprises at least one antigen binding site, said antigen binding site comprising either

- in sequence the hypervariable regions CDR1, CDR2, and CDR3, of which each of the hypervariable regions are at least 50%, preferably 80, 90, 95, 96, 97, 98, 99% homologous to their equivalent hypervariable regions CDR1-11C7 (SEQ ID NO: 8), CDR2-11C7 (SEQ ID NO: 9) and CDR3-11C7 (SEQ ID NO: 10); or
- in sequence the hypervariable regions CDR1', CDR2', and CDR3', of which each of the hypervariable regions are at least 50%, preferably 80, 90, 95, 96, 97, 98, 99%

homologous to their equivalent hypervariable regions CDR1'-11C7 (SEQ ID NO: 11), CDR2'-11C7 (SEQ ID NO: 12) and CDR3'-11C7 (SEQ ID NO: 13).

Furthermore, a Binding Molecule which is capable of binding the human NogoA, human NiG, human NiG-D20, or human NogoA_623-640 with a dissociation constant < 1000nM and comprises

- a first antigen binding site comprising in sequence the hypervariable regions CDR1, CDR2, and CDR3, of which each of the hypervariable regions are at least 50%, preferably 80, 90, 95, 96, 97, 98, 99% homologous to their equivalent hypervariable regions CDR1-11C7 (SEQ ID NO: 8), CDR2-11C7 (SEQ ID NO: 9) and CDR3-11C7 (SEQ ID NO: 10); and
- a second antigen binding site comprising in sequence the hypervariable regions CDR1', CDR2', and CDR3', of which each of the hypervariable regions are at least 50%, preferably 80, 90, 95, 96, 97, 98, 99% homologous to their equivalent hypervariable regions CDR1'-11C7 (SEQ ID NO: 11), CDR2'-11C7 (SEQ ID NO: 12) and CDR3'-11C7 (SEQ ID NO: 13).

This dissociation constant may be conveniently tested in various assays including, for example, the biosensor affinity method described in the example 7. In addition, the binding and functional effect of the Binding Molecules may be shown in a bioassay, e.g. as described below.

The constant part of a human heavy chain may be of the γ1; γ2; γ3; γ4; α1; α2; δ or ε type, preferably of the γ type, more preferably of the γ4; type, whereas the constant part of a human light chain may be of the κ or λ type (which includes the λ1; λ2; and λ3 subtypes) but is preferably of the κ type. The amino acid sequence of all these constant parts are given in Kabat et al (Supra).

Conjugates of the binding molecules of the invention, e. g. enzyme or toxin or radioisotope conjugates, are also included within the scope of the invention.

"Polypeptide", if not otherwise specified herein, includes any peptide or protein comprising amino acids joined to each other by peptide bonds, having an amino acid sequence starting at the N-terminal extremity and ending at the C-terminal extremity. Preferably the

polypeptide of the present invention is a monoclonal antibody, more preferred is a chimeric (also called V-grafted) or humanised (also called CDR-grafted) monoclonal antibody. The humanised (CDR-grafted) monoclonal antibody may or may not include further mutations introduced into the framework (FR) sequences of the acceptor antibody.

A functional derivative of a polypeptide as used herein includes a molecule having a qualitative biological activity in common with a polypeptide to the present invention, i.e. having the ability to bind to the human NogoA, human NiG, human NiG-D20, or human NogoA_623-640. A functional derivative includes fragments and peptide analogs of a polpypeptide according to the present invention. Fragments comprise regions within the sequence of a polypeptide according to the present invention, e.g. of a specified sequence. The term "derivative" is used to define amino acid sequence variants, and covalent modifications of a polypeptide according to the present invention. e.g. of a specified sequence. The functional derivatives of a polypeptide according to the present invention, e.g. of a specified sequence, e.g. of the hypervariable region of the light and the heavy chain, preferably have at least about 65%, more preferably at least about 75%, even more preferably at least about 85%, most preferably at least about 95, 96, 97, 98, 99% overall sequence homology with the amino acid sequence of a polypeptide according to the present invention, e.g. of a specified sequence, and substantially retain the ability to bind the human NogoA, human NiG, human NiG-D20, or human NogoA_623-640.

The term "covalent modification" includes modifications of a polypeptide according to the present invention, e.g. of a specified sequence; or a fragment thereof with an organic proteinaceous or non-proteinaceous derivatizing agent, fusions to heterologous polypeptide sequences, and post-translational modifications. Covalent modified polypeptides, e.g. of a specified sequence, still have the ability bind to the human NogoA, human NiG, human NiG-D20, or human NogoA_623-640 by crosslinking. Covalent modifications are traditionally introduced by reacting targeted amino acid residues with an organic derivatizing agent that is capable of reacting with selected sides or terminal residues, or by harnessing mechanisms of post-translational modifications that function in selected recombinant host cells. Certain post-translational modifications are the result of the action of recombinant host cells on the expressed polypeptide. Glutaminyl and asparaginyl residues are frequently post-translationally deamidated to the corresponding glutamyl and aspartyl residues. Alternatively, these residues are deaminated under mildly acidic conditions. Other post-translational

modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl, tyrosine or threonyl residues, methylation of the α-amino groups of lysine, arginine, and histidine side chains, see e.g. T. E. Creighton, Proteins: Structure and Molecular Properties, W. H. Freeman & Co., San Francisco, pp. 79-86 (1983). Covalent modifications e.g. include fusion proteins comprising a polypeptide according to the present invention, e.g. of a specified sequence and their amino acid sequence variants, such as immunoadhesins, and N-terminal fusions to heterologous signal sequences.

"Homology" with respect to a native polypeptide and its functional derivative is defined herein as the percentage of amino acid residues in the candidate sequence that are identical with the residues of a corresponding native polypeptide, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent homology, and not considering any conservative substitutions as part of the sequence identity. Neither N- or C-terminal extensions nor insertions shall be construed as reducing identity or homology. Methods and computer programs for the alignment are well known.

"Amino acid(s)" refer to all naturally occurring L-α-amino acids, e.g. and including D-amino acids. The amino acids are identified by either the well known single-letter or three-letter designations.

The term "amino acid sequence variant" refers to molecules with some differences in their amino acid sequences as compared to a polypeptide according to the present invention, e.g. of a specified sequence. Amino acid sequence variants of a polypeptide according to the present invention, e.g. of a specified sequence, still have the ability to bind to human NogoA or human NiG or more preferably to NogoA_623-640. Substitutional variants are those that have at least one amino acid residue removed and a different amino acid inserted in its place at the same position in a polypeptide according to the present invention, e.g. of a specified sequence. These substitutions may be single, where only one amino acid in the molecule has been substituted, or they may be multiple, where two or more amino acids have been substituted in the same molecule. Insertional variants are those with one or more amino acids inserted immediately adjacent to an amino acid at a particular position in a polypeptide according to the present invention, e.g. of a specified sequence. Immediately adjacent to an amino acid means connected to either the α-carboxy or α-amino functional group of the amino acid. Deletional variants are those with one or more amino acids in a polypeptide

according to the present invention, e.g. of a specified sequence, removed. Ordinarily, deletional variants will have one or two amino acids deleted in a particular region of the molecule.

A binding molecule of the invention may be produced by recombinant DNA techniques. In view of this, one or more DNA molecules encoding the binding molecule must be constructed, placed under appropriate control sequences and transferred into a suitable host organism for expression.

In a very general manner, there are accordingly provided

(i) DNA molecules encoding a single domain Binding Molecule of the invention, a single chain Binding Molecule of the invention, a heavy or light chain or fragments thereof of a Binding Molecule of the invention; and

(ii) the use of the DNA molecules of the invention for the production of a Binding Molecule of the invention by recombinant means.

The present state of the art is such that the skilled man will be able to synthesize the DNA molecules of the invention given the information provided herein i.e. the amino acid sequences of the hypervariable regions and the DNA sequences coding for them. A method for constructing a variable domain gene is for example described in EP 239 400 and may be briefly summarized as follows: A gene encoding a variable domain of a monoclonal antibody of whatever specificity is cloned. The DNA segments encoding the framework and hypervariable regions are determined and the DNA segments encoding the hypervariable regions are removed so that the DNA segments encoding the framework regions are fused together with suitable restriction sites at the junctions. The restriction sites may be generated at the appropriate positions by mutagenesis of the DNA molecule by standard procedures. Double stranded synthetic CDR cassettes are prepared by DNA synthesis according to the sequences given CDR1-11C7, CDR2-11C7, CDR3-11C7, CDR1'-11C7, CDR2'-11C7 and CDR3'-11C7 above. These cassettes are provided with sticky ends so that they can be ligated at the junctions to the framework by standard protocol for achieving a DNA molecule encoding an immunoglobulin variable domain.

Furthermore, it is not necessary to have access to the mRNA from a producing hybridoma cell line in order to obtain a DNA construct coding for the monoclonal antibodies of the

invention. Thus PCT application W0 90/07861 gives full instructions for the production of a monoclonal antibody by recombinant DNA techniques given only written information as to the nucleotide sequence of the gene.

The method comprises the synthesis of a number of oligonucleotides, their amplification by the PCR method, and their splicing to give the desired DNA sequence.

Expression vectors comprising a suitable promoter or genes encoding heavy and light chain constant parts are publicly available. Thus, once a DNA molecule of the invention is prepared it may be conveniently transferred in an appropriate expression vector.

DNA molecules encoding single chain antibodies may also be prepared by standard methods, for example, as described in W0 88/1649.

In a particular embodiment of the invention, the recombinant means for the production of some of the Binding Molecules of the invention includes first and second DNA constructs as described below:

The first DNA construct encodes a heavy chain or fragment thereof and comprises

a) a first part which encodes a variable domain comprising alternatively framework and hypervariable regions, said hypervariable regions comprising in sequence DNA-CDR1-11C7 (SEQ ID NO: 15), DNA-CDR2-11C7 (SEQ ID NO: 16) and DNA-CDR3-11C7 (SEQ ID NO: 17); this first part starting with a codon encoding the first amino acid of the variable domain and ending with a codon encoding the last amino acid of the variable domain, and
b) a second part encoding a heavy chain constant part or fragment thereof which starts with a codon encoding the first amino acid of the constant part of the heavy chain and ends with a codon encoding the last amino acid of the constant part or fragment thereof, followed by a non-sense codon.

Preferably, the second part encodes the constant part of a human heavy chain, more preferably the constant part of the human γ4 chain. This second part may be a DNA fragment of genomic origin (comprising introns) or a cDNA fragment (without introns).

The second DNA construct encodes a light chain or fragment thereof and comprises

a) a first part which encodes a variable domain comprising alternatively framework and hypervariable regions; said hypervariable regions comprising in sequence DNA-CDR1'-11C7 (SEQ ID NO: 17), DNA-CDR2'-11C7 (SEQ ID NO: 18) and DNA-CDR3'-11C7 (SEQ ID NO: 19), this first part starting with a codon encoding the first amino acid of the variable domain and ending with a codon encoding the last amino acid of the variable domain, and

b) a second part encoding a light chain constant part or fragment thereof which starts with a codon encoding the first amino acid of the constant part of the light chain and ends with a codon encoding the last amino acid of the constant part or fragment thereof followed by a non-sense codon.

Preferably, the second part encodes the constant part of a human light chain, more preferably the constant part of the human κ chain.

The first or second DNA construct advantageously comprises a third part which is located upstream of the first part and which encodes part of a leader peptide; this third part starting with the codon encoding the first amino acid and ending with the last amino acid of the leader peptide. This peptide is required for secretion of the chains by the host organism in which they are expressed and is subsequently removed by the host organism. Preferably, the third part of the first DNA construct encodes a leader peptide having an amino acid sequence substantially identical to the amino acid sequence of the heavy chain leader sequence as shown in SEQ ID NO: 21 (starting with the amino acid at position -19 and ending with the amino acid at position –1). Also preferably, the third part of the second DNA construct encodes a leader peptide having an amino acid sequence as shown in SEQ ID NO: 23 (light chain, starting with the amino acid at position -18 and ending with the amino acid at position –1).

Each of the DNA constructs are placed under the control of suitable control sequences, in particular under the control of a suitable promoter. Any kind of promoter may be used, provided that it is adapted to the host organism in which the DNA constructs will be transferred for expression. However, if expression is to take place in a mammalian cell, it is particularly preferred to use the promoter of an immunoglobulin gene.

The desired antibody may be produced in a cell culture or in a transgenic animal. A suitable transgenic animal may be obtained according to standard methods which include micro injecting into eggs the first and second DNA constructs placed under suitable control sequences transferring the so prepared eggs into appropriate pseudo- pregnant females and selecting a descendant expressing the desired antibody.

When the antibody chains have to be produced in a cell culture, the DNA constructs must first be inserted into either a single expression vector or into two separate but compatible expression vectors, the latter possibility being preferred.

Accordingly, the invention also provides an expression vector able to replicate in a prokaryotic or eukaryotic cell line which comprises at least one of the DNA constructs above described.

Each expression vector containing a DNA construct is then transferred into a suitable host organism. When the DNA constructs are separately inserted on two expression vectors, they may be transferred separately, i.e. one type of vector per cell, or co- transferred, this latter possibility being preferred. A suitable host organism may be a bacterium, a yeast or a mammalian cell line, this latter being preferred. More preferably, the mammalian cell line is of lymphoid origin e.g. a myeloma, hybridoma or a normal immortalized B-cell, but does not express any endogeneous antibody heavy or light chain.

It is also preferred that the host organism contains a large number of copies of the vectors per cell. If the host organism is a mammalian cell line, this desirable goal may be reached by amplifying the number of copies according to standard methods. Amplification methods usually consist of selecting for increased resistance to a drug, said resistance being encoded by the expression vector.

In another aspect of the invention, there is provided a process for producing a multi-chain binding molecule of the invention, which comprises (i) culturing an organism which is transformed with the first and second DNA constructs of the invention and (ii) recovering an active binding molecule of the invention from the culture.

Alternatively, the heavy and light chains may be separately recovered and reconstituted into an active binding molecule after in vitro refolding. Reconstitution methods are well-known in the art; Examples of methods are in particular provided in EP 120 674 or in EP 125 023. Therefore a process may also comprise

(i) culturing a first organism which is transformed with a first DNA construct of the invention and recovering said heavy chain or fragment thereof from the culture and

(ii) culturing a second organism which is transformed with a second DNA construct of the invention and recovering said light chain or fragment thereof from the culture and

(iii) reconstituting in vitro an active binding molecule of the invention from the heavy chain or fragment thereof obtained in (i) and the light chain or fragment thereof obtained in (ii).

In a similar manner, there is also provided a process for producing a single chain or single domain binding molecule of the invention which comprises

(i) culturing an organism which is transformed with a DNA construct respectively encoding a single chain or single domain binding molecule of the invention and

(ii) recovering said molecule from the culture.

The binding molecules of the invention exhibit very good nerve repair activity as shown, for example, in the granule cell neurite outgrowth model.

1. Granule cell neurite outgrowth assay (*in vitro)*

Neurite outgrowth from dissociated cerebellar granule cells are determined as described (Niederöst et al. (1999) J.Neurosci. 19: 8979-8989). Briefly, cerebella are removed from decapitated postnatal day 5 – 7 rats and dissociated by trypsin treatment. To reduce fibroblast contamination, the cells are preplated onto bacterial dishes. 75'000 cells are then cultured per well in 4–well Greiner tissue culture (Huber & Co AG,Rheinach, Basel) dishes (well surface: 1 cm2) in medium (Neurobasal with B27 serum replacement, Invitrogen). Culture dishes are coated with poly-L-lysine (Sigma). Chaps extracted proteins from total spinal cord homogenates of adult rats (Spillmann et al. (1998) J. Biol. Chem. 273: 19283-19293) is coated at protein concentrations of 0.5 till 8 µg per well over night at 4°C and washed. The binding molecules of the invention are then pre-incubated for 30 min on the test substrate and removed before the cells are added. Cerebellar granule cells are added and incubated for 24 hours. To stop the experiment, 2 ml of 4 % buffered formaldehyde is

slowly added to the culture dishes. Cultures are then stained by immunofluorescence for the growth-associated protein GAP-43 and with Hoechst for cell nuclei (Granule cells are stained with Hoechst in order to see if all the cells have neurites (neurite visualised with anti-GAP-43)). Three pictures are taken randomly at a defined distance of the upper, lower and lateral edge of each well with a 40x objectif on a Zeiss Axiophot Fluorescence Microscope. All the neurites in a field are counted on number-coded, randomly arranged photographs. The response (outgrowth of the granule cell neurites) is dose-dependent in the range of about 0.1 – 10 µg total protein per well (the specific activities of a given preparation vary within this range).

Enhancement of neurite outgrowth of cerebellar granule cell in the non-permissive environment of the above prepared spinal cord extract by preincubation with a binding molecule of the invention may be observed. E.g. a typical profile for the neutralizing effect of the mouse 11C7-IgG1 antibody in the granule cell neurite outgrowth model is given below:

Assay 1:

rat myelin coated at 1µg per well	Neurites per field	Percentage
no antibody	80,5	100 %
+mouse IgG	86,5	108 %
11C7 250 µg/ml	160	199 %

Assay 2:

rat myelin (prep. 2) coated at 8 ug per well	Neurites per field	Percentage
no antibody	20	100 %
+mouse IgG	17,3	86,5 %
11C7 250 µg/ml	31	155 %
11C7 75µg/ml	26	130 %
11C7 7,5 µg/ml	26	130 %

The neutralizing activity of the molecules of the invention may also be estimated by measuring the regenerative sprouting and neurite outgrowth in the *in vivo* spinal cord injury model as follows:

2. Spinal cord injury model (*in vivo*)

Adult Lewis rats are injured microsurgically by transecting the dorsal half of the spinal cord bilaterally at the level of the 8th thoracic vertebra. Laminectomy, anesthesia and surgery are

described in Schnell and Schwab 1993 (Eur.J. Neurosci. 5: 1156 – 1171). Controls or binding molecules of the invention are applied in two different ways: either by implanting 10^6 freshly harvested hybridoma cells into one side of the cerebral cortex (grafted animals) or, alternatively, by an implanted intraventricular canula linked to a subcutaneously implanted 2ml Alzet (Alza Corporation, Palo Alto) pump (pump animals). – *Hybridoma grafted animals*: Rats are immunosuppressed for 7 – 10 days with cyclosporin A and sacrificed by transcardial perfusion with 4% buffered formalin 14 days after injury. – *Pump animals*: Binding molecules of the invention (e.g. at 3.3 mg/ml for mouse 11C7) are filled into 2 ml pumps delivering 0.5 µl/h into the lateral ventricle for 2 weeks. Pumps are implanted at the time of the spinal cord lesion, and rats are sacrificed 2 weeks later.

Neuroanatomical tracing: The motor and sensory corticospinal tract is traced by injecting the anterograde tracer biotin dextran amine (BDA) into the cortex of the side opposite to the pump or the graft. BDA is transported to the spinal cord within 10 – 14 days and visualized using diaminobenzidine (DAB) as a substrate as described in Brösamle et al., (2000 J.Neurosci. 20: 8061-8068).

Evalutation of anatomical results: Two methods of evaluation are used: a semi-quantitative and a quantitative one. *Semi-quantitative estimation of intensity of sprouting and regeneration:* Complete sagittal section series of number-coded, randomly mixed animals are evaluated for the presence and density of regenerating sprouts rostral to the lesion using the following definitions: regenerative sprouts are fibers emanating from the transected CST; they are long, irregular in their course, much less branched than the normal grey matter collaterals, and they growth towards and ventrally or laterally around the lesion. Regenerative sprouts often end in a growth cone which can be small and bulbouse or large and branched. Density of sprouting is rated on a scale of 0 – 3 for each animal. – *Long distance regeneration:* fibers that can be followed through the lesion into the caudal spinal cord are considered long-distance regenerating fibers. Their maximal distance from the lesion site can be measured, but is often a minimal distance as some unlesioned fibers from the small ventral funiculus CST are often present; their branches mix with those of regenerating axons and make distinction difficult.

Fiber counts (quantitative assay): A line positioned at -0.5 mm rostral to the end of the transected CST is posed on alternating sections of the grey matter, and all intersections with

CST fibers (normal collaterals or sprouts) are counted. Similar lines are positioned caudal to the lesion at a distance of +0.5, +2 and +5 mm from the lesion center. Intersecting fibers are counted and the 3 levels are added to a sum reflecting CST fibers in the caudal spinal cord. These caudal fibers are divided by the number of fibers -0.5 mm rostral to the CST end to obtain a ratio.

Two weeks after a spinal cord injury destroying about 40 % of the spinal cord segment T8, mainly in the dorsal half, including both main CSTs: tracing of the CST in control animals show a moderate degree of reactive sprouting of the tract. This phenomenon corresponds to the spontaneous sprouting in response to injury well known in the literature. Injured rats being treated with the binding molecules of the invention or with pumps delivering the binding molecules of the invention may show an enhanced sprouting at the lesion site and regeneration of damaged axons neurite outgrowth of damaged neurites.

Therefore the invention also provides

(i) the use of the binding molecules of the invention in the nerve repair of a mammalian nervous system, in particular human nervous system,

(ii) a method of repairing nerves of a mammalian nervous system, in particular human nervous system which comprises administering an effective amount of the binding molecules of the invention to a patient in need of such treatment, or

(iii) a pharmaceutical composition for nerve repair of a mammalian nervous system, in particular human nervous system which comprises the binding molecules of the invention and a pharmaceutically acceptable carrier or diluent.

In particular, the binding molecules of the invention are useful for axonal regeneration and improved sprouting after nerve fiber damage. Thus the molecules of the invention have a wide utility in particular for human subjects. For example the binding molecule of the invention are useful in the treatment of various diseases of the peripheral (PNS) and central (CNS) nervous system, i.e. more particularly in neurodegenerative diseases such as Alzheimer disease, Parkinson disease, Amyotrophic lateral sclerosis (ALS), Lewy like pathologies or other dementia in general, diseases following cranial, cerebral or spinal trauma, stroke or a demyeliating disease. Such demyelinating diseases include, but are not

limited to, multiple sclerosis, monophasic demyelination, encephalomyelitis, multifocal leukoencephalopathy, panencephalitis, Marchiafava-Bignami disease, pontine myelmolysis, adrenoleukodystrophy, Pelizaeus-Merzbacher disease, Spongy degeneration, Alexander's disease, Canavan's disease, metachromatic leukodystrophy and Krabbe's disease. In one example, administration of the binding molecules of the invention can be used to treat a demyelinating disease associated with NogoA protein. In another example, cells which express the binding molecules of the invention may be transplanted to a site spinal cord injury to facilitate axonal growth throughout the injured site. Such transplanted cells would provide a means for restoring spinal cord function following injury or trauma. Such cells could include olfactory ensheathing cells and stem cells of different lineages of fetal nerve or tissue grafts.

In addition, the Binding Molecules of the invention are useful for the treatment of degenerative ocular disorders which may directly or indirectly involve the degeneration of retinal or corneal cells including ischemic retinopathies in general, anterior ischemic optic neuropathy, all forms of optic neuritis, age-related macular degeneration, diabetic retinopathy, cystoid macular edema (CME), retinitis pigmentosa, Stargardt's disease, Best's vitelliform retinal degeneration, Leber's congenital amaurosis and other hereditary retinal degenerations, pathologic myopia, retinopathy of prematurity,and Leber's hereditary optic neuropathy, the after effects of corneal transplantation or of refractive corneal surgery, and herpes keratitis.

Furthermore, it was shown that NogoA plays a role in psychiatric conditions, in particular schizophrenia and depression. Hence, the binding molecules of the invention are useful for the treatment of psychiatric conditions, in particular schizophrenia and depression.

The Binding Molecules of the invention can be provided alone, or in combination, or in sequential combination with other agents. For example, the binding molecules of the invention can be administered in combination with anti-inflammatorv agents such as but not limited to corticosteroids following stroke or spinal cord injury as a means for blocking further neuronal damage and inhibition of axonal regeneration, Neurotrophic factors such as NGF, BDNF or other drugs for neurodegenerative diseases such as Exelon™ or Levodopa. As used herein, two agents are said to be administered in combination when the two agents are

administered simultaneously or are administered independently in a fashion such that the agents will act at the same time.

For the treatment of psychiatric conditions, in particular schizophrenia or depression, the Binding Molecules of the invention can be provided alone or in combination in particular with other agents selected from the group consisting of (a) anti-epileptic drugs selected from barbiturates and derivatives thereof, benzodiazepines, carboxamides, hydantoins, succinimides, valproic acid and other fatty acid derivates and other anti-epileptic drugs, (b) conventional antipsychotics, (c) atypical antipsychotics and (d) antidepressants.

The term "barbiturates and derivatives thereof" as used herein includes, but is not limited to Phenobarbital and primidon. The term "benzodiazepines" as used herein includes, but is not limited to clonazepam, diazepam and lorazepam. The term "carboxamides" as used herein includes, but is not limited to carbamazepine, oxcarbazepine and 10-hydroxy-10,11-dihydrocarbamazepine. The term "hydantoins" as used herein includes, but is not limited to phenytoin. The term "succinimides" as used herein includes, but is not limited to ethosuximide and mesuximide. The term "valproic acid and other fatty acid derivates" as used herein includes, but is not limited to valproic acid sodium salt, tiagabine hydrochloride monohydrate and vigrabatrine. The term "other anti-epileptic drugs" as used herein includes, but is not limited to levetiracetam, lamotrigine, gabapentin and felbamate.

The term "conventional antipsychotics" as used herein includes, but is not limited to haloperidol and fluphenazine.

The term "atypical antipsychotics" as used herein relates to clozaril, risperidone, olanzapine, quetiapine, ziprasidone and aripiprazol.

The term "antidepressants" as used herein includes, but is not limited to selective serotonin reuptake inhibitors (SSRI's), or selective serotonin and norepinephrine reuptake inhibitors (SNRI-s). An SSRI's suitable for the present invention can be selected from fluoxetine, fuvoxamine, sertraline, paroxetine, citalopram and escitalopram.

The structure of the active ingredients identified by code nos., generic or trade names may be taken from the actual edition of the standard compendium "The Merck Index" or from

databases, e.g. Patents International (e.g. IMS World Publications). The corresponding content thereof is hereby incorporated by reference. Any person skilled in the art is fully enabled to identify the active ingredients and, based on these references, likewise enabled to manufacture and test the pharmaceutical indications and properties in standard test models, both *in vitro* and *in vivo*.

For the indications mentioned above, the appropriate dosage will, of course, vary depending upon, for example, the particular molecule of the invention to be employed, the mode of administration and the nature and severity of the condition being treated. The Binding Molecules of the invention are conveniently administered by pumps or injected as therapeutics at the lesioned site, e.g. they can be administered directly into the CNS intracranially or into the spine intrathecally to the lesioned site.

Pharmaceutical compositions of the invention may be manufactured in conventional manner. E.g. a composition according to the invention comprising the molecules of the invention is preferably provided in lyophilized form. For immediate administration it is dissolved in a suitable aqueous carrier, for example sterile water for injection or sterile buffered physiological saline.

To aid in making up suitable compositions, the binding molecules of the invention and optionally a second drug enhancing the effect of the Binding Molecules of the invention, may be packaged separately within the same container, with instructions for mixing or concomitant administration. Optional second drug candidates are provided above.

The synergistic effect of a combination of the binding molecules of the invention and growth factors such as NGF may be demonstrated in vivo by the spinal cord injury model described above.

Brief description of the drawing

Figure 1: Sequence Comparison: Sequence comparison of the NiG from different species, showing the immunogenic peptide sequence for the 11C7 mAb.

The invention will be more fully understood by reference to the following examples. They should not, however, be construed as limiting the scope of the invention.

In the following examples all temperatures are in degree Celsius (°C).

The monoclonal antibody of attention in the Examples is a Binding Molecule according to the present invention comprising the variable part of the light chain (SEQ ID NO: 3) and the variable part of the heavy chain (SEQ ID NO: 2).

The following abbreviations are used:

ELISA	enzyme linked immuno-sorbant assay
FACS	fluorescence activated cell sorting
FITC	fluorescein isothiocyanate
FBS	foetal bovine serum
HCMV	human cytomegalovirus promoter
IgG	immunoglobulin isotype G
MAb	monoclonal antibody
PBS	phosphate-buffered saline
PCR	polymerase chain reaction

Example 1: NiG-D20 (SEQ ID NO: 24) is one of the neurite outgrowth inhibitory fragments of NogoA

Methods:

a) Rat Nogo-A deletion library: Deletion constructs are made using internal restriction sites, by ExonucleaseIII/Mung Bean Nuclease treatment and by PCR with rat Nogo-A-specific primers on rat Nogo- (method as in WO00/31235): rat Nogo-A (aa 1-1163; DNA as shown hereafter related to the amino acids of rat NogoA (SEQ ID NO: 26), e.g. aa 1-1163 means that the cDNA construct encodes for polypeptide which starts at the amino acid1 and ends at amino acid 1163 of the rat polypeptide sequence of NogoA), rat Nogo-B (aa 1-172 + 976-1163), rat Nogo-C (Nogo-C N-terminal 11 aa + aa 976-1163), rat Nogo-66 (aa 1019-1083), rat GST-Nogo-66 (aa 1026-1091), rat NiR-G (aa 1-979), rat NiR (1-172), rat NiR-D1 (aa 1-31), rat NiR-D2 (aa 59-172), rat NiR-D3 (aa 1-31 + 59-172), rat EST-Nogo1 (aa 762-1163), rat NiG (aa 174-979), rat NiG-D1 (aa174-909), rat NiG-D2 (aa 174-865), rat NiG-D3 (aa 172-723), rat NiG-D4 (aa 172-646), rat NiG-D5 (aa 293-647), rat NiG-D6 (aa 763-975), rat NiG-D7 (aa 174-235 + 294-979), rat NiG-D8 (aa 218-653), rat NiG-D9 (aa 172-259 + 646-974), rat NiG-D10 (aa 293-979), rat NiG-D11 (aa 209-268), rat NiG-D12 (aa 198-233), rat NiG-D13 (aa 174-216), rat NiG-D14 (aa 174-260), rat NiG-D15 (aa 174-190 + 493-979), rat NiG-D16 (aa 174-190 + 621-979), rat NiG-D17 (aa 174-190 + 259-979), rat NiG-D18 (aa 174-190 + 263-979), rat NiG-D19 (aa 763-865), rat NiG-D20 (aa 544-725), rat NiG-D21 (aa 812-918), rat NiG-D22 (aa 866-975), rat NiG-D23 (aa 914-975), rat NiG-D24 (aa 544-685), rat NiG-D25 (aa 614-725), rat NiG-D26 (aa 544-613), rat NiG-D27 (aa 581-648), rat NiG-D28 (aa 614-685), rat NiG-D29 (aa 648-725), rat NiG-D30 (aa 682-725), rat NiG-D31 (aa 544-580), rat NiG-D32 (aa 581-613), rat NiG-D33 (aa 614-648), rat NiG-D34 (aa 648-685), rat NiG-D35 (aa 260-556), rat NiG-D36 (aa 260-415). NiR-G and NiR-a are derived from Nogo-A-pET28 by restriction enzyme digestions. NiG is derived from NiR-G by restriction digestion and MungBean Nuclease treatment. NiG-D1, -D3, -D4, -D5, -D7, -D8, -D9, -D10 derived from NiG-pET28 by restriction enzyme digestions. NiG-D15, -D16, -D17, -D18 derived from NiG-pET28 by Exonuclease III digestion. NiR-b, NiR-D1, -D2, -D3 derived by PCR with NiR-a-pET28 as a template. NiG-D2, -D6, -D11, -D12, -D13, -D14, -D19, -D20, -D21, -D22, -D23, -D24, -D25, -D26, -D27, -D28, -D29, -D30, -D31, -D32, -D33, -D34, -D35, -D36 derived by PCR using NiG-pET28 as a template. All constructs subcloned into pET28. pET28 used for all the constructs mentioned aboved. pGEX-6P used for GST-Nogo66 and pET26 for periplasmic expression of rat NiG. Human GST-Nogo-66 (aa 1055-1120 of human Nogo-A)

is cloned by PCR on human NogoA DNA (SEQ ID NO: 4) as a template. Deletion constructs are then cloned into pET28 vector (Novagen), pGEX-6P (Amersham Pharmacia Biotech) and pET26 vector (Novagen). Human GST-Nogo-66 corresponds to the GST-nogo protein published by GrandPré et al. (supra). Synthetic rat peptide 4 EELVQKYSNSALGHVNSTIKELRRL (SEQ ID NO: 27) corresponds to the human peptide 4 (Human peptide 4 has been shown to be the inhibitory region of the Nogo-66 domain (GrandPré et al., 2000)). The orthologous rat peptide has a single mismatch C->S (see peptide 4 sequence in GrandPré et al., 2000, supra). Synthetic Pro/Ser-rich peptide PSSPPPSSPPPSSPPPS (SEQ ID NO: 28) as well as rat peptide 4 have been produced and HPLC-purified by Primm SA. Human NogoA_623-640 (SEQ ID NO: 6) is synthesised and purified by Research Genetics Inc.

b) Generation of human Nogo-A expression constructs (pRK7-hNogo-A): A human cDNA library constructed in lambda gt10 (Clontech) is screened with duplicate filter sets using standard procedures. Fragments of human Nogo-A are amplified by PCR from human whole brain cDNA (Clontech) using a standard protocol and subsequently cloned into pBluescript, digested and isolated, or used as screening probes directly. A 400bp XhoI/SmaI fragment is used as 5' probe, the 3' probe is amplified with primers CA-NA-2F: 5'-AAG CAC CAT TGA ATT CTG CAG TTC C-3' (SEQ ID NO: 29) and CA-NA-3R: 5'-AAC TGC AGT ACT GAG CTC CTC CAT CTG C-3' (SEQ ID NO: 30). Positive clones are isolated, subcloned and sequence confirmed. To obtain a full length human Nogo-A cDNA, overlapping clones are assembled using an unique EcoRI restriction site in the human Nogo-A sequence and subcloned into Bluescript vector, named Pbsnogoa. To obtain pRK7–hNogo-A, the full length cDNA was inserted into the eukaryotic expression vector pRK-7 by directional cloning.

c) Generation of human NiG (hNiG) expression plasmids (pET28a-hNiG) for bacterial production: A hNiG encoding DNA fragment is subcloned into BamHI/XhoI of pET28a (Novagen), after PCR amplification of the respective coding region from Pbsnogoa, in frame with the N-terminal His- and T7-tag for bacterial expression, using primer sets: forward 5'-GTC GCG GAT CCA TGG AGA CCC TTT TTG CTC TTC-3' (SEQ ID NO: 31); reverse 5'-GTT CTC GAG TTA TGA AGT TTT ACT CAG-3' (SEQ ID NO: 32). The final plasmid is termed pET28a-hNiG. hNiG was then expressed in E.coli BL21 pRP by induction with 1 mM Isopropyl-beta-D-thiogalactopyranoside (IPGT).

d) Generation of mouse NiG-exon3 (mNiG-exon3) expression plasmid: The region encoding mouse exon 3 is amplified from mouse genome BAC template with primers: forward 5'-GTG CGG ATC CAT GGA TTT GAA GGA GCA GC-3' (SEQ ID NO: 33); reverse 5'-GTT TCT CGA GTG AAG TTT TAT TCA GCT C-3' (SEQ ID NO: 34) and subcloned into the BamHI/XhoI cloning sites of pET28a. The final plasmid construct is named pET28a-mNiG-exon3.

Cloning of monkey NiG: PolyA RNA is isolated from frozen monkey brain tissue and cDNA are synthesised using an oligo dT primer. Two overlapping fragments covering the 5' and the 3' region of the cDNA are amplified by PCR using sequence-specific primers and a proof-reading enzyme. The primers are designed using the known sequence of the human NiG cDNA. For amplification of the 5' fragment the primers are 5'-TCCACCCCGGCCGCGCCCAA-3' (SEQ ID NO: 35) and 5'-AATGATGGGCAAAGCTGTGCTG-3' (SEQ ID NO: 36), for the 3'-fragment 5'-GGTACAAAGATTGCTTATGAAACA-3' (SEQ ID NO: 37) and 5'-AGCAGGGCCAAGGCAATGTAGG-3' (SEQ ID NO: 38). The two fragments are then subcloned and for each fragment at least 4 independent clones were sequenced. The full length cDNA is assembled by overlapping PCR using the primers mentioned above and the resulting product is cloned and sequenced again.

e) Production of recombinant NogoNiG proteins and the Nogo-A-deletion library as defined above: The bacterial Nogo-A-deletion library is expressed in Escherichia coli. Proteins are extracted either by repeated sonication in sonication buffer (20 mM Tris, 50 mM NaH_2PO_4, 100 mM NaCl, pH 8.0) with 0.75 mg/ml Lysozyme, by solubilisation with B-Per™ (Pierce) or with 8 M urea. NiG expressed with pelB-leader is obtained from the periplasmic space according to the Novagen protocol for periplasmic protein purification. Supernatants of pET28-constructs are purified using the Co^{2+}-Talon™ Metal Affinity Resin (Clontech) in a batch procedure. 8 M urea and B-Per™ solubilised lysates are brought to non-denaturing conditions by increasingly substituting the buffer with sonication buffer during the resin-batch procedure. Proteins are eluted with 250 mM imidazole in sonication buffer on a gravity column (BioRad). NiG proteins are further purified by gel filtration on Superdex 200 HiLoad 16/60. Supernatants of pGEX-6P constructs are purified with G-sepharose column in a batch procedure according to manufacturer indications (Amersham Pharmacia). Cleavage of GST-Nogo-66 is done by incubating solubilised GST-Nogo-66 with PreScission protease and

subsequent HPLC purification. Gel electroelution is performed by preparative SDS-PAGE of IMAC-purified recombinant Nogo and elution with BioRad Electro-Eluter into 50 mM Tris, pH 7.4, 100 mM NaCl, 0.2% (w/v) CHAPS for 1 hr at 250 mA and followed by 30 s of reversed electrode polarities. Protein concentrations of chromatography-purified proteins are determined using Pierce Coomassie Stain and BSA as standard protein. Protein concentrations of gel eluted proteins are estimated based on band intensity of silver-stained gels (Merril CR, Dunau ML, Goldman D (1981) A rapid sensitive silver stain for polypeptides in polyacrylamide gels. Analyt.Biochem. 110:201-207) with BSA as a standard.

f) Production of recombinant NogoA fragments in CHO cells: A 3119 bp fragment resulting from a partial HincII digest of rat Nogo-A cDNA, NiR-G, is cloned into pSecTag2 expression vectors (Invitrogen, Groningen, The Netherlands). Transfection of pNiR-G into CHO cells results in intracellular, cytoplasmic expression of NiR-G. Stable NiR-G CHO cell lines are selected with 250 µg/ml Zeocin (Invitrogen). Recombinant NiR-G from cell lysate is purified over a Ni^{2+}-NTA column (Qiagen AG, Basel, Switzerland). Rat NiG-D20 and Nogo-66 are cloned into pAPtag5 vector by PCR. Transfection of pNiG-D20-AP into CHO cells results in NiG-δ20-AP that was secreted into the culture supernatant. Stable pNiG-D20-AP and pNogo-66-AP cell lines were selected with 250 µg/ml Zeocin (Invitrogen). Both cell lines are adapted to serum-free medium (Gibco) conditions and grown in a cell-line chamber (Integra). Supernatants are tenfold concentrated prior to use, and the concentration of fusion protein is assessed as described elsewhere (Flanagan JG, Leder P (1990) The kit ligand: a cell surface molecule altered in steel mutant fibroblasts. Cell 63:185-194).

g) 3T3 fibroblast and CHO spreading assays: The 3T3 spreading assays are performed as described previously (Spillmann AA, Bandtlow CE, Lottspeich F, Keller F, Schwab ME (1998) Identification and characterization of a bovine neurite growth inhibitor (bNI-220). J.Biol.Chem. 273:19283-19293). CHO spreading assays are performed essentially the same way as for 3T3 fibroblasts. Briefly, CHO cells are split 1:2. 24 hrs later they are trypsinised in PBS-EDTA for 30 s and ~8'000 CHO cells are plated onto culture dishes precoated with 5, 1, 0.5 and 0.2 µg/well NiG or Nogo-66. After 30-45 min the cells are fixed with 4% (w/v) PFA, 5% (w/v) sucrose and then analysed as described Spillmann et al, supra). ~100 cells are counted per well with light microscopy; criterion of spreaded cells: (a) attachement to the dish AND (b) extended morphology indicative for lamellipodia; under light microscopy the cells appear darker and larger than not spreaded, round cells; non-spreaded cells are

considered those cells that are (a) not attached to the dish OR (b) attached to the dish, but small, rounded, without detectable lamellipodia protruding on the dish. The ratio between spreaded and not spreaded cells defines the degree of non-permissiveness of the substratum.

h) PC12 Neurite outgrowth assays: PC12 neurite outgrowth assays are performed as described previously (Rubin BP, Spillmann AA, Bandtlow CE, Keller F, Schwab ME (1995) Inhibition of PC-12 cell attachment and neurite outgrowth by detergent solubilized CNS myelin proteins. Europ. J. Neurosci. 7: 2524-2529). PC12 cells (a PC12 cell clone able to grow independently of laminin obtained from Moses Chao, New York) are primed for two days with 50-100 ng/ml NGF (Harlan Bioproducts, Indianapolis) to DMEM, 5% foetal calf serum, 10% horse serum, 100 U/ml Penicillin and 0.5 mg/ml Streptomycin (Pen-Strep from Gibco-BRL). PC12 cells are detachde mechanically, trypsinised for 5 minutes with 0.05% trypsin (Sigma) in HBSS (Gibco) and plated at a density of 3,000-5,000 cells/cm2 in culture medium with 100 ng/ml NGF. Assays were stopped after 24 hrs by adding 4% (w/v) PFA, 5% (w/v) sucrose in PBS, pH8. Cell culture dishes were coated for PC12 cells the same way as for 3T3 cells.

i) Retinal ganglion cell stripe assays: The retinal ganglion cell stripe assay is performed according to Vielmetter (see Vielmetter J, Stolze B, Bonhoeffer F, Stuermer CA (1990) In vitro assay to test differential substrate affinities of growing axons and migratory cells. Exp. Brain Res. 81:283-287) with modifications (see Schmalfeldt M, Bandtlow CE, Dours-Zimmermann MT, Winterhalter KH, Zimmermann DR (2000) Brain derived versican V2 is a potent inhibitor of axonal growth. J. Cell Sci. 113:807-816). Explants are evaluated after fixation with 4% (w/v) PFA, 0.1% (v/v) glutaraldehyde in PBS for 10 min at RT. For immunostainings, fixed explants are blocked for 1 hr at RT with RNO-blocking solution (0.5% (w/v) BSA, 0.3% (w/v) TopBlock (Juro Supply), 0.1% (w/v) NaN_3 in PBS), permeabilised for 10 min with 0.05% (v/v) Tx-100 in RNO-blocking solution, frozen for one minute at –20 °C and incubated with primary antibodies (AS Bianca for NiR, AS Laura for Nogo-A, NiR-G, NiG, NiG-D3 and NiG-D20, Novagen mAb anti-T7 for Nogo-C and beta-Gal control protein). After washing with PBS, FITC- and TRITC (FITC: Fluorescein-IsoThioCyanate: TRITC: Tetramethyl Rhodamine IsoThiocyanate)-conjugated antibodies (Jackson ImmunoResearch

Laboratories) are added (1:150) to the explants. The samples are coverslipped in 50% (v/v) glycerol, 25 mM $NaHCO_3$, 40 mM NaCl, 1% (w/v) p-Phenylendiamine (Sigma).

Results:

a) Two regions in the N-terminal part of Nogo-A are inhibitory for spreading of 3T3 fibroblasts: In order to identify the regions of Nogo-A responsible for the inhibition of 3T3 fibroblast spreading, a library of 50 Nogo deletion constructs is made and recombinant proteins are expressed in bacteria (see method 1a). The apparent EC_{50} for inhibition of 3T3 fibroblast spreading was approximately 400-500 ng/0.1ml Nogo-A coated overnight per cm^2 of culture dish (~4 pmol/cm^2). Treatment of Nogo-A or its fragments with 8 M urea results in a strong decrease of inhibitory activity, indicating that conformation is important. The analysis of Nogo fragments in the fibroblast spreading assay reveals that at least two stretches of the Nogo-A protein mediate inhibition of the spreading of freshly plated fibroblasts, namely NiR-D2 (aa 59-172) and NiG-D20 (aa 544-725). All the fragments derived from the NiG-region displaying inhibitory activity (e.g. NiG-D4 and NiG-D8) partially overlap with NiG-D20. Minor inhibitory activity at high protein concentration is seen for NiG-D19 within the NiG-D6 region. Nogo-C, Nogo-66 and rat Peptide 4 (shown to be the inhibitory region of Nogo-66 by GrandPré et al., 2000) are not inhibitory for fibroblast spreading. These data show that the anti-spreading activity of Nogo-A on 3T3 fibroblasts resides in two defined stretches located at the N-terminus (NiR-D2) and within the Nogo-A-specific part (NiG-D20) of the protein. Non-specific physico-chemical properties (acidity of the fragments, structural effects due to proline and serine residues) are not responsible for this effect. The C-terminal RTN domain is *not* involved in the inhibition of fibroblast spreading.

b) NiG-D20 Region of Nogo-A is inhibitory for neurite outgrowth: To determine whether the fragments of Nogo-A that are non-permissive for cell spreading are also inhibitory for neurite outgrowth, a series of bacterially produced Nogo-A fragments as well as eukaryotically produced Nogo-AP chimeras in different neuronal assays are tested. In the stripe assay (method 1), neurites avoid laminin/Nogo-A coated stripes, growing on the laminin-only stripes, whereas stripes coated with laminin/beta-Galactosidase are not circumvented. Full-length Nogo-A is strongly non-permissive for retinal ganglion cell (RGC) neurite outgrowth, while the N-terminal part (NiR) had only marginal effects. Nogo-C activity is indistinguishable from the control protein beta-Galactosidase. The Nogo-A-specific region NiG-D20 appears to

contain the main region responsible for the non-permissive activity on RGC neurite outgrowth; the growth cones stop when encountering NiG-D20-coated stripes. The nonpermissive effect is concentration-dependent. At lower Nogo-A concentrations the number of crossing fibers increased. No obvious difference is observed between nasal and temporal RGC neurites concerning their responsiveness to Nogo-A regions. A laminin-independent, NGF-responsive clone of PC12 cells is primed with 50 ng/ml NGF for 24 hrs and then plated onto dishes coated with bacterially produced Nogo fragments at 0.1-3 $\mu g/cm^2$. Neurite outgrowth is scored one day later. The Nogo-A-specific region (NiG) and its fragment NiG-δ20 strongly inhibited PC12 neurite outgrowth. In contrast, the N-terminal fragment NiR has only minor activity, detectable only at high protein concentration. Nogo-C and Nogo-66 are inactive.

Example 2: Presence of binding site(s) for NiR-G and NiG-D20 on 3T3 fibroblasts and rat cortical brain membranes:

Methods:

a) Radioactive labelling and binding experiments: IMAC-purified NiG-D20 is iodinated by ANAWA Trading SA (Wangen, Switzerland) (2,030 Ci/mmol) using Lactoperoxidase and purified by reverse-phase HPLC. Membranes from rat brain cortex are prepared as described (Olpe HR, Karlsson G, Pozza MF, Brugger F, Steinmann M, Van Riezen H, Fagg G, Hall RG, Froestl W, Bittiger H (1990) CGP 35348: a centrally active blocker of GABAB receptors. Eur.J.Pharmacol. 187:27-38). Binding is performed for 1 hr at RT essentially as described (Kaupmann K, Huggel K, Heid J, Flor PJ, Bischoff S, Mickel SJ, McMaster G, Angst C, Bittiger H, Froestl W, Bettler B (1997) Expression cloning of GABA(B) receptors uncovers similarity to metabotropic glutamate receptors. Nature 386:239-246.) using 1.5 ml tubes preincubated for 2 hrs with 1% (w/v) bovine serum albumin to reduce non-specific binding. Membrane homogenates in HEPES buffer pH 7.4 (125 mM NaCl, 5 mM KCl, 0.6 mM $MgCl_2$, 1.8 mM $CaCl_2$, 20 mM HEPES, 6 mM dextrose) containing protease inhibitors (Rôche Diagnostics, Mannheim, FRG) are incubated with 1.3 nM iodinated NiG-D20 in the absence or presence of increasing concentrations of unlabelled NiG-D20.

b) Flow cytometry: Flow cytometry and cell sorting are performed on a Cytomation MoFlo high-speed cell sorter (Fort Collins, Colorado). The flow cytometer is equipped with an argon-ion/UV Enterprise II laser tuned to 488 nm with 130 mW of power. Fluorescein (FITC)

fluorescence is collected through a 530/40 nm bandpass filter. For analysis 3T3 fibroblasts are detached with Cell Dissociation Buffer (Gibco). The pre-formed complex used to detect binding of NiR-G to 3T3 fibroblasts is prepared as follows: NiR-G and anti-Myc antibody (9E10) are incubated at a 1:1 molar ratio for 30 min at 4 ºC. Next, FITC conjugated $F(ab)_2$ Goat Anti Mouse IgG is added and incubated for additional 30 min at 4 ºC . The resulting molar ratio of the trimeric complex is 1:1:0.5. The complex is added to $1x10^6$ 3T3 fibroblasts in a final volume of 0.1 ml, incubated for 2 hrs at 4 ºC, washed, and analysed by flow cytometry.

Results:

Presence of binding site(s) for Nogo-A-specific active fragments on 3T3 fibroblasts and rat cortical brain membranes: Since the NiR-D2 and NiG-D20 regions of Nogo-A are inhibitory for cell spreading and neurite outgrowth despite the absence of Nogo-66 and independently of NgR, the presence of a separate, Nogo-A-specific receptor has to be postulated. Thus binding studies are performed of multimerised, myc-tagged and IMAC-purified NiR-G to living 3T3 fibroblasts that are analysed by flow cytometry. Ab-complexed NiR-G is binding efficiently to 3T3 cells as seen by a fluorescence shift of over 90% of the 3T3 cells. In contrast, 3T3 cells are not labelled after incubation with the 9E10 primary mouse anti-myc mAb complexed with a FITC-conjugated secondary $F(ab)_2$ goat anti-mouse IgG nor with the secondary Ab alone. To test binding of NiG-D20 to rat cortical membranes, [^{125}I]-labelled NiG-D20 in a radioligand binding assay is used. At a concentration of 1.3 nM of [^{125}I]-NiG-D20, evidence for a specific NiG-D20 binding sites on brain membranes as shown by a concentration-dependent competition of radioligand binding by unlabelled NiG-D20 is found. These results show that aminoterminal fragments of Nogo-A can bind to the surface of 3T3 cells and to rat cortical membranes, demonstrating the presence of membrane-bound, Nogo-A-specific binding sites or receptor(s).

Example 3: Generation of mouse 11C7-IgG1

Mice (C3H- and C57Bl6/J-strains) are immunised subcutaneously with the synthetic peptide SYDSIKLEPENPPPYEEA (= rat NogoA_623-640; SEQ ID NO: 1), corresponding to a particular epitope in NiG-D20. This epitope is highly conserved in human, cynomologus monkey and mouse NiG-D20 Nogo-A specific region and starts at amino acid 623 and ends

at amino acid 640 of the human NogoA amino acid sequence (SEQ ID NO: 5) (See also sequence alignment: Figure 1).

mAb 11C7 has been obtained out of a fusion of rat NogoA_623-640 with the carrier protein Key hole limped hemagglutinin (KLH) immunised mice. Monoclonal antibodies have been screened by ELISA on rat NogoA_623-640-KLH, rat NogoA_623-640 free peptide and a nonrelated peptide-KLH. In a further screen, the mAbs have been tested by ELISA on NiR-G versus b-Galactosidase, both expressed as his-tagged proteins and purified by metal affinity chromatography . Subsequently, the mAbs have been tested for recognition of Nogo-A on Western blot of oligodendrocyte and brain lysates (rat origin). Antibodies are tested for recognition of the protein in immunocytochemistry of rat Nogo-A-transfected CHO or COS cells and of endogenous Nogo-A of rat oligodendrocytes (permeabilised cells). They have also been tested for surface binding to living rat oligodednrocytes. Species crossreactivity is tested on recombinant NiG of rat, mouse, human and bovine origin by ELISA and on endogenous rat, mouse, human and monkey Nogo-A by Western blot of tissue or cell extracts.

Western blot analysis: SDS-PAGE and Westernblotting are performed as described earlier (Huber AB, Weinmann O, Brosamle C, Oertle T, Schwab ME (2002) Patterns of Nogo mRNA and protein expression in the developing and adult rat and after CNS lesions. J. Neurosci. 22: 3553-3567), blocking is done with 3% (w/v) Top Block (Juro Supply, Lucerne, Switzerland). Antibodies are diluted as follows: Purified monoclonal 11C7 or hybridoma supernatants 1:150. Secondary antibodies are HRP-conjugate anti-mouse ((Pierce; 1:5000,) 1:50,000). Hybridisation with the 11C7 antibody is carried out over night at 4°C. For detection the ECL detection reagents from Amersham Pharmacia are used.

Results:

The 11C7 mAb identifies the 190 kD Nogo-A band on a Western blot of oligodendrocyte cell culture homogenate. 11C7 also identifies human NiG, Cynomolgus NiG cell lysate and rat NiG-D20 in western blots. 11C7 mAb is characterised as a IgG1 isotype (IsoStrip Kit, Roche).

Example 4: Characterisation of the mouse 11C7 mAb

Immunocytochemistry: Optic nerve oligodendrocytes are prepared as described (Schwab, Caroni, 1988, Neuron). Three to five day-old cultures grown on poly-L-lysine coated coverslips are washed twice with PBS, fixed in 4% (w/v) paraformaldehyde (PFA), 5% (w/v) sucrose in PBS for 15 min at room temperature (RT) and non-specific binding is blocked with 10% (v/v) FCS. Cells were then incubated with mouse 11C7 (1:100). Secondary antibodies are goat-anti-mouse TRITC (Jackson ImmunoResearch Laboratories). For cell surface staining, two day-old rat optic nerve cultures are incubated with monoclonal antibody in medium for 25 min at RT. Secondary alkaline phosphatase conjugated antibodies (Milan Analytica, Lausanne) are used at 1:7,500 in 0.1 M maleic acid with 1% (w/v) blocking reagent (1 hr). The cultures are washed twice with maleic acid buffer, once with alkaline phosphatase buffer (0.1 M Tris-HCl pH 9.5, 0.1 M NaCl, 5 mM $MgCl_2$) and the staining is developed for 3 hrs at room temperature with 0.175 mg/ml BCIP (Sigma) and 0.338 mg/ml NBT (Sigma) in alkaline phosphatase buffer.

NogoA_623-640 epitope of Nogo-A present at the cell surface of cultured oligodendrocytes: Living cultures of oligodendrocytes incubated with mouse 11C7 mAb stain the differentiated oligodendrocyte cell bodies and their radial processes. The control mouse IgG and the antibodies against the intracellular protein CNPase do not stain the living cells. Pre-incubation of mouse 11C7 with the corresponding immunogenic peptide (= rat NogoA_623-640 SED ID NO: 1) reduces staining to background levels (competitive assay). Cell surface staining is present on all major and small processes and on the cell body. Thus, the Nogo-A specific part of the molecule recognised by mouse 11C7 mAb is exposed to the extracellular space on the plasma membrane of oligodendrocytes.

Production and Purification of mouse 11C7 mAb: A 10-L glas bioreactor is used for continuous-mode cultivation of the hybridoma clone producing the mouse 11C7 mAb. The bioreactor is equipped with a marine impeller placed in a center tube for gentle agitation, a spin filter for cell retention, and coiled silicone tubing for bubble-free aeration. The hybridoma cells are cultivated in our RPMI based serum free medium. The medium is inoculated with cells at 3.7 x 10^5/ml. After 28 hours continuous medium flow through the bioreactor is started with a rate of 0.5 fermentor volumes /day (5 liters/day). Another 24 hours later the flow rate is increased to its final level of 1 fermentor volume/day (10 liters/day). After 1 week the culture reaches a steady state with 11 x 10^5 cells/ml and the process is continued for another week. The titer of the mouse 11C7 mAb is determined daily

by HPLC. A total of 150 liters culture supernatant is harvested from the bioreactor, sterile filtered for removal of cells and cell debris. 150 L culture supernatant are concentrated to about 6 L using a Pellikon tangential flow device (Millipore ; 10 kDa cut-off). The concentrated supernatant is purified in 3 runs over a 220 ml bed volume column of Protein A Sepharose Cl-4B (Pharmacia ; 11 cm bed height). Briefly, the culture supernatant after pH correction to 8.1 is loaded at 4 ml/min and the column washed to base-line at 8 ml/min using 100 mM Na_2HPO_4, pH 8.1. Bound material is finally eluted at 8 ml/min using 50 mM NaH_2PO_4, pH 3.0, 140 mM NaCl and immediately neutralized (pH 7.0) with 5 N NaOH and sterile filtered. Absorbance is monitored at 280 nm. Portion of the purified material are eventually further concentrated by ultrafiltration and/or dialyzed against PBS. All the buffers used in the purification are filtered on a 10 kDa ULTRASETTE™ tangential flow device (Filtron Technology Corporation) in order to remove possible endotoxin contaminations. For the same reason the Protein A resin is extensively washed with 20% ethanol and all tubings/pumps treated with 0.1 M NaOH prior to use. Protein concentration is measured spectrophotometrically at 280 nm using a reference absorption of 1.35 for 1 mg/ml. Purity is routinely assessed by SDS-PAGE under reducing conditions using 4-20% Novex gradient gels. Endotoxin content is measured by the classical Limulus Amoebocyte Lysate (LAL) reaction according to the manufacturer instructions (Endotell AG, Allschwil, Switzerland).

Generation of F_{ab} fragments: A portion of mouse 11C7 mAb is extensively dialyzed against 100 mM Na-actetate, pH 5.5, 2 mM EDTA and adjusted to a concentration of 6 mg/ml. F_{ab} fragments are generated by papain digestion (1:200 w/w ratio) in the presence of 0.25 mM cysteine. The reaction is allowed to proceed for 16 hours at 37 °C and then stopped by the addition of the specific papain inhibitor E64 (N-[N-(L-3-trans-carboxirane- 2-carbonyl)-L-leucyl]-agmatine) in large excess (10 μM). The digested antibody is then passed over a column of protein A Sepharose Fast Flow in order to remove intact material and Fc fragments. The F_{ab} fraction is extensively dialysed against PBS and concentrated to about 3 mg/ml. (Papain and E64 are from Roche Molecular Biochemicals).

HPLC, Mass Spectrometry and N-terminal amino acid sequencing of V_L and V_H regions:

a) Reduction and Alkylation: Purified, dried 11C7 antibody are dissolved in 40 µl of 8M urea, 0.4M NH_4HCO_3, pH 8.3. 60 ug DTT (Calbiochem), pre-dissolved in 10 ul of the same buffer as the protein, are added. Reduction is performed at 50°C for 30 min under argon (100 fold molar excess of DTT over protein thiols). After reduction, the sample is cooled

to room temperature. 304 ug of iodoacetamide (Sigma Ultra, I-1149) dissolved in the same buffer as the protein is added. Carboxamidomethylation is carried out at room temperature for 15 min in the dark. 1 µl β-mercaptoethanol is added to quench the reaction.

b) Isolation of Heavy- and Light-Chain: Carboxamidomethylated heavy and light chains of antibody are isolated by Reverse Phase High Pressure Liquid Chromatography (RP-HPLC) on a Hewlett Packard 1090M HPLC System with DR5 pumping system and diode-array UV detector. The conditions for chromatography are: PerSeptive Biosystems Poros 2.1x100 mm column packed with R1/H material; flow is 0.5 ml/min; solvents: (A) 0.1% TFA in water and (B) 0.09% TFA / acetonitril/water 9:1; gradient 25-70% B in 8 minutes at 80°C; detection at 218 / 280 nm.

c) LC-ESI-MS: Mass spectrometry is carried out using a Q-Tof (Micromass, Manchester, UK) quadrupole time-of-flight hybrid tandem mass spectrometer equipped with a Micromass Z-type electrospray ionization source (ESI). Acquisition mass range is typically m/z 500-2000. Data are recorded and processed using MassLynx software. Calibration of the 500-2500 m/z scale is achieved by using the multiple-charged ion peaks of horse heart myoglobin (MW 16951.5).

d) HPLC-MS of heavy and light chain: Separation of reduced and carboxamidomethylated heavy and light chain is performed on a HP1100 HPLC system (Hewlett Packard, Palo-Alto, CA, USA) employing a 1mmx150mm LC Packings column packed with Perseptive Biosystems POROS R1/H. The column is held at 60°C. Sample volumes of 10 µl are injected onto the column using a CTC PAL autosampler (CTC, Zwingen, Switzerland) fitted with a Valco model C6UW HPLC valve (Valco, Houston, TX, USA) and a 10 µl injection loop. HPLC was controlled by MassLynx software (Micromass, Manchester, UK). UV detection is at 214 nm. Eluent A is water containing 0.05% TFA. Eluent B is a 1:9 mixture of water : acetonitrile containing 0.045% TFA. A gradient from 20% B to 90% B is run in 20 minutes at 80 °C. The flow rate is typically 60 µl/min. The total flow from the LC system is introduced into the UV detection cell, then the ESI source without any splitting. The HPLC system is controlled and the signal from the UV detector is processed using MassLynx software (Micromass, Manchester, UK). The following 5 signals are detected:

Table 1:

Measured:	**Signal interpretation**
A= 50959.0 Da	H-Chain with carboxamidomethyl-cysteine (CAMCys)*
B= 51119.5 Da	Signal A+162 Da (= hexose)**

C= 51086.0 Da	Signal A+ 127 (Lys), H-Chain with CAMCys*
D= 51251.0 Da	Signal C+162 Da (= hexose)**
E= 24464.8 Da	L-Chain with CAMCys
	*There are two types of H-chain present, one with and one without Lys at the C-terminal end. The ratio of both forms is approximately 50 : 50%. **Both types of H-chains have two corresponding glycosylated forms (+162)

d) N-terminal amino acid sequencing of V_L and V_H regions: Collected H+L chains peaks form HPLC are used for sequence analysis. Amino acid sequences are determined on a Hewlett Packard G1000A N-terminal Protein Sequencing System. The system performs automated Edman chemistry on protein samples retained on miniature adsorptive biphasic columns. An optimized chemistry method (double couple 3.0) is used to enhance chemical efficiency; minimize lags and herewith extend sequence analysis to about 50 residues. Analysis of PTH-amino acids is performed on an on-line Hewlett Packard HP1090 HPLC System equipped with a ternary pumping system and a narrowbore (2.1mm x 25cm) PTH column.

Results:

From mass analysis homogeneous heavy and light chain of mouse 11C7-IgG1 are determined. The H-chain is single glycosylated and there are two forms with a difference on the C-terminal Lysine. Total mass analysis of heavy and light chain shows a single mass for both chains. HPLC chromatography of mouse 11C7-IgG1 shows a single peak. After HPLC purification followed by reduction and alkylation pure heavy and light chain are available. N-terminal sequence degradation is performed on light-chain and heavy-chain. 45 to 55 amino acids from the N-terminal sequence of L-chain and H-chain are identified by sequence degradation.

Light Chain

1 5 10 15 20 25 30

▼ ▼ ▼ ▼ ▼ ▼ ▼

DVLLTQTPLTLSITIGQPASISCKSSQSLL

31 35 40 45 50 55 60

▼ ▼ ▼ ▼ ▼ ▼ ▼

HSDGKTYLNWLLQRPGQ

Heavy Chain

1 5 10 15 20 25 30

▼ ▼ ▼ ▼ ▼ ▼ ▼

EVKLLESGGGLVQPGGSLKLSCVVSGFDFR

31 35 40 45 50 55 60

▼ ▼ ▼ ▼ ▼ ▼ ▼

RNWMSWVRQAPGKGLEWIGEINPD

Example 5: Cloning of the heavy and light chain genes of mouse 11C7 mAb

Total RNA is prepared from 10^7 hybridoma cells (clone 11C7) using TriPure reagent (Roche diagnostics, Germany, Cat.# 1667157) according to the manufacturers instructions. For cDNA synthesis, mRNA is isolated from above prepared total RNA using Oligotex Resin (Qiagen, Germany, cat. # 70022).

cDNA is generated by reverse transcription using the following conditions: 2 µl mRNA, 2 µl 10 x reverse transcription buffer, 2 µl $(dT)_{20}$ primer (10 µM), 0.5 µl RNasin (Promega, 40 U/ml), 2 µl dNTPs (5 mM each), 1 µl Omniscript™ reverse transcriptase (Qiagen, Cat # 205110), 10.5 µl ddH_2O, Reaction:1hr at 37°C. For PCR amplification of cDNA encoding for the V_H and V_L the proofreading enzyme ProofStart™ DNA polymerase is used.

PCR of light and heavy chain: Reaction mix: 2 µl cDNA , 5 µl 10 x reaction buffer, 3 µl dNTPs (5 mM each), 2 µl 5'primer (10 µM) (see Table 2), 2µl 3'primer (10 µM) (see Table 2), 1 µl ProofStart (Qiagen, Cat # 202203), 36 µl ddH_2O. PCR conditions: 95°C/5 min, (95°C/40 sec, 53°C/1 min, 72°C 1 min) x 35, 72°C/ 10 min. The resulting PCR products are ligated directly into pCRbluntTOPO (Invitrogen). The ligation mix is transfected into TOP 10 cells (Invitrogen) and several clones are picked. The nucleotide sequences of the variable part of the heavy chain of the 11C7 mAb (V-H, SEQ ID NO: 43) and of the light chain of the 11C7 mAb (V-L, SEQ ID NO: 44) cDNas are determined on an ABI sequencer. The subsequent amino acid sequence of V-H and V-L are shown in SEQ ID NO: 2 (V-H) and SEQ ID NO: 3 (V-L). Primers used for PCR amplification of the V_H and V_L cDNAs; all primers are synthesized by MWG Biotech, Germany.

Table2:

Primer	Sequence	SEQ ID

		NO:
5'-V_L leader	AATATGAGTCCTGCCCAGTTCCTGTTTC	39
3'-$C\kappa$	TTAGGAATTCCTAACACTCTCCCCTGTTGAAG	40
5'-V_H leader	AATATGGATTTTGGGCTGATTTTTTTTATTG	41
3'-C_H hinge	AATTGGGCAACGTTGCAGGTGACG	42

Example 6: Binding of 11C7 and Fab to Nogo-A domains using ELISA

Greiner 96 well PS plates (#655161) are coated with 0.4-2ug/ml Nogo protein fragments in PBS (100ul/well) covered and incubated 4 hours at room temperature. Plates are flicked and refilled with 200ul/well blocking buffer (PBS+2% BSA), covered and incubated. 1h at RT or overnight at 4 °C, then washed 4 times with water and PBS. Different concentrations of mouse 11C7 mAb or 11C7 Fab are diluted in PBS +2% BSA (100 ul/well), and incubated 2h at RT or overnight at 4 °C. Wash step is repeated and Goat anti-mouse IgG conjugated with horse radish peroxidase (HRP) at a dilution of 1:5000 (ICN #55550) in PBS/0.1%BSA /0.1%Nonidet 40 (100 ul/well) is added and incubated. 2h at RT or overnight at 4 °C and wash step is repeated. HRP reaction is started by adding 100 ul/well BM blue POD (Roche #1484281) and incubated in the dark at RT for 15 minutes . H2SO4 50ul/well 1M is added to stop HRP substrate reaction and the optical density is determinated using a microplate reader (Packard Spectra Count) set to 450nm.

The mouse 11C7 mAb binds to human NiG, rat NiG, mouse NiG, rat NiG-D20 and peptide 472 at very low concentrations of 0.02 to 2.5 nM. Binding to human NiG, rat NiG, mouse NiG at very low concentration is confirmed by the very high affinity (Kd 0.1 – 0.44nM Biosensor affinity measurements) and is consistent with the fact that 472 peptide with the exception of 2-3 amino acids is identical in human compared to rat and mouse equivalent region. The specificity of the binding is indicated by the fact that the mouse 11C7 mAb does not show any binding at all to rat NiG-D6 and Nogo-66 fragments over the same concentration range. The Fab monovalent fragment bound to human NiG and rat NiG-D20 at concentrations 0.025 to 25nM and showed no binding to rat NiG-D6 and Nogo-66 fragments over the same concentration range. The Kd measured by Biosensor was 7.14 nM for human NiG.

Example 7: Biosensor affinity measurements for mouse 11C7-IgG1 and Fab to Nogo-A domains

The affinity of the mouse 11C7 mAb and of the 11C7 Fab are measured by surface plasmon resonance (SPR) using a BIAcore 2000 optical biosensor (Biacore, Uppsala, Sweden) according to the manufacture's instructions (see Figure 2). Recombinant human, mouse, and rat NIG are covalently attached to three separate flow cells of a CM5 sensor chip using amine-coupling chemistry. Briefly; the carboxymethlyladed dextran matrix is activated by injecting 35ul of a solution containing 0.025M NHS and 0.1M EDC. For the immobilization on the sensor chip the recombinant mouse, human, and rat NIG are diluted in 0.01M citrate buffer at a pH varying between 3.5 and 4.5 and injected at a flow rate of 5ul/min to achieve coupling levels allowing affinity measurements. The deactivation of the remaining NHS-ester group is performed by injection of 35ul of 1M ethanolamine hydrochloride (pH 8.5). The surface of the sensor chip is regenerated by injecting 5ul 0.1M HCl. For the measurement of the affinity the antibodies are injected at different concentration, ranging from 0.50nM to 100nM at a flow rate of 200 ul/min. After each injection the sensor chip surface is regenerated with the injection of 10 ul 0.1M HCl without loss of binding activity on the surface. The kinetic constants, *ka* and *kd* and the affinity constants KA and KD are evaluated using the BIAevaluations 3.0 software supplied by the manufacturer.

Affinity measurement in BIAcore: The kinietc and the affinity binding constants of the mouse 11C7 mAb and the 11C7 derived monovalent Fab fragment to recombinat NogoA are measured in real time using surface plasmon resonance (SPR) technology (Biacore). For this analysis recombinant human, mouse and rat NIGs are coupled on three independent sensor chip surfaces and different concentrations of the antibodies are injected. Kinetic parameters of the binding interactions are derived from the sensorgrams by non-linear curve fitting. The affinity constants at equilibrium of mouse 11C7-IgG1 are KD= 0.1nM, KD= 0.4nM and KD= 0.19nM for human, rat, and mouse NIG respectively (table 3). For the 11C7 derived Fab fragment the affinity constant to human NIG is KD= 7.14nM. The lower affinity of the Fab fragment results from a decrease of both kinetic constants, association and dissociation (*ka, kd).* Lower affinity of the Fab fragment compared to the complete antibody is probably related to the avidity effect, which is lacking in the monomeric Fab.

Table 3:

11C7	Ka (1/Ms)	kd (1/s)	KA (M^{-1})	KD (M)
HumanNIG	4.48×10^{5}	4.6×10^{-5}	9.73×10^{9}	1.03×10^{-10}
Rat NIG	8.76×10^{5}	3.89×10^{-4}	2.25×10^{9}	4.44×10^{-10}
Mouse NIG	5.52×10^{5}	1.06×10^{-4}	5.2×10^{9}	1.92×10^{-10}
11C7 Fab	Ka (1/Ms)	kd (1/s)	KA (M^{-1})	KD (M)
HumanNIG	7.29×10^{4}	5.28×10^{-4}	1.4×10^{8}	7.14×10^{-9}

Claims:

1.) A binding molecule which is capable of binding to the human NogoA polypeptide (SEQ ID NO: 5) or human NiG (SEQ ID NO: 7) or human NiG-D20 (SEQ ID NO: 24) or human NogoA_623-640 (SEQ ID NO: 6) with a dissociation constant < 1000nM.

2.) A binding molecule which is capable of binding to the human NogoA polypeptide (SEQ ID NO: 5) or human NiG (SEQ ID NO: 7) or human NiG-D20 (SEQ ID NO: 24) or human NogoA_623-640 (SEQ ID NO: 6) with a dissociation constant < 1000nM and comprises at least one antigen binding site, said antigen binding site comprising either

- in sequence the hypervariable regions CDR1, CDR2, and CDR3, of which each of the hypervariable regions are at least 50% homologous to their equivalent hypervariable regions CDR1-11C7 (SEQ ID NO: 8), CDR2-11C7 (SEQ ID NO: 9) and CDR3-11C7 (SEQ ID NO: 10); or
- in sequence the hypervariable regions CDR1', CDR2', and CDR3', of which each of the hypervariable regions are at least 50% homologous to their equivalent hypervariable regions CDR1'-11C7 (SEQ ID NO: 11), CDR2'-11C7 (SEQ ID NO: 12) and CDR3'-11C7 (SEQ ID NO: 13).

3.) A binding molecule which is capable of binding to the human NogoA polypeptide (SEQ ID NO: 5) or human NiG (SEQ ID NO: 7) or human NiG-D20 (SEQ ID NO: 24) or human NogoA_623-640 (SEQ ID NO: 6) with a dissociation constant < 1000nM and comprises

- a first antigen binding site comprising in sequence the hypervariable regions CDR1, CDR2, and CDR3, of which each of the hypervariable regions are at least 50% homologous to their equivalent hypervariable regions CDR1-11C7 (SEQ ID NO: 8), CDR2-11C7 (SEQ ID NO: 9) and CDR3-11C7 (SEQ ID NO: 10); and
- a second antigen binding site comprising in sequence the hypervariable regions CDR1', CDR2', and CDR3', of which each of the hypervariable regions are at least 50% homologous to their equivalent hypervariable regions CDR1'-11C7 (SEQ ID NO: 11), CDR2'-11C7 (SEQ ID NO: 12) and CDR3'-11C7 (SEQ ID NO: 13).

4.) A binding molecule which comprises at least one antigen binding site, said antigen binding site comprising either

- in sequence the hypervariable regions CDR1-11C7 (SEQ ID NO: 8), CDR2-11C7 (SEQ ID NO: 9) and CDR3-11C7 (SEQ ID NO: 10); or
- in sequence the hypervariable regions CDR1'-11C7 (SEQ ID NO: 11), CDR2'-11C7 (SEQ ID NO: 12) and CDR3'-11C7 (SEQ ID NO: 13); or
- direct equivalents thereof.

5.) A binding molecule comprising

- a first antigen binding site comprising in sequence the hypervariable regions CDR1-11C7 (SEQ ID NO: 8), CDR2-11C7 (SEQ ID NO: 9) and CDR3-11C7 (SEQ ID NO: 10); and
- a second antigen binding site comprising in sequence the hypervariable regions CDR1'-11C7 (SEQ ID NO: 11), CDR2'-11C7 (SEQ ID NO: 12) and CDR3'-11C7 (SEQ ID NO: 13); or
- direct equivalents thereof.

6.) The binding molecule according to claims 1 to 5 which comprises at least

- one immunoglobulin heavy chain or fragment thereof which comprises (i) a variable domain comprising in sequence the hypervariable regions regions CDR1-11C7 (SEQ ID NO: 8), CDR2-11C7 (SEQ ID NO: 9) and CDR3-11C7 (SEQ ID NO: 10) and (ii) the constant part or fragment thereof of a human heavy chain; and
- one immunoglobulin light chain or fragment thereof which comprises (i) a variable domain comprising in sequence the hypervariable regions CDR1'-11C7 (SEQ ID NO: 11), CDR2'-11C7 (SEQ ID NO: 12) and CDR3'-11C7 (SEQ ID NO: 13) and (ii) the constant part or fragment thereof of a human light chain; or
- direct equivalents thereof.

7. The binding molecule according to claim 6 in which the constant part or fragment thereof of the human heavy chain is of the γ4 type and the constant part or fragment thereof of the human light chain is of the κ type.

8. The binding molecule according to claims 1 to 7, which is a chimeric or humanised monoclonal antibody.

9. A binding molecule comprising polypeptide sequences as shown in SEQ ID NO: 2 and SEQ ID NO: 3.

10. A polynucleotide comprising polynucleotides encoding a binding molecule according to any of claims 1 to 9.

11. A polynucleotide comprising either

- polynucletide sequences as shown in SEQ ID NO: 14, SEQ ID NO: 15 and SEQ ID NO: 16; or
- polynucletide sequences as shown in SEQ ID NO: 17, SEQ ID NO: 18 and SEQ ID NO: 19.

12. An expression vector comprising polynucleotides according to any one of claims 10 or 11.

13. An expression system comprising a polynucleotide according to any one of claims 10 or 11, wherein said expression system or part thereof is capable of producing a polypeptide of any one of claims 1 to 9, when said expression system or part thereof is present in a compatible host cell.

14. An isolated host cell which comprises an expression system according to claim 13.

15. The use of a binding molecule according to any one of claims 1 to 9 as a pharmaceutical.

16. The use of a binding molecule according to any one of claims 1 to 9 in the treatment of nerve repair.

17. A pharmaceutical composition comprising a binding molecule according to any one of claims 1 to 9 in association with at least one pharmaceutically acceptable carrier or diluent.

18. A method of treatment of diseases associated with nerve repair comprising administering to a subject in need of such treatment an effective amount of a binding molecule according to any one of claims 1 to 9.