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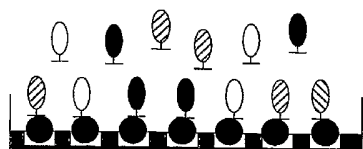
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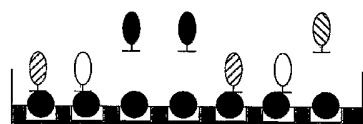
(54) Title: NEURON TARGETING PEPTIDES



Step 1: Apply Random 12mer
Phage Library to G_{T1B}



Step 2: Wash to Remove
Unbound Phage



Step 3: Elute Phage with Glycine
or rTTC



Step 4: Amplify and Repeat Step 1

(57) Abstract: A neuron targeting peptide includes about 5 to about 30 amino
acids and an amino acid sequence that preferentially binds to isolated G_{T1b}



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NEURON TARGETING PEPTIDES

TECHNICAL FIELD

The present invention relates to peptides capable of preferentially binding to particular cell types, and more particularly peptides capable of preferentially binding to neurons, as well as uses of such peptides.

BACKGROUND OF THE INVENTION

Currently, treatment for Amyotrophic Lateral Sclerosis (ALS) is largely palliative. The administration of neurotrophic growth factors as a means to protect motor neurons has been extensively explored. Ciliary neurotrophic factor (CNTF), brain-derived neurotrophic factor (BDNF), insulin-like growth factor (IGF), and glial cell-line-derived neurotrophic factor (GDNF) slow motor neuron degeneration. Insulin-like Growth Factor-I (IGF-I) protects motor neurons (MNs) in both organotypic slice as well as dissociated cultures.

Clinical trials of subcutaneous and intrathecal neurotrophic factors have been attempted in ALS. CNTF peripheral injections caused weight loss, fever, cough, and fatigue, and subcutaneous injections of BDNF caused injection site reactions, changes in bowel function and other behavioral side effects. Similarly, recombinant GDNF presented a short plasma life, poor access to motor neurons and severe side effect. Trials of IGF-I in sporadic ALS have had mixed results. The failure of these many trials may be attributed in part to insufficient trophic factor delivery to motor neurons and nonspecific delivery to non-motor systems.

Viral gene transfer may offer an alternative approach to motor neuron protection with several advantages over protein-based therapies. Gene-based therapies allow for prolonged expression of neuroprotective factors minimizing or potentially eliminating the need for repeated dosing. Several viral vectors have been demonstrated to undergo retrograde axonal transport, lending them to application in motor neuron therapies. Retrograde axonal transport of adeno-associated virus (AAV) vectors from peripheral injection sites to spinal motor neurons has recently been characterized. Dramatic therapeutic effects have recently been achieved by employing this approach to IGF-I gene delivery in the

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SOD1 mouse model of ALS. However, only a small fraction of peripherally administered vector reaches spinal motor neurons. This poor efficiency may result in part from a tendency of various AAV serotypes to bind and infect the muscle cells that surround the axon terminal at the neuromuscular junction. Alternatively, low axon terminal binding affinity could undermine effective motor neuron gene delivery. Thus, like trophic factor therapies, AAV therapies may be limited by inefficient binding to motor neuron terminals and nonspecific binding to surrounding cell types.

SUMMARY OF THE INVENTION

The present invention relates generally to peptides with selective and/or preferential neuronal binding affinity. The peptides of the present invention can comprise about 5 to about 30 amino acids and include an amino acid sequence that preferentially binds to isolated neuron receptors. The isolated neuron receptors can comprise isolated G_{T1b} (e.g., isolated mammalian trisialoganglioside G_{T1b}). The amino acid sequence of the peptide can be identified by biopanning phage displayed peptides against G_{T1b}.

In one aspect of the invention, the peptide can comprise about 5 to about 20 amino acids and include the amino acid sequence LST. The about 5 to about 20 amino acid peptide can also include amino acids, such as histidine, tryptophan, and arginine. These amino acids need not be provided in any particular order within the amino acid sequence of the peptide. Still other amino acids that can further be included in the peptide comprise tyrosine and leucine.

By way of example, the peptide can include an amino acid sequence selected from the group consisting of:

HLNILSTLWKYR (SEQ ID NO: 1);
SYQLSTHRWPLH (SEQ ID NO: 2); and
a derivative thereof.

In another aspect of the invention the peptide can comprise about 7 to about 20 amino acids and include an amino acid sequence selected from the group consisting of:

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5 KGTINPF (SEQ ID NO: 3);
XGAINPF (SEQ ID NO: 4);
KLTANPT (SEQ ID NO: 5);
NVHRGLH (SEQ ID NO: 6);
LTPWASS (SEQ ID NO: 7);
MLSNARH (SEQ ID NO: 8); and
a derivative thereof.

10 The peptide in accordance with the present invention can be used as a targeting peptide for targeting neurons, such as motor neurons and sensory neurons. The peptide targets neurons by preferentially binding to a ganglioside (*e.g.*, trisialoganglioside (G_{T1b})) receptor of the neuron. The targeting peptides once bound to the G_{T1b} receptor undergo retrograde transport to the cell body.

15 In certain aspects of the invention, the targeting peptide may be operably coupled or conjugated to a diagnostic agent and/or therapeutic agent. The ability to selectively target neurons with the peptides, diagnostic agents, and/or therapeutic agents provides a significant advantage for the increased efficacy and potency of treatments.

20 The attachment can be a covalent attachment, and the therapeutic agent and/or diagnostic agent can be a second molecule, such as a drug, a radioisotope, a peptide, a protein, that is used in the treatment of a neuromuscular disorder. These molecules or substances can be virtually any molecule that may yield a therapeutic or diagnostic benefit to a subject within the scope of the invention. An exemplary therapeutic agent that is operably coupled to the peptide can be a neurotrophic agent, such as a neurotrophic growth factor.

25 In another embodiment of the invention, the therapeutic agent and/or diagnostic agent can comprise a macromolecular complex. The macromolecular complex can be a vector, a bacteriophage, a bacterium, a liposome, a microparticle, a nanoparticle (*e.g.*, a gold nanoparticle), a magnetic bead, a yeast cell, a mammalian cell, a cell or a microdevice. These are representative examples only
30 and macromolecular complexes within the scope of the present invention may include virtually any complex that may be attached to a targeting peptide and administered to a subject. In certain embodiments, the macromolecule may be a

vector, such as a virus. Viruses can be produced that express or incorporate the targeting peptides. These viruses can then be used for targeted gene therapy for the treatment of neuromuscular disorders or other disease states that are associated with neurons.

5 A further aspect of the invention relates to a pharmaceutical composition that includes a neuron targeting peptide or a variant thereof and a pharmaceutically acceptable carrier. The pharmaceutical composition can be used in a therapeutic kit.

10 Yet another aspect of the invention relates to a method of targeting at least one of a diagnostic agent and/or therapeutic agent to cells with a G_{T1b} receptor. The method comprises operably linking at least one diagnostic agent or therapeutic agent to a neuron targeting peptide or variant thereof. The peptide can include an amino acid sequence in accordance with the present invention.

15 A further aspect of the invention relates to the use of neuron targeting peptides or conjugates thereof in accordance with the present invention as a toxin antagonist, such as a bacterial toxin or neurotoxin (*e.g.*, clostridial neurotoxin). The neuron targeting peptides and conjugates thereof can be administered to a subject upon exposure of the subject to a clostridial neurotoxin. The neuron targeting peptides and conjugates thereof can be administered to a subject to serve
20 as competitors to the clostridial neurotoxin at the neuronal receptor (*e.g.*, G_{T1b} receptor).

 Another aspect of the invention relates to a method of targeting at least one neuron. The method comprises identifying a polypeptide that binds to a ganglioside receptor. The ganglioside receptor can include a G_{T1b} receptor. The
25 identified polypeptide can be then administered to a patient. Optionally, the identified peptide can be conjugated or operatively attached to a therapeutic agent and/or prior to administration of the polypeptide to a patient.

 A further aspect of the invention relates to a method of treating a neurotoxic disease. In the method, a peptide that binds to a ganglioside receptor is
30 administered to a patient identified with a neurotoxic disease. The ganglioside receptor can comprise a G_{T1b} receptor. The peptide can act as a toxin antagonist.

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Yet another aspect of the invention relates to a method of identifying a neuron targeting peptide. The method includes exposing an isolated neuroreceptor to a phage display library. The phages can present a peptide comprising about 5 to about 30 amino acids. The isolated neuroreceptor can comprise isolated G_{T1b} that is coated on a substrate. The exposed phage library can be eluted with a first non-specific eluent. The exposed phage library can then be eluted with a second specific eluant.

BRIEF DESCRIPTION OF THE DRAWINGS

Further features of the present invention will become apparent to those skilled in the art to which the present invention relates from reading the following description of the invention with reference to the accompanying drawings in which:

Fig. 1 illustrates a phage display biopanning strategy. (A) Schematic depicting a single round of biopanning. (B) Flow chart detailing the four-staged biopanning. The initial round utilized pooled phage eluted from three wells with Glycine-HCl (pH 2.2), and three subsequent rounds eluted with rTTC.

Fig. 2 illustrates G_{T1b} binding by phage peptides. Phage peptides Tet1 and Tet2 and PLP were incubated at 10^9 pfus in G_{T1b} -coated wells or uncoated plates. ELISA was performed with an anti-phage HRP-conjugated antibody. The OD measured at 490 nm is depicted for all conditions. Both Tet1 and Tet2 phage bind G_{T1b} better than plate alone, but Tet1 binding is markedly greater than Tet2 binding. ANOVA revealed significant effects of G_{T1b} plate coating ($P < 0.001$), phage clone ($P < 0.001$), and a G_{T1b} by phage clone interaction ($P < 0.001$).

Fig. 3 illustrates that phage peptides and rTTC compete for G_{T1b} binding. Phage peptides and increasing rTTC concentrations (0 ng/ μ l, 25 ng/ μ l, 50 ng/ μ l, 75 ng/ μ l) were simultaneously incubated with G_{T1b} . rTTC did not hinder Tet1 binding except at the highest concentration (75 ng/ μ l). Tet2 showed a steady decrease in binding with increased rTTC concentration.

Fig. 4 illustrates phage clone cellular binding affinity and specificity. (A) Tet1, Tet2, random library (RL), and PLP were bound to PC12 cells and collected to obtain the cell-associated fraction. This cell-associated fraction

(Output) is divided by the phage titer added to the well (Input) to obtain the Bound Phage Ratio. Error bars represent SEM for 6 experiments. (B) Bound Phage ratios were obtained for Tet1 and Tet2 phage clones on HEK293 or differentiated PC12 cells. Error bars represent SEM over 6 experiments for PC12 cells and 4 experiments for 293 cells.

Fig. 5 illustrates phage binding visualized by immunofluorescence. Phage clones were exposed to HEK293 and PC12 cells and visualized under phase contrast and fluorescent microscopy. No fluorescence is detected on either 293 cells (A, B) or PC12 cells (G, H) grown without phage exposure. Phage binding is detected on PC12 (I, J) but not HEK293 cells (C, D) exposed to Tet1 phage. More robust Tet2 phage binding is detected on PC12 (K, L) than on HEK293 cells (E, F). While Tet1 phage binding appears to be relatively evenly distributed over the PC12 cells, Tet2 phage appears in aggregates on a fraction of cells imaged.

Fig. 6 illustrates fluorescein-conjugated synthetic peptide binding to neuronal membranes in vitro. Differentiated PC12 cells, primary motor neurons, and dorsal root ganglion cells were grown in culture. Fluorescein-conjugated synthetic Tet1 and Tet2 peptides were applied to the cells and binding was visualized under fluorescent microscopy. The figure shows the cells as seen under phase contrast as well as the respective frame under fluorescence. Tet1 demonstrated strong binding to all three cell lines (B, F, J) while Tet2 showed minimal binding (D, H, L).

Fig. 7 illustrates the effect of neuronal differentiation on Tet1 peptide binding and uptake. Undifferentiated PC12 cells (A–C) lack cell processes apparent on differentiated PC12 cells (D–F). Fluorescent microscopy reveals a concentration of Tet1 in the cytoplasm, suggesting internalization after binding (B and E). The process of binding and uptake appears to be enhanced in differentiated cells (E and F) in comparison with undifferentiated cells (B and C) which contain a lower concentration of membrane G_{T1b} . Finally, DAPI nuclear staining suggests that internalized Tet1 remains predominantly in the cytoplasm (C and E).

Fig. 8 illustrates fluorescein-conjugated synthetic peptide binding to neuronal membranes in DRG tissue. 20- μ m tissue sections were exposed to Tet1 or Tet2 for 1 h. No appreciable binding could be detected after Tet2 exposure. In

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contrast, Tet1 failed to bind to muscle tissue (A), but bound avidly to neurons in the DRG (B) and Spinal Cord Ventral Horn (C).

Fig. 9 illustrates fluorescein-conjugated Tet1 retrograde transport in vivo in sciatic nerve and lumbar spinal cord.

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DETAILED DESCRIPTION

The present invention relates generally to peptides and particularly to targeting peptides as well as to uses of such targeting peptides. By targeting peptide it is meant a peptide comprising a contiguous sequence of amino acids, which is characterized by selective localization to an organ, tissue, or cell type, which includes specific binding with an extracellular protein or molecule that is specifically expressed or produced in a specific tissue or cell type(s). Selective localization may be determined, for example, by methods wherein the putative targeting peptide sequence is incorporated into a protein that is displayed on the outer surface of a phage.

The peptides in accordance with the present invention have a preferential and/or selective binding affinity for nerve cells and particularly for a ganglioside (*e.g.*, trisialoganglioside (G_{T1b})) receptor on the axon of the neuron. The peptides in accordance with the present invention can therefore be used as neuron targeting peptides by preferentially binding to the G_{T1b} receptor on the axon of a neuron. Once bound to the receptor, the targeting peptide and any other molecule or macromolecule conjugated to the targeting peptide, undergoes retrograde transport to the cell body.

The peptides of the present invention can comprise about 5 to about 30 amino acids and include an amino acid sequence that preferentially binds to an isolated neuron receptor. The isolated neuron receptor can comprise G_{T1b} . The amino acid sequence of the peptide can be identified by biopanning phage displayed peptides against G_{T1b} . The biopanning strategy can be performed *in vitro* and the peptide displayed by the phage can comprise about 5 to about 30 amino acids. The G_{T1b} can be coated on a substrate (*e.g.*, plate or bead) or provided in solution.

In accordance with one aspect of the invention, the neuron targeting peptide can include about 5 to about 20 amino acids and comprise an at least three amino acid motif having the amino acid sequence LST. The about 7 to about 20 amino acid peptide can also include amino acids, such as histidine (H), tryptophan (W), and arginine (R). These amino acids can be provided in any particular order within the amino acid sequence of the peptide. Still other amino acids that can further be included in the peptide comprise tyrosine (Y) and leucine (L).

In a further aspect of the invention the neuron targeting peptide and can include an amino acid sequence selected from the group consisting of:

HLNILSTLWKYR (SEQ ID NO: 2);

SYQLSTHRWPLH (SEQ ID NO: 3); and derivatives thereof.

In another aspect of the invention the neuron targeting peptide can comprise about 7 to about 20 amino acids and include an amino acid sequence selected from the group consisting of:

KGTINPF (SEQ ID NO: 3);

XGAINPF (SEQ ID NO: 4);

KLTANPT (SEQ ID NO: 5);

NVHRGLH (SEQ ID NO: 6);

LTPWASS (SEQ ID NO: 7);

MLSNARH (SEQ ID NO: 8); and

a derivative thereof.

The present invention also embraces neuron targeting peptide derivatives, such as an analog, variant, mimetic, or fragment, with a selective and/or preferential neuronal binding affinity, but with limited binding affinity to non-neuron cells. Derivatives are defined as any modified form of the neuron targeting peptides described above, which also substantially retains the activity of the neuron targeting peptides disclosed herein. Such variants may take the form of amino acid substitutions that are generally based on the relative similarity of the amino acid side-chain substituents. For example, the amino acid substitution may be in the form of like for like substitution, such as a substitution of one polar amino acid residue for another polar amino acid residue or like for non-like, such as a

substitution of a polar amino acid residue for a non-polar residue as discussed in more detail below.

Replacement amino acid residues may be selected from the residues of alanine (A), arginine (R), asparagine (N), aspartic acid (D), cysteine (C), glutamic acid (E), glutamine (Q), glycine (G), histidine (H), isoleucine (I), leucine (L), lysine (K), methionine (M), phenylalanine (F), proline (P), serine (S), threonine (T), tryptophan (W), tyrosine (Y), and valine (V). The replacement amino acid residue may additionally be selected from unnatural amino acids. Within the above definitions of the peptides of the present invention, the specific amino acid residues of the peptide may be modified in such a manner that retains their ability to bind to a ganglioside receptor of a neuron cell. Thus, homologous substitution may occur, that is like-for-like substitution such as basic for basic, acidic for acidic, polar for polar, etc. Non-homologous substitution may also occur, that is from one class of residue to another or alternatively involving the inclusion of unnatural amino acids such as ornithine (O), diaminobutyric acid (B), norleucine, pyriylalanine, thienylalanine, naphthylalanine and phenylglycine and the like. Within each peptide, more than one amine acid residue may be modified at a time.

As used herein, amino acids are classified according to the following classes: basic (H, K, and R); acidic (D and E), polar (A, F, G, I, L, M, P, V, and W) non-polar (C, N, Q, S, T, and Y) and homologous and non-homologous substitution is defined using these classes. Thus, homologous substitution is used to refer to a substitution from within the same class, whereas non-homologous substitution refers to a substitution from a different class or by an unnatural amino acid.

It will be appreciated that biologically functional equivalents or derivatives, or even improvements, of the neuron targeting peptides in accordance with the present invention, can be made, generally using a neuron targeting peptide, such as a peptide defined by SEQ ID NO: 1, SEQ ID NO: 3, or SEQ ID NO: 6, as a starting point. Modifications and changes may be made in the structure of such a peptide and still obtain a molecule having like or otherwise desirable

characteristics. For example, certain amino acids may be substituted for other amino acids in the peptide without appreciable loss of interactive binding capacity.

Since it is the interactive capacity and nature of the neuron targeting peptide that defines that peptides' biological functional activity, certain amino acid sequence substitutions can be made in a amino acid sequence of a peptide and nevertheless obtain a peptide with like (agonistic) properties. It is thus contemplated that various changes may be made in the sequence of the neuron targeting peptides without appreciable loss of their neuron targeting affinity.

It is also well understood by the skilled artisan that, inherent in the definition of a "biologically functional equivalent" peptide, is the concept that there is a limit to the number of changes that may be made within a defined portion of the molecule and still result in a molecule with an acceptable level of equivalent biological activity. Biologically functional equivalent peptides are thus defined herein as those peptides in which certain, not most or all, of the amino acids may be substituted. Of course, a plurality of distinct peptides with different substitutions may easily be made and used in accordance with the invention.

Optionally, the neuron targeting peptides of the invention may include modifications of one or more amino acid residue(s) by way of addition of moieties (i.e., glycosylation, alkylation, acetylation, amidation, phosphorylation and the like). The neuron targeting peptide can also be linear or cyclized by, for example, flanking the peptide at both extremities by cysteine residues. Exemplary modifications can include those that allow or improve the coupling of a neuron targeting peptide of the invention to a therapeutic agent as described hereinafter (i.e., addition of sulfhydryl, amine groups).

The neuron targeting peptides in accordance with the present invention can be identified, for example, by phage display biopanning against immobilized trisialogangliosides (G_{T1b}). Phage display is an effective method for identifying novel peptides with specific binding properties. In this technique, a constrained or random library of oligonucleotides is inserted into one of the genes encoding phage coat proteins. The resulting library of phage presents the peptides encoded by the oligonucleotides on their surface, creating a physical link between the DNA sequence and the binding properties of the encoded peptide. Biopanning strategies

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that select for specific binding properties are repeated to enrich for phage presenting peptides with these properties. An exemplary biopanning strategy is illustrated in Fig. 1 and described in Example 1.

Because of their relatively small size, the neuron targeting peptides identified by the biopanning strategy of the invention can be synthesized in solution or on a solid support in accordance with conventional techniques. Various automatic synthesizers are commercially available and can be used in accordance with known protocols. Short peptide sequences, usually from about 5 up to about 35 to 50 amino acids, can be readily synthesized by such methods. Alternatively, recombinant DNA technology may be employed wherein a nucleotide sequence which encodes a peptide of the invention is inserted into an expression vector, transformed or transfected into an appropriate host cell, and cultivated under conditions suitable for expression. In addition, fragments of the neuron targeting peptides can be chemically synthesized using techniques known in the art, such as conventional Merrifield solid phase f-MOC or t-BOC chemistry.

In certain embodiments the neuron targeting peptides in accordance with the invention may be isolated or purified. Peptide purification techniques are well known to those of skill in the art. These techniques involve, at one level, the homogenization and crude fractionation of the cells, tissue or organ to peptide and non-peptide fractions. The peptide of interest may be further purified using chromatographic and electrophoretic techniques to achieve partial or complete purification (or purification to homogeneity). Analytical methods particularly suited to the preparation of a pure peptide are ion-exchange chromatography, gel exclusion chromatography, polyacrylamide gel electrophoresis, affinity chromatography, immunoaffinity chromatography and isoelectric focusing. An example of receptor protein purification by affinity chromatography is disclosed in U.S. Pat. No. 5,206,347, the entire text of which is incorporated herein by reference. A particularly efficient method of purifying peptides is fast pressure liquid chromatography (FPLC) or even high pressure liquid chromatography (HPLC).

A purified peptide is intended to refer to a composition, isolatable from other components, wherein the peptide is purified to any degree relative to its

naturally-obtainable state. An isolated or purified peptide, therefore, also refers to a peptide free from the environment in which it may naturally occur. Generally, "purified" will refer to a peptide composition that has been subjected to fractionation to remove various other components, and which composition substantially retains its expressed biological activity. Where the term "substantially purified" is used, this designation will refer to a composition in which peptide forms the major component of the composition, such as constituting about 50%, about 60%, about 70%, about 80%, about 90%, about 95%, or more of the peptides in the composition.

There is no general requirement that the neuron targeting peptide always be provided in their most purified state. Indeed, it is contemplated that less substantially purified products will have utility in certain embodiments. Partial purification may be accomplished by using fewer purification steps in combination, or by utilizing different forms of the same general purification scheme. For example, it is appreciated that a cation-exchange column chromatography performed utilizing an HPLC apparatus will generally result in a greater "-fold" purification than the same technique utilizing a low pressure chromatography system. Methods exhibiting a lower degree of relative purification may have advantages in total recovery of the peptide product, or in maintaining the activity of an expressed peptide.

In accordance with another aspect of the invention, the neuron targeting peptides can be operably linked, conjugated with or attached to a molecule, such as a therapeutic agent or diagnostic agent. The neuron targeting peptide can specifically deliver or target therapeutic agents and/or diagnostic agents to the neuron cells and particularly the G_{T1b} receptor of the neuron cells.

The therapeutic agent can include any organic chemical, such as a drug, a peptide including a variant or a modified peptide or a peptide-like molecule, a protein, an antibody or a fragment thereof such as a Fab (ab for antigen binding), a $F(ab')_2$, a Fc (c for crystallisable) or a scFv (sc for single chain and v for variable). Antibody fragments are described in detail in immunology manuals (such as Immunology, third edition 1993, Roitt, Brostoff and Male, ed Gambli, Mosby). The therapeutic agent may also be a nucleic acid molecule e.g. DNA, or

RNA, antisense or sense, oligonucleotide, double-stranded or single-stranded, circular or linear.

Examples of therapeutic agents that can be operably linked, conjugated with or attached to the neuron targeting peptides include a neurotrophic agent and/or neurotrophic growth factor, such as a ciliary neurotrophic factor (CNTF),
5 brain-derived neurotrophic factor (BDNF), insulin-like growth factor (IGF), and glial cell-line-derived neurotrophic factor (GDNF) NGF, α -FGF, β -FGF, PDGF, BDNF, CNTF, NT-3, NT 4/5, and mixtures thereof. Other therapeutic agents can comprise a neurotoxin light chain peptide, such as described in US Patent
10 Application Publication 2005/0019346, which is herein incorporated by reference in its entirety. Still other therapeutic agents can include a pharmaceutical agents such as synthetic anticholinergic agents, antihistamines, dopamine agonists, decarboxylase, dopamine-releasing agent, glycerol monoethers and derivatives, substituted alkanediols and derivatives, benzazole, orphenadrine citrate, and
15 cyclobenzaprine. In general, these aspects of the invention contemplate the use of any pharmacological agent or therapeutic agent that can be conjugated neuron targeting peptide and delivered in active form to the targeted neurons. It will be appreciated that the therapeutic agents conjugated to the neuron targeting peptides are not limited to the therapeutic agents described above and that other therapeutic
20 agents and other agents, which do not have therapeutic properties, can be conjugated to the neuron targeting peptides.

Conjugates of neuron targeting peptides and therapeutic agent may be readily prepared as fusion proteins using molecular biological techniques. Any fusion protein may be designed and made using any of the therapeutic agents
25 disclosed herein and those known in the art. The fusion protein technology is readily adapted to prepare fusion proteins in which the two portions are joined by a selectively cleavable peptide sequence.

The use of recombinant DNA techniques to achieve such ends is now standard practice to those of skill in the art. These methods include, for example,
30 *in vitro* recombinant DNA techniques, synthetic techniques and *in vivo* recombination/genetic recombination. DNA and RNA synthesis may, additionally, be performed using an automated synthesizers.

The preparation of such a fusion protein generally entails the preparation of a first and second DNA coding region and the functional ligation or joining of such regions, in frame, to prepare a single coding region that encodes the desired fusion protein. In the present context, the nucleotide sequence for the neuron targeting peptide can be joined in frame with a DNA sequence encoding a therapeutic agent. It is not generally believed to be particularly relevant which portion of the construct is prepared as the N-terminal region or as the C-terminal region.

Once the desired coding region has been produced, an expression vector is created. Expression vectors contain one or more promoters upstream of the inserted DNA regions that act to promote transcription of the DNA and to thus promote expression of the encoded recombinant protein. This is the meaning of "recombinant expression".

To obtain a so-called "recombinant" version of the conjugate of the neuron targeting peptide, it is expressed in a recombinant cell. The engineering of DNA segment(s) for expression in a prokaryotic or eukaryotic system may be performed by techniques generally known to those of skill in recombinant expression. It is believed that virtually any expression system may be employed in the expression of conjugate constructs comprising the neuron targeting peptide.

Such peptides may be successfully expressed in eukaryotic expression systems, however, it is envisioned that bacterial expression systems will be particularly useful for the large-scale preparation and subsequent purification of the conjugates comprising neuron targeting peptides. cDNAs may also be expressed in bacterial systems, with the peptide being expressed as fusions with β -galactosidase, ubiquitin, and the like. It is believed that bacterial expression will have advantages over eukaryotic expression in terms of ease of use and quantity of materials obtained thereby.

In terms of microbial expression, U.S. Pat. Nos. 5,583,013; 5,221,619; 4,785,420; 4,704,362; and 4,366,246 are incorporated herein by reference for the purposes of even further supplementing the present disclosure in connection with the expression of genes in recombinant host cells.

Recombinantly produced, the conjugates comprising the neuron targeting peptide and a therapeutic agent may be purified and formulated for human administration. Alternatively, nucleic acids encoding the conjugates may be delivered via gene therapy. Although naked recombinant DNA or plasmids may be employed, the use of liposomes or vectors is preferred. The ability of certain viruses to enter cells via receptor-mediated endocytosis and to integrate into the host cell genome and express viral genes stably and efficiently have made them attractive candidates for the transfer of foreign genes into mammalian cells. Preferred gene therapy vectors for use in the present invention will generally be viral vectors.

Retroviruses have promise as gene delivery vectors due to their ability to integrate their genes into the host genome, transferring a large amount of foreign genetic material, infecting a broad spectrum of species and cell types and of being packaged in special cell-lines. Other viruses, such as adenovirus, herpes simplex viruses (HSV), cytomegalovirus (CMV), and adeno-associated virus (AAV), such as those described by U.S. Pat. No. 5,139,941 (incorporated herein by reference), may also be engineered to serve as vectors for gene transfer.

Of course, in using viral delivery systems, one will desire to purify the virion sufficiently to render it essentially free of undesirable contaminants, such as defective interfering viral particles or endotoxins and other pyrogens such that it will not cause any untoward reactions in the cell, animal or individual receiving the vector construct. A preferred means of purifying the vector involves the use of buoyant density gradients, such as cesium chloride gradient centrifugation.

In accordance with another aspect of the invention, conjugates of the neuron targeting peptides and therapeutic agents can be either directly linked or indirectly linked to a binding region binding peptide to form a bispecific peptide construct. The preparation and use of bispecific peptides in general is well known in the art, and is further disclosed herein

The bispecific peptide constructs can be formed by attaching, in frame, nucleic acid sequences encoding the neuron targeting peptide to nucleic acid sequences encoding the binding peptide and therapeutic agent to create an

expression unit or vector. Recombinant expression results in translation of the new nucleic acid, to yield the desired bispecific peptide construct.

Depending on the specific therapeutic agent used as part of the fusion protein, it may be desirable to provide a peptide spacer, which is capable of folding
5 into a disulfide-bonded loop structure, operably attaching the neuron targeting peptide with the therapeutic agent. Proteolytic cleavage within the loop would then yield a heterodimeric peptide wherein the neuron targeting peptide and the therapeutic agent are linked by only a single disulfide bond. A non-cleavable peptide spacer may also be provided to operably attach the neuron targeting
10 peptide and the therapeutic agent of the fusion protein. It will be appreciated that the compositions are thus "linked" in any operative manner that allows each region to perform its intended function without significant impairment.

In accordance with another aspect of the invention, the neuron targeting peptides can be conjugated to therapeutic agents using biochemical cross-linkers.
15 Biochemical cross-linkers can be used to form molecular bridges that tie together functional groups of two different molecules. To link two different peptides or proteins in a step-wise manner, hetero-bifunctional cross-linkers can be used that eliminate unwanted homopolymer formation.

Hetero-bifunctional cross-linkers contain two reactive groups: one
20 generally reacting with a primary amine group and the other generally reacting with a thiol group. Through the primary amine reactive group, the cross-linker may react with the lysine residue(s) of one peptide or protein (*e.g.*, the neuron targeting peptide) and through the thiol reactive group, the cross-linker, already tied up to the first peptide or protein, reacts with the cysteine residue (free
25 sulfhydryl group) of the other protein.

Compositions therefore generally have, or are derivatized to have, a functional group available for cross-linking purposes. This requirement is not considered to be limiting in that a wide variety of groups can be used in this manner. For example, primary or secondary amine groups, hydrazide or hydrazine
30 groups, carboxyl alcohol, phosphate, or alkylating groups may be used for binding or cross-linking.

The spacer arm between the two reactive groups of cross-linkers may have various length and chemical compositions. A longer spacer arm allows a better flexibility of the conjugate components while some particular components in the bridge (*e.g.*, benzene group) may lend extra stability to the reactive group or an increased resistance of the chemical link to the action of various aspects (*e.g.*, disulfide bond resistant to reducing agents). The use of peptide spacers, such as L-Leu-L-Ala-L-Leu-L-Ala, is also contemplated.

It is preferred that a cross-linker having reasonable stability in blood will be employed. Numerous types of disulfide-bond containing linkers are known that can be successfully employed to conjugate the neuron targeting peptide and the therapeutic agent. Linkers that contain a disulfide bond that is sterically hindered may prove to give greater stability *in vivo*, preventing release of the agent prior to binding at the site of action. These linkers are thus one preferred group of linking agents.

One example of a cross-linking reagent is SMPT, which is a bifunctional cross-linker containing a disulfide bond that is "sterically hindered" by an adjacent benzene ring and methyl groups. It is believed that steric hindrance of the disulfide bond serves a function of protecting the bond from attack by thiolate anions, such as glutathione, which can be present in tissues and blood, and thereby help in preventing decoupling of the conjugate prior to the delivery of the attached therapeutic agent. It is contemplated that the SMPT agent may also be used in connection with the bispecific ligands.

The SMPT cross-linking reagent, as with many other known cross-linking reagents, lends the ability to cross-link functional groups such as the SH of cysteine or primary amines (*e.g.*, the epsilon amino group of lysine). Another possible type of cross-linker includes the hetero-bifunctional photoreactive phenylazides containing a cleavable disulfide bond such as sulfosuccinimidyl-2-(p-azido salicylamido) ethyl-1,3'-dithiopropionate. The N-hydroxy-succinimidyl group reacts with primary amino groups and the phenylazide (upon photolysis) reacts non-selectively with any amino acid residue.

Other useful cross-linkers, not considered to contain or generate a protected disulfide, include SATA, SPDP and 2-iminothiolane. The use of such cross-

linkers is well understood in the art. Once conjugated, the conjugate is separated from unconjugated targeting and therapeutic agents and from other contaminants. A large a number of purification techniques are available for use in providing conjugates of a sufficient degree of purity to render them clinically useful.

5 Purification methods based upon size separation, such as gel filtration, gel permeation or high performance liquid chromatography, will generally be of most use. Other chromatographic techniques, such as Blue-Sepharose separation, may also be used.

10 Although it is contemplated that any linking moiety will have reasonable stability in blood, to prevent substantial release of the attached therapeutic agent before targeting to the neuron, in certain aspects, the use of biologically-releasable bonds and/or selectively cleavable spacers or linkers is contemplated. "Biologically-releasable bonds" and "selectively cleavable spacers or linkers" still have reasonable stability in the circulation.

15 Neuron targeting peptides in accordance with the present invention may thus be linked to one or more therapeutic agents via a biologically-releasable bond. "Biologically-releasable bonds" or "selectively hydrolyzable bonds" include all linkages that are releasable, cleavable or hydrolyzable only or preferentially under certain conditions. This includes disulfide and trisulfide bonds and acid-labile
20 bonds, as described in U.S. Pat. Nos. 5,474,765 and 5,762,918, each specifically incorporated herein by reference.

The use of an acid sensitive spacer for attachment of a therapeutic agent to a neuron targeting peptide in accordance with the present invention is particularly contemplated. In such embodiments, the therapeutic agents or drugs are released
25 within the acidic compartments inside a cell. It is contemplated that acid-sensitive release may occur extracellularly, but still after specific targeting.

The neuron targeting peptides may also be derivatized to introduce functional groups permitting the attachment of the therapeutic agent through a biologically releasable bond. The neuron targeting peptide may thus be derivatized
30 to introduce side chains terminating in hydrazide, hydrazine, primary amine or secondary amine groups. Therapeutic agents may be conjugated through a Schiff's

base linkage, a hydrazone or acyl hydrazone bond or a hydrazide linker (U.S. Pat. Nos. 5,474,765 and 5,762,918, each specifically incorporated herein by reference).

5 Additionally, as described in U.S. Pat. Nos. 5,474,765 and 5,762,918, each specifically incorporated herein by reference, the neuron targeting peptides in accordance with the present invention may be operably attached to the therapeutic agent(s) through one or more biologically releasable bonds that are enzyme-sensitive bonds, including peptide bonds, esters, amides, phosphodiester and glycosides.

10 Other aspects of the invention concern the use of peptide linkers that include at least a first cleavage site for a peptidase and/or proteinase that is preferentially located within a disease site. The peptide-mediated delivery of the attached therapeutic agent thus results in cleavage specifically within the disease site, resulting in the specific release of the active agent. Certain peptide linkers will include a cleavage site that is recognized by one or more enzymes involved in remodeling.

15 In another aspect of the invention, the therapeutic agent that is operably linked to the neuron targeting peptide can comprise a macromolecular complex, such as a vector, a bacteriophage, a bacterium, a liposome, a microparticle, a nanoparticle (*e.g.*, a gold nanoparticle), a magnetic bead, a yeast cell, a mammalian cell, a cell or a microdevice. An example of a macromolecular complex comprises a vector. The vector conjugated to the neuron targeting peptide can be used to deliver at least one therapeutic gene or gene of interest to a neuron cell (*e.g.*, gene or nucleotide sequence encoding a neurotoxin light chain peptide as disclosed in US Patent Application Publication No. 2005/0019346). In the context of the invention, the vector that is conjugated or operably linked to the neuron targeting peptide can be a plasmid, a synthetic (non viral) or a viral vector.

25 Plasmid denotes an extrachromosomal circular DNA capable of autonomous replication in a given cell. Plasmids can be designed for amplification in bacteria and expression in eukaryotic host cell. Such plasmids can be purchased from a variety of manufacturers. Examples of plasmids include but are not limited to those derived from pBR322 (Gibco BRL), pUC (Gibco BRL), pBluescript (Stratagene), pREP4, pCEP4 (Invitrogene), pCI (Promega) and p Poly (Lathe et al.,

Gene 57 (1987), 193-201). It is also possible to engineer such a plasmid by molecular biology techniques (Sambrook et al., Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor (1989), NY). A plasmid may also comprise a selection gene in order to select or identify the transfected cells (*e.g.*, by
5 complementation of a cell auxotrophy, antibiotic resistance), stabilizing elements (*e.g.* *cer* sequence; Summers and Sherrat, Cell 36 (1984), 1097-1103) or integrative elements (*e.g.*, LTR viral sequences).

The vector can be from viral origin and may be derived from a variety of viruses, such as herpes viruses, cytomegaloviruses, foamy viruses, lentiviruses,
10 AAV (adeno-associated virus), poxviruses, adenoviruses and retroviruses. Such viral vectors are well known in the art. The term viral vector as used in the present invention encompasses the vector genome, the viral particles (*i.e.*, the viral capsid including the viral genome) as well as empty viral capsids.

A viral vector which is particularly appropriate for the present invention is
15 AAV. Large neuron targeting peptides up to 30 amino acids in length can be conjugated, operably linked, and/or inserted at the N-terminus of the AAV2 VP1, VP2 and/or VP3 capsid protein. The full length nucleotide sequence of the wild type AAV2 vector is set out as SEQ ID NO: 9. The amino acid sequence of VP1 capsid protein (SEQ ID NO: 10) is encoded by the nucleotides 2203-4410 of
20 SEQ ID NO: 9, the amino acid sequence of VP2 capsid protein (SEQ ID NO: 11) is encoded by nucleotides 2614-4410 of SEQ ID NO: 9 and the amino acid sequence of VP3 capsid protein (SEQ ID NO: 12) is encoded by nucleotides 2809-4410 of SEQ ID NO: 9.

AAV vectors (viral particles) can be used to encode capsid proteins
25 of AAV vectors that comprise insertions of neuron targeting peptides in accordance with the present invention. One such technique is described in US Patent Application Publication No. 2002/0192832, herein incorporated by reference in its entirety. Exemplary, AAV vectors are AAV2 vectors. DNA encoding the insertion of the neuron targeting peptide can follow the cap gene
30 DNA encoding amino acid position 139 and/or position 161 in the VP1/VP2 capsid region, and/or amino acid position 459, 584, 588 and/or 657 in the VP3 region.

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Particular peptide insertions following AAV2 VP1 amino acid 588 are well tolerated and can alter AAV2 natural tropism.

While the capsid sites/regions amenable to insertions have been described herein with respect to AAV2, those skilled in the art will understand that
5 corresponding sites in other parvoviruses, both autonomously-replicating parvoviruses and other AAV dependent viruses, are also sites/regions amenable to insertions in those viruses. The neuron targeting peptides of interest may impart a different binding/targeting ability to the vector. As a result, the vectors of the invention exhibit altered characteristics in comparison to wild type AAV, including
10 but not limited to, altered cellular tropism and/or antigenic properties. The invention also contemplates cells, plasmids and viruses, which comprise polynucleotides encoding the capsid proteins of the invention.

It is contemplated that in addition to the neuron targeting peptides of interest, amino acids serving as linker/scaffolding sequences may be included in
15 the AAV vector capsid insert to maintain the functional conformation of the capsid. The linker/scaffolding sequences are short sequences, which flank the insertion of interest in the mutated capsid protein. For example, the insertion may have the amino acids TG at its amino terminus and the tripeptide ALS, GLS or LLA at its carboxy terminus.

20 Techniques to produce AAV vectors, in which an AAV genome to be packaged, rep and cap genes, and helper virus functions are provided to a cell are standard in the art. Production of AAV vectors requires that the following components are present within a single cell (denoted herein as a packaging cell): a rAAV construct consisting of a DNA of interest flanked by AAV inverted
25 terminal repeats, an AAV helper construct containing the capsid gene (which may or may not comprise an insert) and the rep gene, and an adenovirus helper plasmid or infected with an adenovirus. The rAAV construct may be delivered to a packaging cell by transfection in a plasmid, infection by a viral genome or may be integrated into the packaging cell genome. The AAV helper construct may be
30 delivered to a packaging cell by transfection of a plasmid or integrated into the packaging cell genome. The adenovirus helper plasmid or adenovirus may be delivered to the packaging cell by transfection/infection. The term "helper virus

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functions" refers to the functions carried out by the addition of an adenovirus helper plasmid or infection of adenovirus to support production of AAV viral particles.

One method of generating a packaging cell with all the necessary components for AAV production is the triple transfection method. In this method a cell such as a 293 cell is transfected with the rAAV construct, the AAV helper construct and a adenovirus helper plasmid or infected with adenovirus. The advantages of the triple transfection method are that it is easily adaptable and straightforward. Another method of generating a packaging cell is to create a cell line, which stably expresses all the necessary components for AAV vector production. For example, a plasmid expressing the rAAV construct, a helper construct expressing the rep and cap proteins (modified or wild type) and a selectable marker, such as Neo, are integrated into the genome of a cell. The packaging cell line is then infected with a helper virus such as adenovirus. The advantages of this method are that the cells are selectable and are suitable for large-scale production of the vector.

AAV vectors of the invention that exhibit an altered cellular tropism may differ from wild type in that the natural tropism of AAV may be reduced or abolished by insertion or substitution of the amino acids of the neuron targeting peptide in the capsid protein of the vector. The insertion or substitution of the amino acids of the neuron targeting peptide can then target the vector to neurons as well as other cell types perhaps not targeted by wild type AAV. Capsid amino acids can be modified to remove wild type tropism and to introduce the new tropism to the G_{T1B} receptor of the neurons.

The present invention also encompasses modified AAV vectors, the capsid protein(s) of which are biotinylated in vivo. For example, the invention contemplates AAV capsids engineered to include the biotin acceptor peptide (BAP). Expression of the E. coli enzyme biotin protein ligase during AAV vector biosynthesis in the presence of biotin results in biotinylation of the AAV capsid proteins as they are made and assembled into viral particles.

In order to biotinylate the AAV viral particles, a system for expressing the biotin ligase enzyme in packaging cell lines is contemplated by the present

invention. The invention provides for plasmids, such as the pCMV plasmid, which direct expression of the biotin ligase gene within the packaging cell line. For production of the biotinylated AAV vector the following components need to be transfected into a packaging cell: a rAAV vector comprising DNA of interest
5 flanked by AAV inverted terminal repeats, an AAV helper construct containing a capsid gene with a BAP insert and the rep gene, adenovirus helper plasmid or infected with adenovirus, and the biotin ligase gene (BirA). In this system, the biotin ligase gene may be expressed by a plasmid including the BirA gene (such as pCMV-BirA) infection with an adenovirus which expresses the BirA gene or by
10 using a packaging cell line that is stably transfected with the BirA gene.

It is contemplated that the biotinylated AAV viral particles will serve as substrates for conjugation of neuron targeting peptides to the surface of vector particles through utilizing avidin/streptavidin-biotin chemistry. In addition, the biotinylated AAV viral particles are contemplated to be useful for visualizing the
15 biodistribution of the viral particles both *in vivo* and *in vitro*. The biotinylated viral particles can be visualized with fluorescence or enzymatically with labeled streptavidin compounds. Biotinylation is also useful for conjugating epitope shielding moieties, such as polyethylene glycol, to the AAV vector. The conjugation of shielding moieties allows the vector to evade immune recognition.
20 Biotinylation of the AAV vector is also contemplated to enhance intracellular trafficking of viral particles through conjugation of proteins or peptides such as nuclear transport proteins. Biotinylation may also be used to conjugate proteins or peptides, which affect the processing of AAV vector genomes such as increasing the efficiency of integration. In addition, biotinylation may also be used to
25 conjugate proteins or peptides that affect the target cells, *e.g.*, proteins that make a target cell more susceptible to infection or proteins that activate a target cell thereby making it a better target for the expression of a therapeutic or antigenic peptide.

In yet a further aspect of the invention, the neuron targeting peptides can be
30 operably linked, conjugated to, or attached to a diagnostic agent, such as a detectable label. "Detectable labels" are compounds or elements that can be detected due to their specific functional properties, or chemical characteristics, the

use of which allows the component to which they are attached to be detected, and further quantified if desired. In polypeptide conjugates for *in vivo* diagnostic protocols or "imaging methods" labels are required that can be detected using non-invasive methods.

5 Many appropriate imaging agents are known in the art, as are methods for their attachment to peptides (see, *e.g.*, U.S. Pat. Nos. 5,021,236 and 4,472,509, both incorporated herein by reference). Certain attachment methods involve the use of a metal chelate complex employing, for example, an organic chelating agent such a DTPA attached to the peptide (U.S. Pat. No. 4,472,509). Monoclonal
10 polypeptides may also be reacted with an enzyme in the presence of a coupling agent such as glutaraldehyde or periodate. Conjugates with fluorescein markers are prepared in the presence of these coupling agents or by reaction with an isothiocyanate.

An example of detectable labels are the paramagnetic ions. In this case,
15 suitable ions include chromium (III), manganese (II), iron (III), iron (II), cobalt (II), nickel (II), copper (II), neodymium (III), samarium (III), ytterbium (III), gadolinium (III), vanadium (II), terbium (III), dysprosium (III), holmium (III) and erbium (III), with gadolinium being particularly preferred.

Ions useful in other contexts, such as X-ray imaging, include but are not
20 limited to lanthanum (III), gold (III), lead (II), and especially bismuth (III). Fluorescent labels include rhodamine, fluorescein and renographin. Rhodamine and fluorescein are often linked via an isothiocyanate intermediate.

In the case of radioactive isotopes for diagnostic applications, suitable examples include carbon¹⁴, chromium⁵¹, chlorine³⁶, cobalt⁵⁷, copper⁶⁷, Eu¹⁵²,
25 gallium⁶⁷, gallium⁶⁸, hydrogen³, iodine¹²³, iodine¹²⁵, iodine¹³¹, indium¹³¹, iron⁵⁹, phosphorus³², rhenium¹⁸⁶, rhenium¹⁸⁸, selenium⁷⁵, sulphur³⁵, technetium^{99m} and yttrium⁹⁰. ¹²⁵I is often being preferred for use in certain embodiments, and technetium^{99m} and indium¹¹¹ are also often preferred due to their low energy and suitability for long range detection.

30 Radioactively labeled neuron targeting peptide derivatives for use in the present invention may be produced according to well-known methods in the art. For instance, intermediary functional groups that are often used to bind

radioisotopic metallic ions to polypeptides are diethylenetriaminepentaacetic acid (DTPA) and ethylene diaminetetracetic acid (EDTA).

Peptides can also be iodinated by contact with sodium or potassium iodide and a chemical oxidizing agent such as sodium hypochlorite, or an enzymatic oxidizing agent, such as lactoperoxidase. Neuron targeting peptides according to the invention may be labeled with technetium^{99m} by ligand exchange process, for example, by reducing pertechnate with stannous solution, chelating the reduced technetium onto a Sephadex column and applying the peptide to this column; or by direct labeling techniques, *e.g.*, by incubating pertechnate, a reducing agent such as SnCl_2 , a buffer solution such as sodium-potassium phthalate solution, and the polypeptide.

Any of the foregoing type of detectably labeled neuron targeting peptides may be used in the imaging or combined imaging and treatment aspects of the present invention. They are equally suitable for use in *in vitro* diagnostics.

In accordance with another aspect of the invention, pharmaceutical compositions can be prepared comprising the neuron targeting peptides or conjugates thereof. The pharmaceutical compositions will generally comprise an effective amount of neuron targeting peptides or conjugates thereof, dissolved or dispersed in a pharmaceutically acceptable carrier or aqueous medium. Combined therapeutics are also contemplated, and the same type of underlying pharmaceutical compositions may be employed for both single and combined medicaments.

The phrases "pharmaceutically or pharmacologically acceptable" refer to molecular entities and compositions that do not produce an adverse, allergic or other untoward reaction when administered to an animal, or a human, as appropriate. Veterinary uses are equally included within the invention and "pharmaceutically acceptable" formulations include formulations for both clinical and/or veterinary use.

As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any

conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. For human administration, preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biologics standards. Supplementary active ingredients can also be incorporated into the compositions.

"Unit dosage" formulations are those containing a dose or sub-dose of the administered ingredient adapted for a particular timed delivery. For example, exemplary "unit dosage" formulations are those containing a daily dose or unit or daily sub-dose or a weekly dose or unit or weekly sub-dose and the like. The neuron targeting peptides or conjugates thereof of the present invention will most often be formulated for parenteral administration, *e.g.*, formulated for injection via the intravenous, intramuscular, sub-cutaneous, transdermal, or other such routes, including peristaltic administration and direct instillation into disease site (intracavity administration). The preparation of an aqueous composition that contains a neuron targeting peptide or a conjugate thereof as an active ingredient will be known to those of skill in the art in light of the present disclosure. Typically, such compositions can be prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for using to prepare solutions or suspensions upon the addition of a liquid prior to injection can also be prepared; and the preparations can also be emulsified.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions; formulations including sesame oil, peanut oil or aqueous propylene glycol; and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases, the form should be sterile and fluid to the extent that syringability exists. It should be stable under the conditions of manufacture and storage and should be preserved against the contaminating action of microorganisms, such as bacteria and fungi.

Neuron targeting peptides or conjugates thereof can be formulated into a sterile aqueous composition in a neutral or salt form. Solutions as free base or pharmacologically acceptable salts can be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Pharmaceutically acceptable salts, include the acid addition salts (formed with the free amino groups of the protein),

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and those that are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, trifluoroacetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like.

Suitable carriers include solvents and dispersion media containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and/or by the use of surfactants.

Under ordinary conditions of storage and use, all such preparations should contain a preservative to prevent the growth of microorganisms. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Prior to or upon formulation, neuron targeting peptides or conjugates thereof should be extensively dialyzed to remove undesired small molecular weight molecules, and/or lyophilized for more ready formulation into a desired vehicle, where appropriate. Sterile injectable solutions are prepared by incorporating the active agents in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as desired, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle that contains the basic dispersion medium and the required other ingredients from those enumerated above.

In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-

drying techniques that yield a powder of the active ingredient, plus any additional desired ingredient from a previously sterile-filtered solution thereof.

Suitable pharmaceutical compositions in accordance with the invention will generally include an amount of the neuron targeting peptide or conjugates thereof
5 admixed with an acceptable pharmaceutical diluent or excipient, such as a sterile aqueous solution, to give a range of final concentrations, depending on the intended use. The techniques of preparation are generally well known in the art as exemplified by Remington's Pharmaceutical Sciences, 16th Ed. Mack Publishing Company, 1980, incorporated herein by reference. Moreover, for human
10 administration, preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biological Standards. Upon formulation, the polypeptide or conjugate solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective.

15 Formulations of neuron targeting peptides or conjugates thereof are easily administered in a variety of dosage forms, such as the type of injectable solutions described above, but other pharmaceutically acceptable forms are also contemplated, *e.g.*, tablets, pills, capsules or other solids for oral administration, suppositories, pessaries, nasal solutions or sprays, aerosols, inhalants, topical
20 formulations, liposomal forms and the like. The type of form for administration will be matched to the disease or disorder to be treated.

Pharmaceutical "slow release" capsules or "sustained release" compositions or preparations may be used and are generally applicable. Slow release formulations are generally designed to give a constant drug level over an extended
25 period and may be used to deliver the neuron targeting peptides or conjugates thereof in accordance with the present invention. The slow release formulations are typically implanted in the vicinity of the disease site.

Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the polypeptide or
30 immunoconjugate, which matrices are in the form of shaped articles, *e.g.*, films or microcapsule. Examples of sustained-release matrices include polyesters; hydrogels, for example, poly(2-hydroxyethyl-methacrylate) or poly(vinylalcohol);

polylactides, *e.g.*, U.S. Pat. No. 3,773,919; copolymers of L-glutamic acid and .gamma. ethyl-L-glutamate; non-degradable ethylene-vinyl acetate; degradable lactic acid-glycolic acid copolymers, such as the Lupron DepotTM (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate); and poly-D-(-)-3-hydroxybutyric acid.

While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated polypeptides remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37°C., thus reducing biological activity and/or changing immunogenicity. Rational strategies are available for stabilization depending on the mechanism involved. For example, if the aggregation mechanism involves intermolecular S-S bond formation through thio-disulfide interchange, stabilization is achieved by modifying sulfhydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, developing specific polymer matrix compositions, and the like.

In certain embodiments, liposomes and/or nanoparticles may also be employed with the neuron targeting peptides or conjugates thereof. The formation and use of liposomes is generally known to those of skill in the art, as summarized below.

Liposomes are formed from phospholipids that are dispersed in an aqueous medium and spontaneously form multilamellar concentric bilayer vesicles (also termed multilamellar vesicles (MLVs)). MLVs generally have diameters of from 25 nm to 4 μ m. Sonication of MLVs results in the formation of small unilamellar vesicles (SUVs) with diameters in the range of 200 to 500 Å, containing an aqueous solution in the core.

Phospholipids can form a variety of structures other than liposomes when dispersed in water, depending on the molar ratio of lipid to water. At low ratios, the liposome is the preferred structure. The physical characteristics of liposomes depend on pH, ionic strength and the presence of divalent cations. Liposomes can show low permeability to ionic and polar substances, but at elevated temperatures

undergo a phase transition which markedly alters their permeability. The phase transition involves a change from a closely packed, ordered structure, known as the gel state, to a loosely packed, less-ordered structure, known as the fluid state. This occurs at a characteristic phase-transition temperature and results in an increase in permeability to ions, sugars and drugs.

Liposomes interact with cells via four different mechanisms: Endocytosis by phagocytic cells of the reticuloendothelial system such as macrophages and neutrophils; adsorption to the cell surface, either by nonspecific weak hydrophobic or electrostatic forces, or by specific interactions with cell-surface components; fusion with the plasma cell membrane by insertion of the lipid bilayer of the liposome into the plasma membrane, with simultaneous release of liposomal contents into the cytoplasm; and by transfer of liposomal lipids to cellular or subcellular membranes, or vice versa, without any association of the liposome contents. Varying the liposome formulation can alter which mechanism is operative, although more than one may operate at the same time.

Nanocapsules can generally entrap compounds in a stable and reproducible way. To avoid side effects due to intracellular polymeric overloading, such ultrafine particles (sized around 0.1 μm) should be designed using polymers able to be degraded *in vivo*. Biodegradable polyalkyl-cyanoacrylate nanoparticles that meet these requirements are contemplated for use in the present invention, and such particles may be easily made.

The neuron targeting peptides or conjugates thereof can also be formulated for topical administration. Topical formulations include those for delivery via the mouth (buccal) and through the skin. "Topical delivery systems" also include transdermal patches containing the ingredient to be administered. Delivery through the skin can further be achieved by iontophoresis or electrotransport, if desired.

Formulations suitable for topical administration in the mouth include lozenges comprising the ingredients in a flavored basis, usually sucrose and acacia or tragacanth; pastilles comprising the active ingredient in an inert basis such as gelatin and glycerin, or sucrose and acacia; and mouthwashes comprising the ingredient to be administered in a suitable liquid carrier.

Formulations suitable for topical administration to the skin include ointments, creams, gels and pastes comprising the ingredient to be administered in a pharmaceutical acceptable carrier. The formulation of neuron targeting peptide or conjugates thereof for topical use, such as in creams, ointments and gels, includes the preparation of oleaginous or water-soluble ointment bases, as is well known to those in the art. For example, these compositions may include vegetable oils, animal fats, and more preferably, semisolid hydrocarbons obtained from petroleum. Particular components used may include white ointment, yellow ointment, cetyl esters wax, oleic acid, olive oil, paraffin, petrolatum, white petrolatum, spermaceti, starch glycerite, white wax, yellow wax, lanolin, anhydrous lanolin and glyceryl monostearate. Various water-soluble ointment bases may also be used, including glycol ethers and derivatives, polyethylene glycols, polyoxyl 40 stearate and polysorbates.

Formulations for rectal administration may be presented as a suppository with a suitable base comprising, for example, cocoa butter or a salicylate. Formulations suitable for vaginal administration may be presented as pessaries, tampons, creams, gels, pastes, foams or spray formulations containing in addition to the active ingredient such carriers as are known in the art to be appropriate.

Local delivery via the nasal and respiratory routes is contemplated for treating various conditions. These delivery routes are also suitable for delivering agents into the systemic circulation. Formulations of active ingredients in carriers suitable for nasal administration are therefore also included within the invention, for example, nasal solutions, sprays, aerosols and inhalants. Where the carrier is a solid, the formulations include a coarse powder having a particle size, for example, in the range of 20 to 500 microns, which is administered, *e.g.*, by rapid inhalation through the nasal passage from a container of the powder held close up to the nose.

Suitable formulations wherein the carrier is a liquid are useful in nasal administration. Nasal solutions are usually aqueous solutions designed to be administered to the nasal passages in drops or sprays and are prepared so that they are similar in many respects to nasal secretions, so that normal ciliary action is maintained. In addition, antimicrobial preservatives, similar to those used in ophthalmic preparations, and appropriate drug stabilizers, if required, may be

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included in the formulation. Various commercial nasal preparations are known and include, for example, antibiotics and antihistamines and are used for asthma prophylaxis.

5 Inhalations and inhalants are pharmaceutical preparations designed for delivering a drug or compound into the respiratory tree of a patient. A vapor or mist is administered and reaches the affected area. This route can also be employed to deliver agents into the systemic circulation. Inhalations may be administered by the nasal or oral respiratory routes. The administration of inhalation solutions is only effective if the droplets are sufficiently fine and
10 uniform in size so that the mist reaches the bronchioles.

Another group of products, also known as inhalations, and sometimes called insufflations, comprises finely powdered or liquid drugs that are carried into the respiratory passages by the use of special delivery systems, such as pharmaceutical aerosols, that hold a solution or suspension of the drug in a
15 liquefied gas propellant. When released through a suitable valve and oral adapter, a metered dose of the inhalation is propelled into the respiratory tract of the patient. Particle size is of major importance in the administration of this type of preparation. It has been reported that the optimum particle size for penetration into the pulmonary cavity is of the order of 0.5 to 7 μm . Fine mists are produced by
20 pressurized aerosols and hence their use is considered advantageous.

The present invention also provides therapeutic kits including neuron targeting peptides or conjugates thereof for use in the present treatment methods. Such kits will generally contain, in suitable container means, a pharmaceutically acceptable formulation of at least one neuron targeting peptide or conjugates
25 thereof. The kits may also contain other pharmaceutically acceptable formulations, either for diagnosis/imaging or combined therapy.

The kits may have a single container (container means) that contains the neuron targeting peptide or conjugates thereof, with or without any additional components, or they may have distinct containers for each desired agent. Where
30 combined therapeutics are provided, a single solution may be pre-mixed, either in a molar equivalent combination, or with one component in excess of the other.

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Alternatively, comprising the neuron targeting peptides or conjugates thereof may be maintained separately within distinct containers prior to administration to a patient.

5 When the components of the kit are provided in one or more liquid solutions, the liquid solution is preferably an aqueous solution, with a sterile aqueous solution being particularly preferred. However, the components of the kit may be provided as dried powder(s). When reagents or components are provided as a dry powder, the powder can be reconstituted by the addition of a suitable solvent. It is envisioned that the solvent may also be provided in another container.

10 The containers of the kit will generally include at least one vial, test tube, flask, bottle, syringe or other container means, into which the neuron targeting peptide or conjugates thereof, and any other desired agent, may be placed and, preferably, suitably aliquoted. Where separate components are included, the kit will also generally contain a second vial or other container into which these are
15 placed, enabling the administration of separated designed doses. The kits may also comprise a second/third container means for containing a sterile, pharmaceutically acceptable buffer or other diluent.

The kits may also contain a means by which to administer the neuron targeting peptides or conjugate to an animal or patient, *e.g.*, one or more needles or
20 syringes, or even an eye dropper, pipette, or other such like apparatus, from which the formulation may be injected into the animal or applied to a diseased area of the body. The kits of the present invention will also typically include a means for containing the vials, or such like, and other component, in close confinement for commercial sale, such as, *e.g.*, injection or blow-molded plastic containers into
25 which the desired vials and other apparatus are placed and retained.

The present invention also relates to a method of targeting at least one neuron. The method comprises identifying a polypeptide that binds to a ganglioside receptor. The peptide can be identified by biopanning phage displayed peptides against isolated G_{T1b}. The identified polypeptide can be then administered
30 to a patient. Optionally, the identified peptide can be conjugated or operatively attached to a therapeutic agent and/or prior to administration of the polypeptide to a patient.

The present invention also provides, methods of, and uses in, targeting therapeutic agents to cells expressing G_{T1b} receptor. These methods comprise contacting, in the presence a population of cells or tissues that includes a G_{T1b} receptors (*e.g.*, neurons) with a composition comprising the neuron targeting peptides or conjugates thereof.

Further methods and uses of the invention are in analyzing the biological roles of the G_{T1b} receptor. In the method, a biological composition or tissue that comprises a population of cells that express G_{T1b} receptors are contacted with a composition comprising a biologically effective amount of at least of at least one of neuron targeting peptide or conjugates thereof. The effect of the neuron targeting peptide or conjugate thereof on at least a first biological response to the neuron targeting peptide or conjugate thereof is then determined, such that an alteration in a biological response in the presence of the at least one of neuron targeting peptide or conjugates thereof is indicative of a response mediated by the G_{T1b} receptor.

Toxin antagonist methods and uses are further provided, including those to specifically inhibit bacterial toxin or neurotoxin (*e.g.*, clostridial neurotoxin) uptake are also provided in accordance with the present invention. These methods can comprise contacting a population of G_{T1b} expressing cells or tissues (*e.g.*, neurons), that have been exposed to a bacterial toxin or neurotoxin (*e.g.*, clostridial neurotoxin) with a composition comprising a biologically effective amount of at a neuron targeting peptide or conjugate thereof under conditions effective to inhibit uptake of the bacterial toxin or neurotoxin by the cells or tissue.

The foregoing methods and uses can be performed *in vitro* and *in vivo*. In the latter case the tissues or cells are located within an animal and the neuron targeting peptides or conjugates thereof are administered to the animal. Where population of G_{T1b} expressing cells are maintained *ex vivo*, the present invention has utility in drug discovery programs. *In vitro* screening assays, with reliable positive and negative controls, are useful as a first step in the development of such drugs. Where the population of G_{T1b} expressing cells is located within an animal or patient, the composition including the neuron targeting peptide or conjugate thereof is administered to the animal as a form of therapy.

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The foregoing treatment methods and uses will generally involve the administration of the pharmaceutically effective composition to the animal or patient systemically, such as by transdermal, intramuscular, intravenous injection and the like. "Administration", as used herein, means provision or delivery of the neuron targeting peptides or conjugates thereof in an amount(s) and for a period of time(s) effective to exert therapeutic effects.

In yet further embodiments, the invention provides methods for, and uses in, delivering selected therapeutic or diagnostic agents to neurons. Such embodiments can be used for delivering selected therapeutic or diagnostic agents to neural tissue.

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLES

Example 1

Introduction

To define peptides with affinity for motor neuron axon terminals, a biopanning strategy was developed to select for the binding characteristics of tetanus toxin. The botulinum and tetanus clostridial toxins (CNTs) are the most potent neurotoxins identified. Their remarkable toxicity results from the combination of their neurospecificity, neuronal binding affinity, and the extraordinary potency of their intracellular activity (K_a ; 10^{-12} to 10^{-13} M). High-affinity selective neural uptake of CNTs derives from the C fragment of the toxins' heavy-chain component. The toxins selectively bind and penetrate axon terminals

in the peripheral nervous system. Tetanus toxin penetrates the terminals of all peripheral neurons including autonomic, sensory, and MNs. Uptake of the labeled C fragment of tetanus toxin occurs most avidly in large motoneurons, followed by preganglionic neurons, with the least uptake in sensory neurons.

5 Tetanus neuronal binding and biological activity depends on the trisialoganglioside (G_{T1b}) receptor.

In order to select for phage with tetanus-like binding properties, a type III 12mer phage library presenting 10^9 random 12 amino acid peptides on the pIII phage coat protein was exposed to G_{T1b} -coated plates and eluted with
10 glycine-hydrochloric acid. Recovered phage were amplified and underwent three more rounds of biopanning, utilizing recombinant tetanus C fragment (rTTC) to elute phage. The oligonucleotide inserts of forty-two phage plaques from the resulting library were excised and sequenced. Four sequences were found to occur more than once, and a single sequence (Tet1) was found in 83% of plaques
15 sequenced. The Tet1 phage clone and a second clone with limited homology (Tet2) were evaluated by anti-phage ELISA for G_{T1b} binding and background plate binding. While both clones possessed enhanced G_{T1b} binding,

Tet1's selectivity for G_{T1b} was greater than Tet2. Next, the individual clones were assessed for competition with rTTC for G_{T1b} binding. rTTC competed
20 with both clones for binding to G_{T1b} . Next, cellular binding of the phage peptides were compared to binding by the random library and peptideless phage (PLP). Both the recovery of infectious phage and immunofluorescence suggest selective binding to differentiated pheochromocytoma cells (PC12) in comparison with kidney epithelial cells (HEK293). As with G_{T1b} binding, Tet1 selectively bound
25 the neuronal cell line with greater efficiency and specificity than Tet2. Synthetic Tet1 and Tet2 fluorescein-conjugated peptides were constructed and allowed to bind to several different neuronal cells lines. Tet1, but not Tet2, bound avidly to PC12 cells, primary motor neurons and dorsal root ganglion (DRG) cells. These findings suggest that Tet1 may provide a means to achieve enhanced and selective
30 neural binding of both viral vectors and neuroprotective proteins.

Materials and Methods

Phage Display biopanning against immobilized trisialogangliosides (G_{T1b}).

Trisialoganglioside (Sigma-Aldrich Co., St. Louis, MO) diluted in 0.1M NaHCO₃ pH 8.6 were immobilized onto 96-well plates by incubating wells with 100 µg/ml overnight at 4°C. The wells were then blocked overnight with phage block buffer (0.1M NaHCO₃ pH8.6, 5 mg/ml BSA, 0.02% NaN₃) at 4°C. The blocking buffer was discarded and the wells were washed 6 times in TBS with 0.1% Tween-20. Each well was exposed to 4 x 10¹⁰ plaque forming units (pfu) of a random 12 amino acid (12mer) pIII peptide phage library and allowed to incubate for 1 h. After washing away unbound phage, bound phage were eluted with a nonspecific general elution buffer, containing 0.2 M Glycine-HCl (pH 2.2) and 1 mg/ml BSA for 1 h, following neutralization with 1 M Tris-HCl (pH 9.1). The non-specific eluates from three separate wells were combined and amplified in ER2738 *E. coli* cells and titered. For the second through fourth rounds of biopanning, G_{T1b} was immobilized and blocked as stated above. Following incubation with 10¹¹ pfus of the previous rounds' eluate, bound phage was eluted with 100 µg/ml of rTTC (Roche Diagnostics Corp., Indianapolis, IN) for 2 h. Following the fourth round of biopanning, the enriched phage pool was plated onto LB/IPTG/Xgal plates and 50 colonies were picked for plasmid preparation. Automated sequencing was performed by SeqWright, Inc. (Houston, TX). The phage display biopanning process is schematically illustrated in Fig. 1.

G_{T1b} binding, competition and displacement ELISAs

The dominant phage clone identified in 83% of sequenced colonies (Tet1) and a second clone bearing homology with Tet1 (Tet2) were amplified for binding studies. G_{T1b} was immobilized and blocked as mentioned previously. For binding studies, 10⁹ pfu of individual phage clones were applied to ELISA wells coated with G_{T1b}. Phage were allowed to bind for 2 h. The wells were washed in TBST (TBS + 0.5% Tween 20) and then incubated with mouse anti-M13 HRP-conjugated antibody (Amersham Pharmacia Biotech Inc, Piscataway, NJ).

Secondary antibody was developed with OPD substrate (Pierce, Rockford, IL) and plates were read using a SpectraMax 190 microplate reader at an absorbance of 490 nm. The signal of bound phage in G_{T1b}-coated wells was divided over signal from uncoated wells to obtain the percentage increase in binding due to the presence of G_{T1b}. In addition, Tet1 and Tet2 binding in G_{T1b}-coated wells were compared to peptideless phage (PLP) to obtain the increase in binding attributable to selected peptides.

Next, phage clone – rTTC competition studies were performed. Individual phage clones were applied at a titer of 10^9 pfu G_{T1b}-coated ELISA wells with varying concentrations of rTTC (0, 25, 50, and 75 ng/ml). Phage and rTTC were incubated for 2 h. Phage binding was measured using mouse anti-M13 HRP-conjugated antibody, developed with OPD substrate and measured as previously described. In addition, the inverse experiment was performed, displacing rTTC from G_{T1b} with individual phage clones. For rTTC displacement, rTTC was allowed to bind for 2 h to G_{T1b}-coated wells. Following removal of rTTC, the wells were incubated with phage clones for 2 h at 10^9 pfu. Anti-tetanus toxin C fragment (Roche, Indianapolis, IL) was applied for 1 h followed by a 1-h incubation with goat anti-mouse IgG₁-FITC secondary antibody (Santa Cruz Biotechnology, Inc, Santa Cruz, CA). Secondary antibody was developed with OPD substrate and measured as previously described. The percentage decrease in rTTC signal from non-phage exposed wells to phage-displaced rTTC was calculated.

Cell Binding Assays

PC12 cells and HEK293 were grown on collagen-coated plates in DMEM supplemented with 10% horse serum and 5 % FBS. PC12 cells were differentiated with 100 ng/ml NGF 2.5S (Invitrogen Corp, Carlsbad, CA) in DMEM with 2% horse serum, and 1% FBS. Binding assays were performed 3 days after differentiation. 10^9 pfu of individual phage clones were applied to the cells in phage wash buffer (PBS, 0.1% BSA, 1mM CaCl₂, 10mM MgCl₂) for 1 h. Wells were exposed to Tet1, Tet2, PLP phage clones, or the random 12mer phage library used to initiate biopanning. The cells were then washed 6 times, for 5 min per wash, with phage wash buffer. The cells were then lysed in lysis buffer

(1% Triton 100, 10 mM Tris, 2 mM EDTA) for 1 h at room temperature. The lysis buffer was collected and the bound phage was titered. The same protocol was repeated for HEK293 cells. The “bound phage ratio” is defined as the ratio of phage retrieved following cell lysis divided by the amount of phage applied to the cells (output/input ratio).

Statistical Analysis

G_{T1b} binding studies and competition assays were replicated in triplicate and individual conditions were compared with a two-way ANOVA. Cell binding assays were performed on six wells of PC12 cells and four wells of HEK 293 cells. Bound phage ratios for Tet1 (N=10), Tet2 (N=10), peptideless phage (N=10), and random library (N=10) were compared in a two-way ANOVA evaluating cell type and phage clone as separate variables. Student's *t* tests were used to compare the binding of Tet1 and Tet2 clones to PC12 and HEK293 cells.

Immunofluorescence Phage Localization *in vitro*

HEK293 cells were grown as previously described. PC12 cells were grown on collagen-coated glass cover slips and differentiated as previously described. 10⁹ pfus of individual phage clones were applied after fresh media was added to the cells and allowed to incubate overnight. HEK293 cells were exposed to Tet1 and Tet2 phage, as well as PBS as a negative control. Differentiated PC12 cells were exposed to PBS, PLP, Random Library, Tet1, and Tet phage. Following removal of media and PBS wash, cells were fixed in 4% paraformaldehyde. The cells were incubated for 15 min in wash buffer (PBS + 0.1% saponin). Cells were then blocked in PBS + 0.1% saponin + 1% BSA for 1 h at 4°C. Mouse anti-M13 antibody (Amersham Pharmacia Biotech Inc, Piscataway, NJ) diluted 1:5000 in blocking buffer was applied to the cells for 1 h. Cells were rinsed three times with wash buffer for 5 min per wash. Goat anti-mouse IgG_{2A} FITC-conjugated antibody (Santa Cruz Biotechnology Inc, Santa Cruz, CA) was diluted 1:200 in blocking buffer and applied to the cells for 1 h. Cells were washed again and mounted with Vectashield mounting medium (Vector Laboratories, Inc., Burlingame, CA). Because Vectashield mounting medium contains DAPI stain, nuclear blue staining

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can be detected. Cells were visualized using a Leica DM R microscope and pictures were captured with a QImaging Retiga EX camera.

Primary Motor Neuron and Dorsal Root Ganglion Culture

Spinal cords were obtained from 15-day Sprague–Dawley rat embryos
5 (Harlan, Indianapolis, IN). Dorsal root ganglia and perineural membranes were removed and cords were cut into approximately 2-mm sections. Cord sections were placed in trypsin/EDTA 1X solution. Cells obtained from the trypsinized tissue were collected and centrifuged through a 6.8% metrizamide column for 15 min at 2000 rpm. The upper layer of the gradient was collected and diluted in
10 Leibowitz L-15 media followed by centrifugation. Pelleted cells were resuspended in complete growth medium made in Neurobasal Medium with the following supplements: 0.2 µg/ml Dglucose, 2.5 mg/ml albumin, 2.5 µg/ml catalase, 0.1 mg/mL biotin, 2.5 µg/ml SOD, 0.01 mg/ml transferrin, 5 µg/ml galactose, 6.3 ng/ml progesterone, 16 µg/ml putrescine, 4 ng/ml selenium, 3 ng/ml β-estradiol, 4
15 ng/ml hydrocortisone, 2 µM L-glutamine, penicillin/streptomycin/ neomycin, and B-27. Cells were plated on glass cover slips in multi well tissue culture plates precoated with poly-L-lysine. Medium was renewed after 1 h and neurite outgrowth was observed.

DRGs collected from rat embryos were incubated in trypsin for for 10 min
20 at 37°C. Following addition of prewarmed calf serum to stop the trypsinization reaction, the cells were collected following centrifugation and resuspended in L-15 media. Following repeated trituration, cells were once again collected following centrifugation and resuspended in plating media (NB media supplemented with 30 nm selenium, 10 nm hydrocortisone, 10 nm β-estradiol, 10 mg/L apo-transferrin,
25 pen/strep/neo, 12.5 ng/ml NGF, 0.05 mM FUDR, 140 µM Lglutamine). After incubation for 2 h to allow for cell attachment, the cells are incubated in feed media (plating media without L-glutamine) for culturing.

In vitro Peptide Binding

PC12, primary motor neurons, or DRGs were cultured as stated above.
30 Following a 1-h incubation in serum free media at 37°C, the cells were fixed in 4%

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paraformaldehyde for 20 min at room temperature. The cells were washed three times for 1 min each in PBS and then blocked in PBS with 0.1% BSA for 1 h at 4°C. Following an additional round of washing, fluoresceinconjugated Tet1 or Tet2 peptides (100 µg/ml), synthesized by EvoQuest Laboratory Services (Invitrogen Corp., Carlsbad, CA), were applied and allowed to bind cells for 1 h. Unbound peptides were washed away and cells were mounted with Vectashield mounting medium. Peptide binding was observed under fluorescence as noted above.

In a separate experiment, PC12 cells were grown to 80% confluency. Half of the wells were then exposed to NGF as previously described to induce differentiation. 24 h later, when morphological changes consistent with differentiation were observed in the NGF group, cultures were fixed and exposed to fluorescein-conjugated Tet1 peptide as described.

Cellular Peptide Binding in Tissue Sections

Animals were anesthetized and underwent intracardiac perfusion with normal saline solution followed by buffered 2% paraformaldehyde. Brains and spinal cords were removed, 24 h postfixed in 2% paraformaldehyde, and cryopreserved in 20% sucrose for additional 24 h. Rodent DRG, lumbar spinal cord, and gastrocnemius muscle tissue sections frozen in optimal cutting temperature gel (OCT; Sakura Finetek USA Inc, Torrance, CA) were cut at 20 µm in transverse sections on a cryostat (Leica Microsystems, Nussloch, Germany). Sections were washed in PBS for 5 min 3 times and placed on glass slides. Peptides (100 µg/ml) were added and allowed to remain for 1 h at room temperature. Specimens were washed for 5 min in PBS then cover slipped and mounted with Biomeda gel mount (Foster City Ca.) for microscopic fluorescent analysis.

Results

Identification of G_{T1b} specific peptides

Peptides specific for G_{T1b} were isolated using a four-round biopanning strategy. The initial round of biopanning was carried out in triplicate, eluting

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bound phage with a nonspecific acidic elution. This stringent elution was performed to prevent the loss of strong-binding phage clones in early rounds. Also, to optimize the capture of G_{T1b} binding phage clones, eluates from the three separate biopanned wells were combined and amplified for use in the second round of biopanning. In the second through fourth rounds of panning, the bound phage was eluted with rTTC to specifically isolate phage peptides that mimic tetanus toxin binding properties. Following the final biopanning round, 42 random phage clones were collected for sequence analysis of the peptide insert. A total of four different peptide sequences were obtained (Table 1). 83% of these phage plaques contained the same phage clone (Tet1). A second sequence (Tet2) present in 5% showed a three amino acid combination homology with Tet2. Both Tet1 and Tet2 contain a leucine, serine, and threonine combination, as well as sharing other amino acids such as histidine, tryptophan and arginine. A blast search run on the sequences of Tet1 and Tet2 revealed homology to a variety of oligonucleotides from unidentified proteins. Tet1 bore homology to an actin-reacting protein, not previously associated with the nervous system. No similarities were detected between the selected peptides and the clostridial toxins.

Table 1

Peptide isolated through novel four-round biopanning process

Clone ID	Peptide Sequence												%
Tet 1	H	L	N	I	L	S	T	L	W	K	Y	R	83% (SEQ ID NO: 1)
Tet 2	S	Y	Q	L	S	T	H	R	W	P	L	H	5% (SEQ ID NO: 2)
Tet 3	T	T	V	Y	P	A	R	W	G	A	H	P	7% (SEQ ID NO: 13)
Tet 4	N	H	V	H	R	M	H	A	T	P	A	Y	5% (SEQ ID NO: 14)

Sequencing from 42 randomly selected clones revealed 4 different peptide sequences labeled Tet1-Tet2 are shown in bold in the left column. The right column contains the percentage of plaques in which the oligonucleotide sequence was deleted.

G_{T1b} binding and rTTC Competition Assays

In order to confirm that phage peptides were selected due to their affinity for G_{T1b} rather than from nonspecific plate binding, individual phage clones were applied to wells with and without G_{T1b} coating. Phage binding was measured via ELISA performed with an anti-M13 phage antibody. Fig. 2 demonstrates that both Tet1 phage and Tet2 phage bound G_{T1b}-coated plates better than uncoated plates, in contrast to PLP for which no significant difference in binding could be detected. Tet1 phage bound both uncoated and coated plates better than Tet2 phage. However, the difference in binding was greater for Tet1 phage. ANOVA revealed significant plate coating ($P < 0.001$), phage clone ($P < 0.001$), and coating by clone interaction ($P < 0.001$). These results verify G_{T1b} as a receptor for the peptides born by both the Tet1 and Tet2 phage clones, but suggest that Tet1- G_{T1b} affinity is greater than Tet2- G_{T1b} affinity.

In order to confirm that the Tet1 and Tet2 peptides bind G_{T1b} at the rTTC receptor site, phage clones were allowed to bind G_{T1b} in the presence of varying amounts of rTTC to assess whether rTTC could compete for binding. Phage clones were applied to immobilized G_{T1b} at approximately 10^9 pfus. Fig. 3 demonstrates that both Tet1 and Tet2 clones undergo rTTC concentration-dependent displacement from G_{T1b}. Tet1 was resistant to rTTC competition at lower concentrations of rTTC (25 and 50 ng/ μ l) but showed decreased binding when incubated at 75 ng/ μ l of rTTC. Tet2 binding decreased progressively with increasing rTTC concentrations. Tet1- G_{T1b} binding was stronger than that of Tet2 at all concentrations of rTTC, replicating the results illustrated in Fig. 2. Peptideless phage again showed minimal binding to G_{T1b} regardless of the rTTC concentration.

To reinforce the finding that Tet1 and Tet2 bind G_{T1b} at the rTTC receptor site, the reciprocal experiment was performed. G_{T1b} binding of rTTC was assessed in the presence of either Tet1 or Tet2 clones at 10^9 pfus. In both cases, rTTC was displaced from G_{T1b} in the presence of both Tet1 and Tet2. Tet1 rTTC displacement (28%) exceeded that of Tet2 (21%). Because the maximal titer of phage was used in this experiment, it was impossible to increase rTTC

displacement by increasing phage titer. Taken together with rTTCs ability to displace Tet1 and Tet2-G_{T1b} binding, these experiments confirm that both peptides bind at the rTTC- G_{T1b} receptor site. Furthermore, Tet1 binds at this receptor with higher affinity than Tet2.

5 **Selective phage peptide binding to neuronal membranes**

The neuronal binding affinity of phage peptides was tested in vitro by applying the phage peptides to differentiated PC12 cells. NGF exposure induces neuronal differentiation in PC12 cells increasing the concentration of membrane G_{T1b} gangliosides. Tet1, Tet2, PLP clones, and the original random 12mer phage
10 library were applied to the PC12 cells. Following rigorous wash steps to remove unbound phage, the cells were lysed and the lysate was titrated to obtain the cell-associated phage fraction. Fig. 4A shows that the Tet1-PC12 bound phage ratio was 23-fold greater than peptideless phage and 13-fold greater than the random phage library. Similarly, Tet2-PC12 bound phage ratio showed a 5-fold increase in
15 binding compared to PLP binding and a 3-fold increase when compared to random library binding. In order to evaluate the neuronal specificity of Tet1 and Tet2 binding, the bound phage ratio for differentiated PC12 cells were compared to those for HEK293 cells. The HEK 293 cell line is derived from human kidney epithelial cells. Fig. 4B demonstrates the neuronal binding specificity of Tet1, but
20 not the Tet2 phage clone. Comparison of Tet1's bound phage ratio for PC12 and HEK293 cells by a Student's *t* test revealed significant neuronal specificity (N=10, P<0.04). In contrast, no significant difference in Tet2 binding could be detected between PC12 and HEK293 cells (N=10, P=0.09). A two-way ANOVA performed on all bound phage ratio data taken from HEK293 cells (N=16) and differentiated
25 PC12 cells (N=24), revealed a significant cell type effect (N=40, P<0.04) and a significant phage clone effect (N=40, P<0.006).

Phage peptide localization by immunofluorescence

To further characterize the neuronal binding of the Tet1 and Tet2 clones, phage binding to differentiated PC12 and HEK293 cells was evaluated by
30 immunofluorescence. FITC-conjugated phage antibodies were used to detect the

presence of bound phage on the surface of these cells in culture. Approximately 10^9 pfu of phage were exposed to both cell lines and allowed to incubate overnight. HEK293 cells were exposed to PBS, Tet1, and Tet2 phage. Differentiated PC12 cells were exposed to PBS, PLP, Random Library, Tet1, or Tet2 phage. Anti-M13 phage primary and FITC-conjugate secondary antibodies were used to visualize the distribution of phage binding. Fig. 5 demonstrates that both Tet1 and Tet2 phage can be detected on PC12 cells under fluorescent microscopy. No binding could be detected by any of the negative control conditions including PBS (Fig. 5), PLP, or Random Library (data not shown). No phage clone binding could be detected on HEK293 cells. Tet1 binding occurred in an evenly distributed pattern, while Tet2 staining appeared to be distributed in aggregates. Further, PC12-Tet2 staining is noted sporadically, occurring on only a fraction of cells visualized.

Fluorescein-conjugated peptide binding to neuronal cell lines and tissue sections

100 μ g/ml of Tet1 and Tet2 were applied to NGF differentiated PC12 cells, primary motor neurons and dorsal root ganglion cells obtained from E15 rat embryos grown in culture. The peptides were allowed to bind for 1 h following blocking, and binding was visualized under fluorescence. Fig. 6 shows visualization of binding of Tet1 and Tet2 via immunofluorescence. Tet1 demonstrated strong binding to all three neuronal cell lines, while Tet2 showed minimal cell binding.

In order to assess the impact of G_{T1b} on binding as well as evaluate the cellular distribution of Tet1 binding, undifferentiated PC12 cells were compared to differentiated PC12 cells. While undifferentiated PC12 membranes contain G_{T1b} , NGF exposure increases the concentration of this ganglioside. Fig. 7 demonstrates that Tet1 binds both differentiated and undifferentiated cells. High-power examination of differentiated cells reveals no obvious enhancement of Tet1 binding to cell processes or growth cones. However, differentiated cells consistently appear to have brighter fluorescence. In particular, this increase appears to occur in the region of the cytoplasm, suggesting uptake and accumulation of the Tet1 peptide in cytoplasm.

Next, 20- μ m-thick sections of gastrocnemius muscle and DRG were exposed to the same concentration of either Tet1 or Tet2 for 1 h. Fig. 8 demonstrates that Tet1 bound avidly to DRG neurons but not to surrounding structural elements. Similarly, Tet1 bound to large neurons of the lumbar spinal cord ventral horn. Counter-staining revealed these cells to have the morphology of spinal cord motor neurons. In contrast, no significant binding was observed in muscle tissue. As with cultured neurons, Tet2 failed to show significant binding in either DRG or muscle tissue.

In vivo fluorescein-conjugated peptide binding to Neuron cells

FITC conjugated Tet1 was administered *in vivo* to sciatic nerve cells and lumbar spinal cord cells. The peptides were allowed to bind for 18 h following administration, and binding was visualized under fluorescence. Fig. 9 shows that Tet1 bound avidly to sciatic nerve cells and lumbar spinal cells and underwent retrograde transport in these cells.

Discussion

Both proteins and viral vectors have proven therapeutic in models of nervous system disease through neuroprotection and alteration in synaptic function. However, their application is impeded by poor blood-brain barrier penetration and binding specificity. In order to define peptides capable of enhanced motor and sensory neuron binding efficiency and specificity, phage display was performed with a novel four-stage biopanning process that selected for phage clones bearing peptides with binding to the G_{T1b} receptor for tetanus toxin. This biopanning process utilized nonspecific stringent elution together with the pooling of multiple wells to enhance the recovery of strong G_{T1b} binding phage in the initial round. Elution with the rTTC was used in the subsequent rounds of biopanning to capture phage with tetanus toxin-like G_{T1b} binding properties.

This biopanning strategy enriched for a single phage clone (Tet1) found in 83% of sequenced plaques. Further analysis of binding characteristics focused on this clone and a second clone (Tet2), which shared a three amino acid sequence with Tet1. Both clones were found to possess enhanced G_{T1b} binding. Similarly,

both clones competed with rTTC for G_{T1b} binding. Tet1 was found to bind G_{T1b} more avidly and compete with rTTC more effectively suggesting stronger, more specific binding to the tetanus receptor. Analysis of cellular binding using phage recovery and immunofluorescence suggested that both clones possessed enhanced neuronal binding in comparison with random library and peptideless phage. Again, neuronal binding of Tet1 bearing phage exceeded that of Tet2 bearing phage. In addition, the Tet1 phage clone possessed neuron-specific binding properties that were not detected in the Tet2 clone. Synthetic Tet1 binds neuronal membranes both in vitro and in vivo. Differentiation of PC12 cells enhances the detected fluorescence further implicating G_{T1b} in the binding process. Interestingly, there is no obvious affinity for cell processes. However, Tet1 appears to accumulate in the cytoplasm of differentiated PC12 cells, supporting the hypothesis that Tet1 is taken up by neurons after binding. Finally, synthetic Tet1 peptide bound neuronal membranes dramatically better than Tet1 bearing phage, suggesting that the phage particle undermines the binding properties of the attached peptide. The ability of the Tet1 peptide to enhance the neuronal binding efficiency and specificity of phage suggests the potential to apply this peptide to neuronal targeting of therapeutic proteins and vectors. As predicted by the phage binding experiments, the Tet1 peptide bound neuronal membranes far more avidly than the Tet2 peptide.

The biopanning strategy described here was designed to identify peptides with the binding properties of the clostridial toxins. Biopanning on immobilized receptors has a high likelihood of identifying peptides with specific binding properties. However, this approach is limited by the potential to eliminate clones that bind to novel neuronal receptors that possess even better affinity. This problem can be overcome by biopanning directly on neuronal cell lines. The application of this approach on non-neuronal cell lines has yielded peptides with substantially higher target cell binding efficiency (100- to 1000-fold enhancement over random phage) than currently observed with Tet1. However, the use of cell lines as biopanning targets skews selection towards binding to receptors present in the highest concentration on the cell membrane. While biopanning directly on cell membranes may isolate peptides with high affinity to unknown receptors, the

binding specificity of these peptides is not guaranteed. Pre-clearing steps utilizing negative biopanning to eliminate unwanted binding characteristics have been employed to control this problem. However, by its nature, negative biopanning is cumbersome. It remains difficult to anticipate all of the potential nonspecific binding characteristics that might confound efforts to utilize the peptides for cellular targeting. The biopanning strategy of the present invention circumvents this disadvantage by focusing on a specific receptor-ligand interaction known to possess high neuronal binding efficiency and specificity. Further, this interaction plays a critical role in shuttling protein from the periphery to motor neurons.

Specific cell surface gangliosides bind clostridial toxins and promote neuronal uptake. Using a photoaffinity reagent, demonstrated a ganglioside interaction site in the 34 aa of the tetanus toxin heavy chain's carboxy terminus. The three-dimensional structure of this domain shows loop regions connecting β -sheets. The locations of these loops suggest a role in toxin:receptor binding. Mutation in the region of the first loop (aa 1274-1279) significantly reduces toxin binding to gangliosides as well as toxin binding to PC12 cells and the retrograde axonal transport of toxin in mice. Extending this mutation to include the region extending from aa 1271-1282 further reduces toxin binding to gangliosides. Additionally, deletion of 6 amino acids in a second loop extending from aa 1214-1219 abolishes both toxin:ganglioside binding and biological activity. In contrast, deletions of 5 amino acids from the carboxyl terminal of rTTC do not affect ganglioside binding, while 10 and 15 amino acid deletions did, suggesting that this region was important for ganglioside binding. Nonetheless, a synthetic peptide consisting of the 20 amino acids from the carboxyl terminal of rTTC failed to demonstrate ganglioside or neuronal membrane binding properties. In addition, antibodies against this peptide failed to compete with gangliosides for rTTC binding. Together, these findings suggest that ganglioside binding is critical to toxin uptake and ultimately retrograde toxin transport. While the carboxy terminal amino acids play a vital part in ganglioside binding, other areas of the rTTC are likely to contribute to the tertiary structure that is necessary for proper ganglioside binding. As such, it is unlikely that a small

peptide sequence taken from rTTC would possess tetanus-like G_{T1b} binding properties.

The finding that none of the peptides identified through phage display bore homology to rTTC is consistent with the absence of a specific contiguous sequence within rTTC that is responsible for G_{T1b} binding. However, both peptides
5 contained amino acids that are thought to play a critical role in rTTC ganglioside binding, including histidine, tryptophan, and tyrosine. The importance of tertiary structure in rTTC-ganglioside interactions may explain why there are no direct homologies between rTTC and either Tet1 or Tet2. Blast searches of the Tet1
10 peptide showed some homology with proteins in the database, only one of which, an actin-interacting protein, has been previously characterized. This protein has not been previously associated with a function in the CNS. A similar search of the Tet2 sequence failed to detect similarities with known proteins in the database.

C fragment alone has been shown to possess tetanus toxin's property of
15 inducing retrograde axonal transport. It has been demonstrated that the addition of C fragment to β -galactosidase in a fusion protein triggered retrograde transport and β -galactosidase activity in the CNS following intramuscular injection. Previous research has suggested that a 15-kDa protein receptor contributes to the membrane translocation of the toxin. However, in a study of different C fragment mutants, a
20 correlation between the properties of retrograde transport and ganglioside binding was observed. Nonetheless, our goal in identifying small neurotropic peptides was to apply them to the creation of fusion proteins and viral vectors with enhanced and specific neuronal binding. If the parent proteins and vectors targeted with Tet1 possess the ability to undergo uptake and retrograde transport, the peptide itself
25 needn't possess this property. We have previously shown that AAV2 is capable of retrograde transport following peripheral delivery. The presence of neural targeting peptides on its coat may enhance this uptake through improved binding to axon terminals. Similarly, neural growth factors have long been recognized to undergo uptake and retrograde transport. Thus, the targeting peptide may not need
30 to retain C fragment's retrograde transport properties to enhance neural delivery of therapeutic proteins and vectors.

Initial cell binding assays were performed with phage clones bearing the peptides. Synthetic Tet1 peptide binding to PC12 cells exceeds that of the Tet1 bearing phage. The fact that only 4 copies of the peptide are expressed on the pIII proteins per phage surface doubtlessly impedes the peptide's binding kinetics. The much larger phage is likely to create steric interference with the peptides' ability to bind G_{T1b}. Nonetheless, peptide bearing phage may approximate the impact of Tet1 capsid insertion on AAV tropism. Further, insertions into the *cap* gene of the AAV would result in the presentation of many more copies of the peptide than occurs on pIII phage proteins, potentially enhancing targeting properties. Finally, because of the enhanced kinetics, synthetic Tet1 peptide may displace rTTC more effectively than the pIII phage clones bearing the peptides.

Enhanced G_{T1b} binding and rTTC displacement may prove to be an important property for broader application of these peptides. If the synthetic versions of Tet1 and Tet2 act as effective competitors to rTTC at the neuronal receptor, it is possible that they may serve as toxin antagonists. Bacterial toxins pose a significant threat due to their extreme potency, ease of production, and stability under normal environmental conditions. Neurotoxins secreted by five known species of the genus *Clostridium*; *C botulinum*, *C tetani*, *C argentinense*, *C butyricum* and *C baratii* are capable of producing fatal flaccid or spastic paralysis. However, the administration of antitoxin within 24 h of the onset of disease can lower death rates and shorten the duration of symptoms. Existing antitoxins are antibodies to the clostridial toxins. Because clostridial toxins are taken up avidly by the peripheral nerve terminals, antitoxins can serve to reduce the available reservoir for continued uptake by susceptible neurons. The Tet1 peptide employed as a toxin antagonist may serve as an alternative to antibody antitoxins.

Example 2

Manipulating AAV2 Tropism for Enhanced Delivery of AAV Vectors to the Spinal Cord

Amyotrophic Lateral Sclerosis (ALS) is a rapidly progressing neurodegenerative disorder. Currently, the pathway of motoneuron degeneration, caused by ALS, is poorly characterized and has severely limited the development

of viable treatment options. Although gene therapy may provide a novel approach for developing effective treatments for ALS the need to deliver therapeutic genes throughout the CNS will require highly efficient and selective gene vector systems. Adeno-Associated Virus (AAV) has been shown to undergo retrograde transport to the spinal cord following peripheral injection. On the basis of its extremely efficient uptake and delivery to motor neurons, we have attempted to develop tetanus toxin as targeting ligand for neurospecific binding of AAV vectors with the goal of increasing the efficiency of retrograde transport and spinal cord delivery of AAV vectors.

It has previously been shown that large peptide ligands can be inserted at the N-terminus of the AAV2 VP2 capsid protein. Accordingly, an AAV clone was constructed to encode the C-terminal fragment of the tetanus toxin heavy chain protein (TTH_c) as an AAV2 VP2 N-terminal fusion. This construct was complemented with an AAV2 VP1, VP3 expression construct to produce infectious AAV2 particles. Concurrently a fusion protein was constructed by genetically fusing the coding sequence of streptavidin (SA_v) with TTH_c. This fusion protein was expressed in bacteria using the pTrcHis-TOPO system and purified by nickel-affinity chromatography. AAV vectors displaying TTH_c and molecular conjugates prepared by binding SA_v-TTH_c to metabolically biotinylated AAV vectors will be assessed for specific and enhanced binding to differentiated pheochromocytoma (PC12) cells, neuroblastoma cells, and primary motor neurons *in vitro*.

A phage display biopanning strategy for isolation of peptides with specific affinity for the trisialoganglioside (G_{T1b}) Clostridial toxin receptor was described in Example 1. This process identified Tet1, a 12 AA peptide with specific and enhanced binding to differentiated pheochromocytoma (PC12) cells, primary motor neurons, and dorsal root ganglion (DRG) cells *in vitro*. Similarly, we have shown that small peptide insertions following AAV2 VP1 amino acid 588 are well tolerated and can alter AAV2 natural tropism. Based upon these findings, an AAV2 construct was made encoding the Tet1 peptide at this position in the viral capsid protein. Recombinant viral vectors packaged with this modified AAV2 capsid maintained wild type particle and infectious titers on HeLa cells indicating

Table 2 lists six different sequences that were obtained. 58% of the randomly selected clones contained a sequence corresponding to SEQ ID NO: 3. Two other sequences, i.e., SEQ ID NO: 4 and SEQ ID NO: 5 contained a similar homology to SEQ ID NO: 3 and differed from SEQ ID NO: 3 by, respectively, 2 and 3 amino acids, 21% of the randomly selected clones contained a sequence corresponding to SEQ ID NO: 6.

Having described the invention, the following is claimed:

1. An isolated neuron targeting peptide comprising about 5 to about 30 amino acids and including an amino acid sequence that preferentially binds to an isolated neuron receptor.
2. The peptide of claim 1, being identified by biopanning phage displayed peptides against the isolated neuron receptor.
3. The peptide of claim 1, the isolated neuron receptor comprising G_{T1b}.
4. The peptide of claim 1, being operably linked to at least one of a therapeutic agent or a diagnostic agent.
5. The peptide of claim 4, the therapeutic agent comprising a neurotrophic agent.
6. The peptide of claim 5, the therapeutic agent comprising a vector for delivering at least one gene of interest to a target cell.
7. The peptide of claim 6, the vector comprising a virus and the peptide being operably linked to the virus by expressing the peptide in the capsid of the virus.
8. The peptide of claim 7, the virus being an adeno-associated virus (AAV).
9. The peptide of claim 8, the virus being genetically modified to include a nucleotide sequence encoding a therapeutic agent.

10. An isolated targeting peptide comprising an amino acid sequence selected from the group consisting of:

HLNILSTLWKYR (SEQ ID NO: 1);
SYQLSTHRWPLH (SEQ ID NO: 2); and
KGTINPF (SEQ ID NO: 3);
XGAINPF (SEQ ID NO: 4);
KLTANPT (SEQ ID NO: 5);
NVHRGLH (SEQ ID NO: 6); and
a derivative thereof.

11. The peptide of claim 10, being operably linked to at least one of a therapeutic agent or targeting agent.

12. The peptide of claim 11 being operably linked to a neurotrophic growth factor selected from the group consisting of ciliary neurotrophic factor (CNTF), brain-derived neurotrophic factor (BDNF), insulin-like growth factor (IGF), and glial cell-line-derived neurotrophic factor (GDNF) NGF, α -FGF, β -FGF, PDGF, BDNF, CNTF, NT-3, NT 4/5, and mixtures thereof.

13. The peptide of claim 11, forming a fusion protein with the neurotrophic growth factor.

14. The peptide of claim 10, being operably linked to a vector for delivering at least one gene of interest to a target cell.

15. The peptide of claim 14, the virus being an adeno-associated virus and the peptide being operably linked to the adeno-associated virus by expressing the peptide in the capsid and/or attaching the peptide to the capsid.

16. The peptide of claim 15, the virus being genetically modified to include a nucleic acid encoding a clostridial neurotoxin light chain peptide.

17. An isolated fusion protein comprising a neuron targeting peptide, the neuron targeting peptide comprising an amino sequence selected from the group consisting of:

HLNILSTLWKYR (SEQ ID NO: 1);
SYQLSTHRWPLH (SEQ ID NO: 2);
KGTINPF (SEQ ID NO: 3);
XGAINPF (SEQ ID NO: 4);
KLTANPT (SEQ ID NO: 5);
NVHRGLH (SEQ ID NO: 6); and
a derivative thereof.

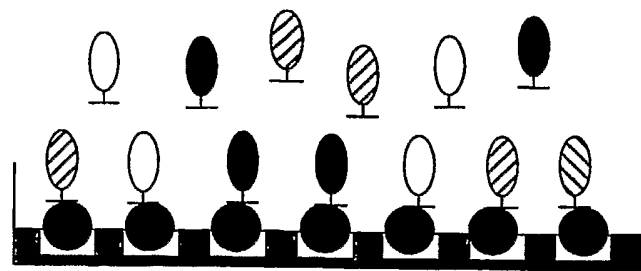
18. An AAV vector comprising a capsid protein with an amino acid insertion, the amino acid insertion including an amino acid sequence selected from the group consisting of:

HLNILSTLWKYR (SEQ ID NO: 1);
SYQLSTHRWPLH (SEQ ID NO: 2);
KGTINPF (SEQ ID NO: 3);
XGAINPF (SEQ ID NO: 4);
KLTANPT (SEQ ID NO: 5);
NVHRGLH (SEQ ID NO: 6); and
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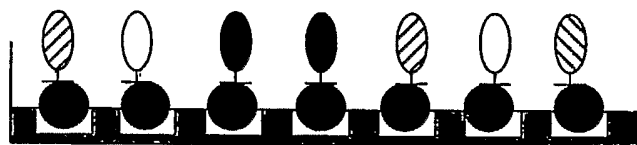
19. A clostridial neurotoxin antagonist comprising a peptide, the peptide including an amino sequence selected from the group consisting of:

HLNILSTLWKYR (SEQ ID NO: 1);
SYQLSTHRWPLH (SEQ ID NO: 2);
KGTINPF (SEQ ID NO: 3);
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NVHRGLH (SEQ ID NO: 6); and
a derivative thereof.

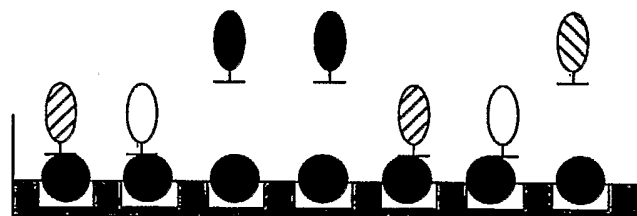
20. A method of identifying a neuron targeting peptide, the method comprising:
- exposing an isolated neuroreceptor to a phage display library, the phages presenting a peptide comprising about 5 to about 30 amino acids;
 - eluting the phage library with a first non-specific eluent; and
 - eluting the phage library with a second specific eluent.
21. The method of claim 20, the isolated neuroreceptor comprising isolated G_{T1b}.
22. The method of claim 21, the isolated neuroreceptor being coated on a substrate.
23. The method of claim 21, the specific eluent comprising recombinant tetanus C fragment.



Step 1: Apply Random 12mer
Phage Library to G_{T1B}



Step 2: Wash to Remove
Unbound Phage



Step: 3 Elute Phage with Glycine
or rTTC



Step: 4 Amplify and Repeat Step 1

Fig. 1A

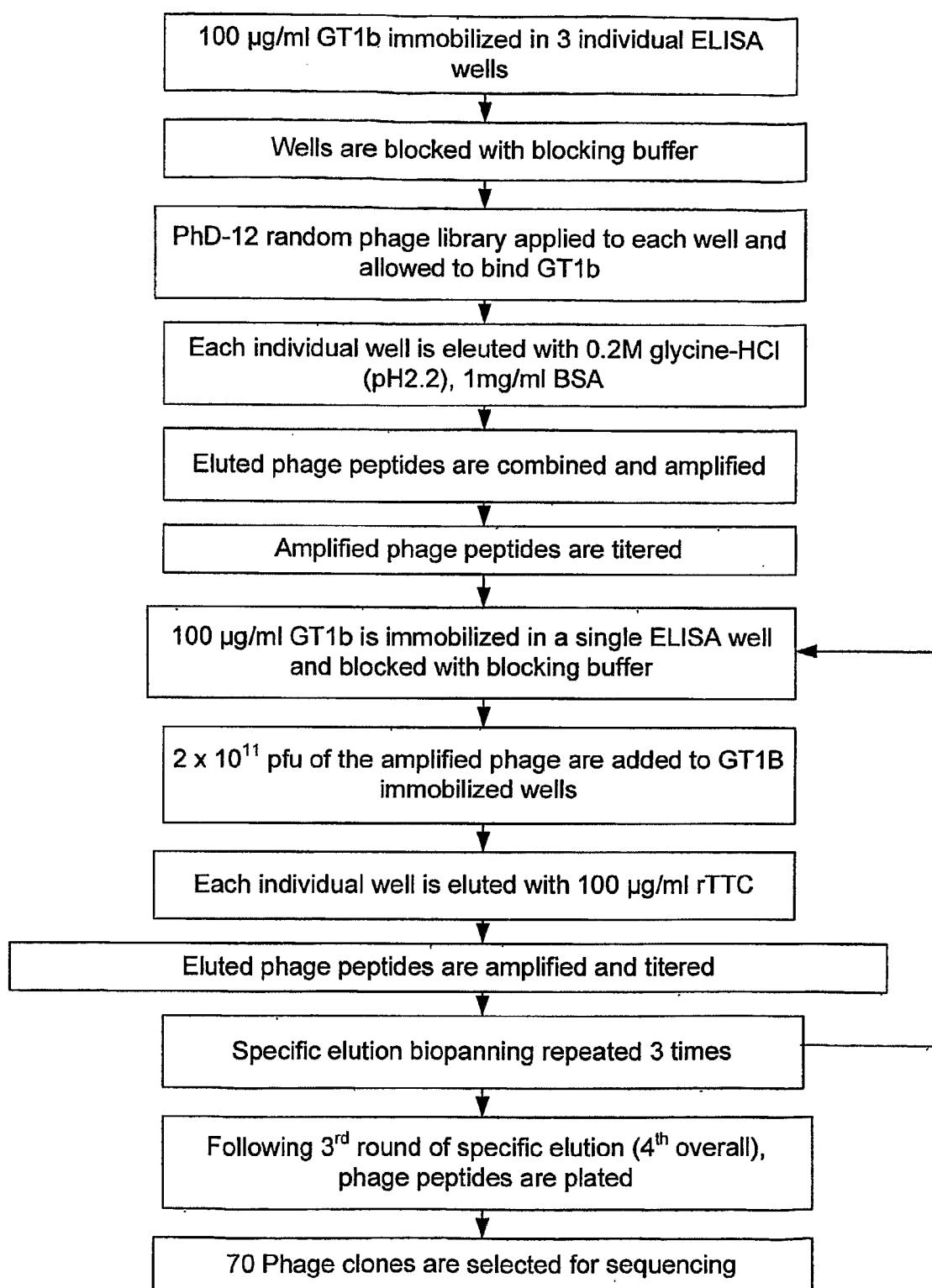
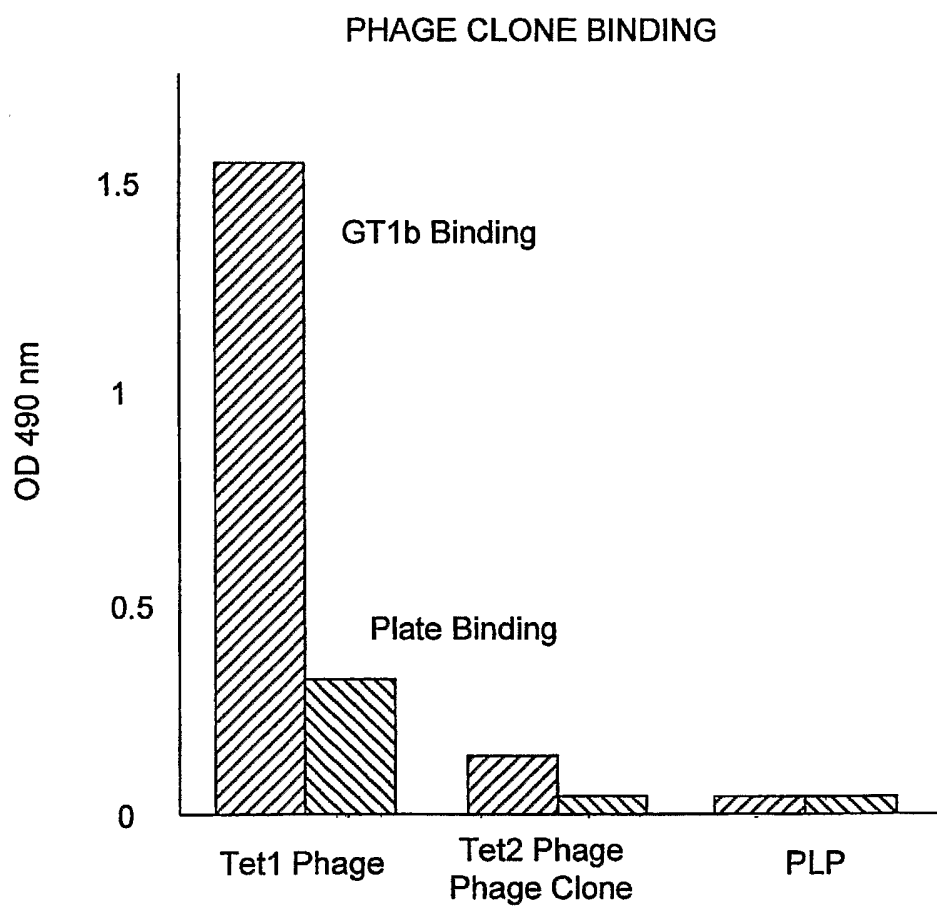


Fig. 1B



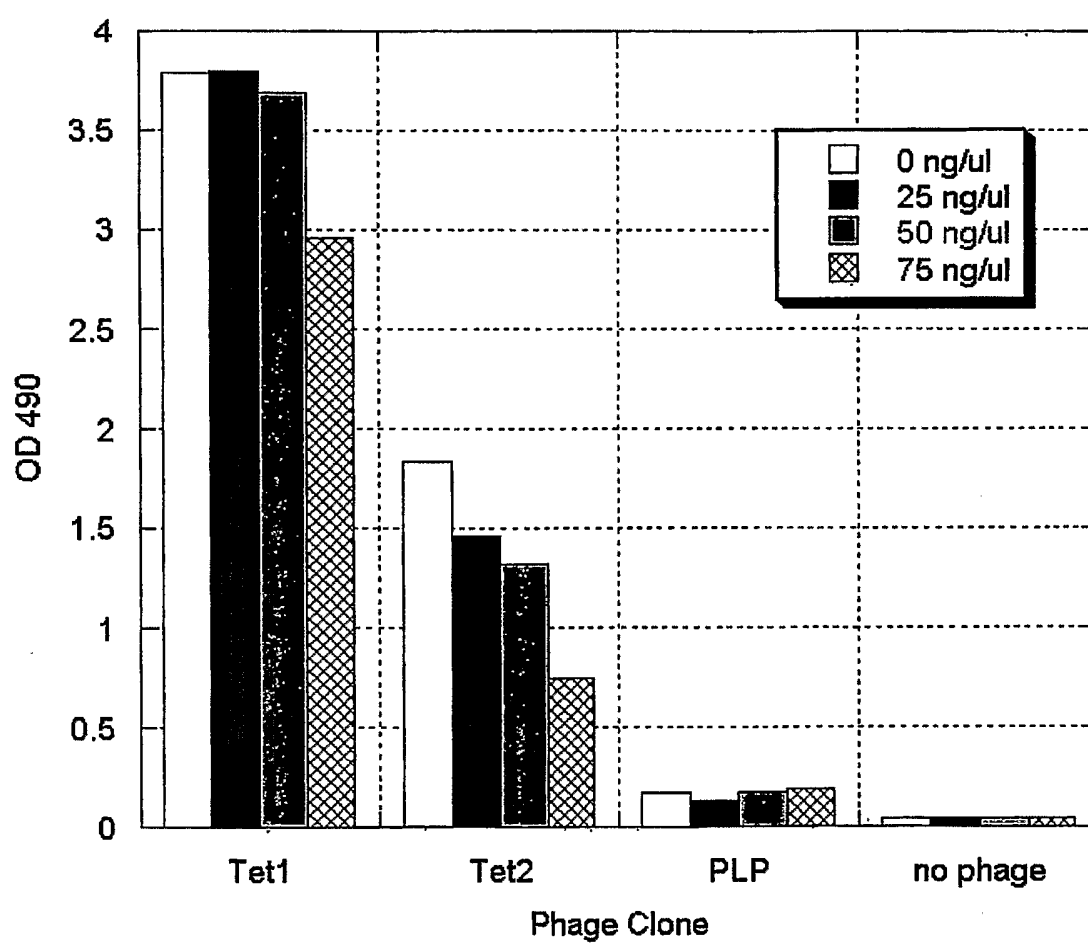


Fig. 3

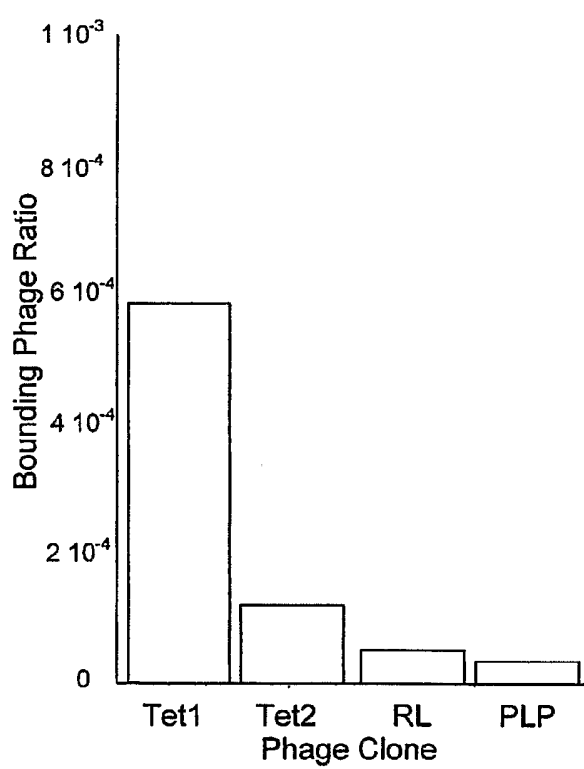


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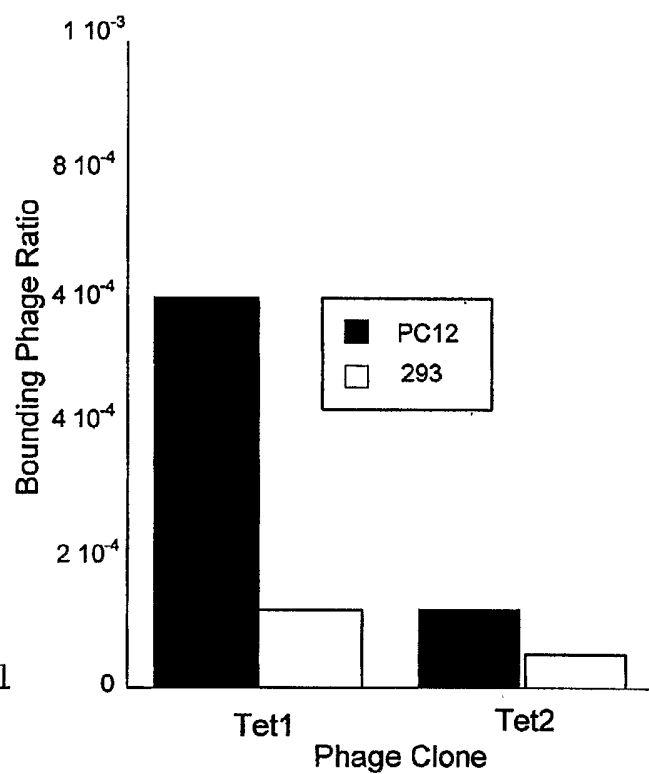


Fig. 4B

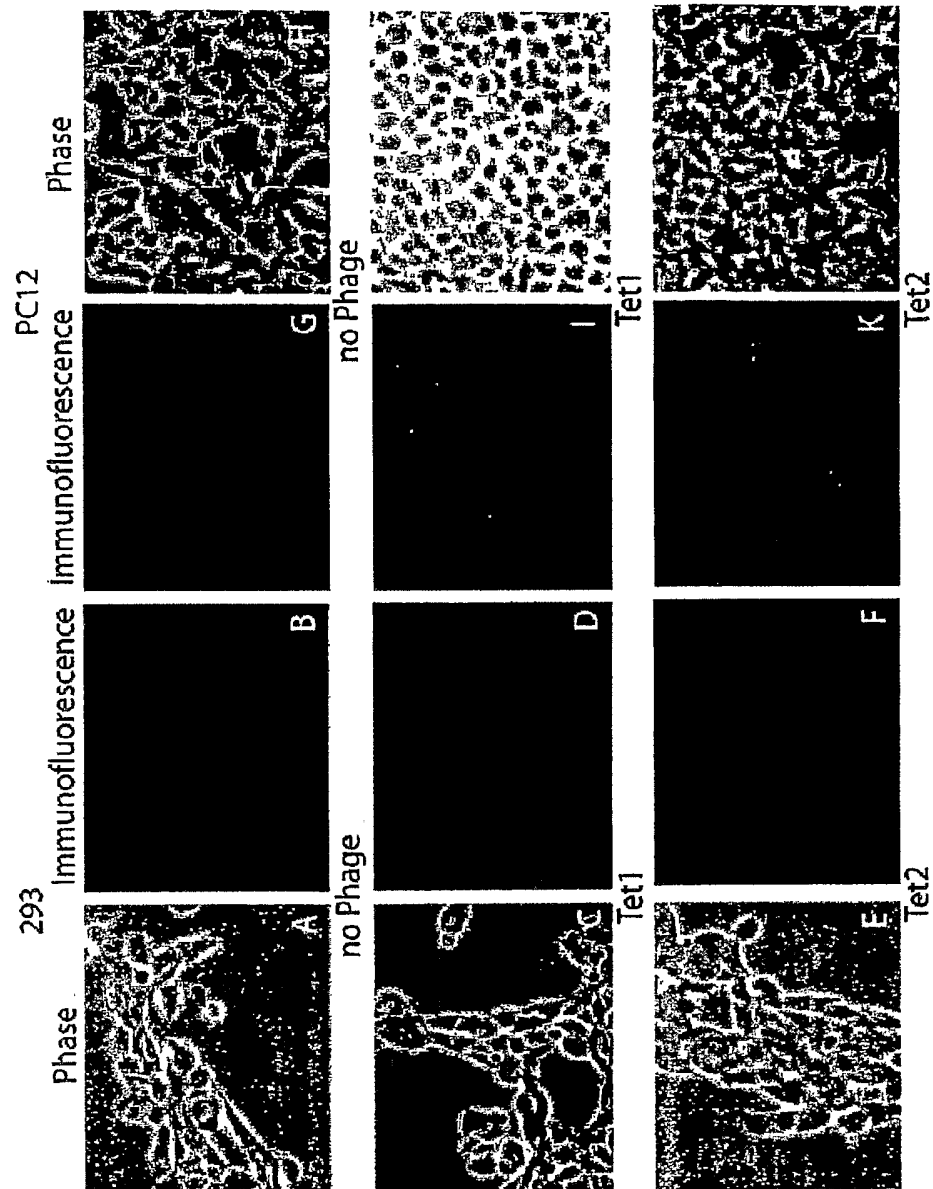


Fig. 5

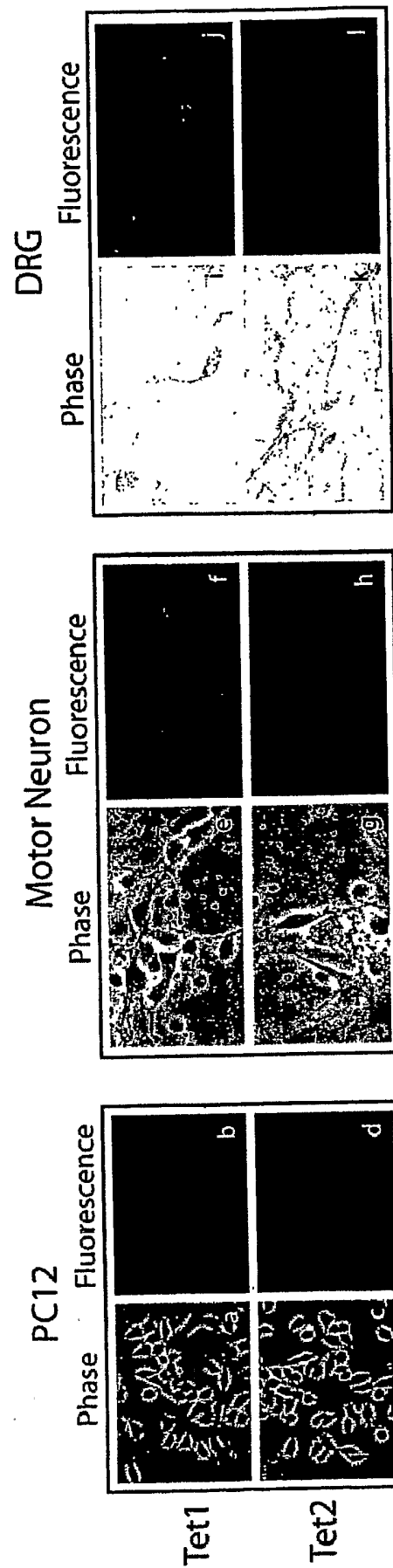


Fig. 6

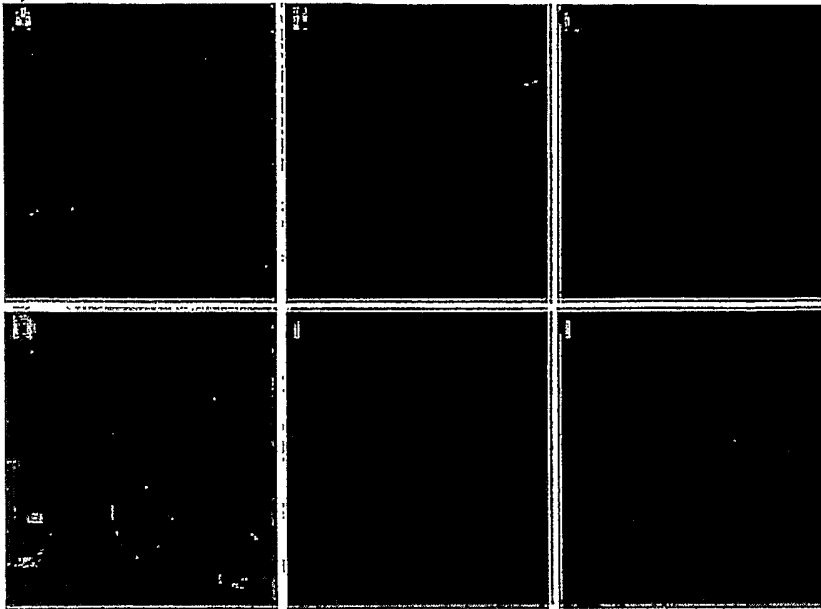


Fig. 7

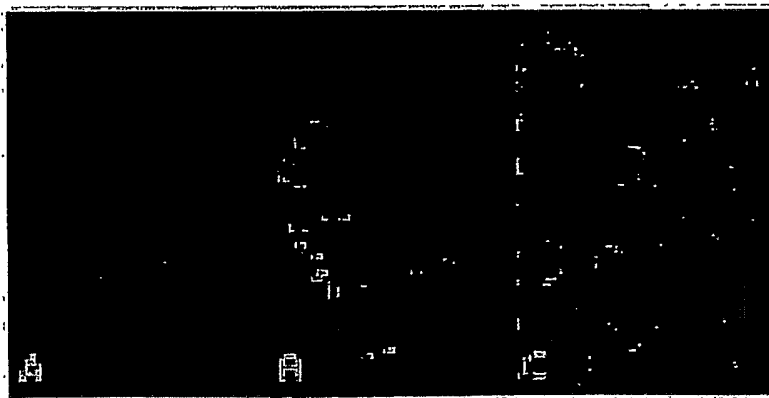


Fig. 8

FITC-conjugated Tet1 peptide

18 hours after injection

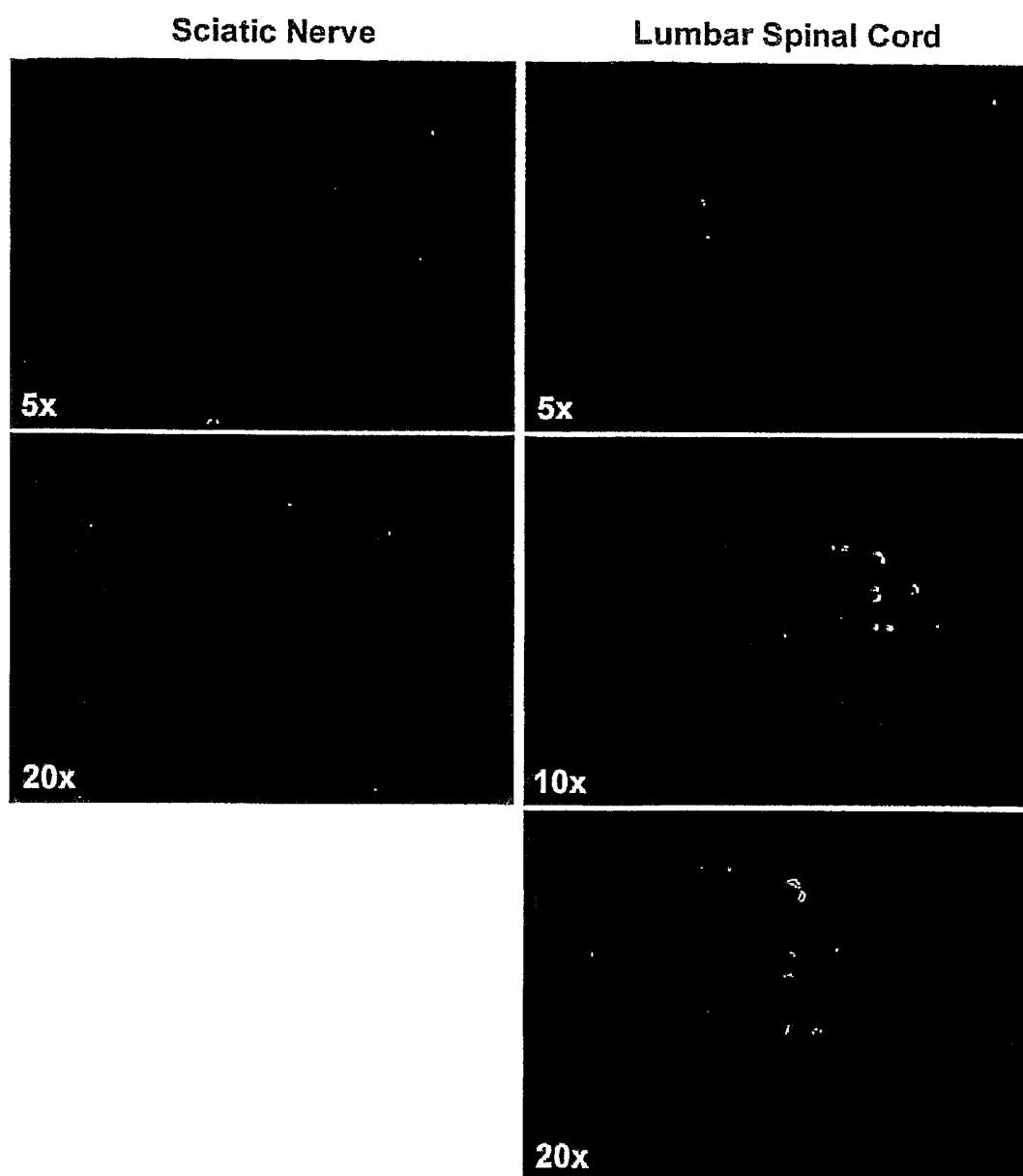


Fig. 9

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tctgttaatg tggactttac tgtggacact aatggcgtgt attcagagcc tcgccccatt 4380
ggcaccagat acctgactcg taatctgtaa ttgcttgta atcaataaac cgtttaattc 4440
gtttcagttg aactttggtc tctgcgtatt tctttcttat ctagtttcca tggctacgta 4500
gataagtagc atggcgggtt aatcatTAAC tacaaggaac ccctagtgat ggagttggcc 4560
actccctctc tgcgcgctcg ctcgctcact gaggcgggc gaccaaaggt cggccgacgc 4620
ccgggctttg cccgggcggc ctcagtgagc gagcgagcgc gcagagaggg agtggccaa 4679

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<210> 10

<211> 735

<212> PRT

<213> adeno-associated virus 2 VP1 capsid protein

<400> 10

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Met Ala Ala Asp Gly Tyr Leu Pro Asp Trp Leu Glu Asp Thr Leu Ser
1           5           10           15

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Glu Gly Ile Arg Gln Trp Trp Lys Leu Lys Pro Gly Pro Pro Pro Pro
          20           25           30

```

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Lys Pro Ala Glu Arg His Lys Asp Asp Ser Arg Gly Leu Val Leu Pro
          35           40           45

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Gly Tyr Lys Tyr Leu Gly Pro Phe Asn Gly Leu Asp Lys Gly Glu Pro
 50 55 60

Val Asn Glu Ala Asp Ala Ala Ala Leu Glu His Asp Lys Ala Tyr Asp
 65 70 75 80

Arg Gln Leu Asp Ser Gly Asp Asn Pro Tyr Leu Lys Tyr Asn His Ala
 85 90 95

Asp Ala Glu Phe Gln Glu Arg Leu Lys Glu Asp Thr Ser Phe Gly Gly
 100 105 110

Asn Leu Gly Arg Ala Val Phe Gln Ala Lys Lys Arg Val Leu Glu Pro
 115 120 125

Leu Gly Leu Val Glu Glu Pro Val Lys Thr Ala Pro Gly Lys Lys Arg
 130 135 140

Pro Val Glu His Ser Pro Val Glu Pro Asp Ser Ser Ser Gly Thr Gly
 145 150 155 160

Lys Ala Gly Gln Gln Pro Ala Arg Lys Arg Leu Asn Phe Gly Gln Thr
 165 170 175

Gly Asp Ala Asp Ser Val Pro Asp Pro Gln Pro Leu Gly Gln Pro Pro
 180 185 190

Ala Ala Pro Ser Gly Leu Gly Thr Asn Thr Met Ala Thr Gly Ser Gly
 195 200 205

Ala Pro Met Ala Asp Asn Asn Glu Gly Ala Asp Gly Val Gly Asn Ser
 210 215 220

Ser Gly Asn Trp His Cys Asp Ser Thr Trp Met Gly Asp Arg Val Ile
 225 230 235 240

Thr Thr Ser Thr Arg Thr Trp Ala Leu Pro Thr Tyr Asn Asn His Leu
 245 250 255

Tyr Lys Gln Ile Ser Ser Gln Ser Gly Ala Ser Asn Asp Asn His Tyr
 260 265 270

Phe Gly Tyr Ser Thr Pro Trp Gly Tyr Phe Asp Phe Asn Arg Phe His
 275 280 285

Cys His Phe Ser Pro Arg Asp Trp Gln Arg Leu Ile Asn Asn Asn Trp
 290 295 300

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Gly Phe Arg Pro Lys Arg Leu Asn Phe Lys Leu Phe Asn Ile Gln Val
 305 310 315 320

Lys Glu Val Thr Gln Asn Asp Gly Thr Thr Thr Ile Ala Asn Asn Leu
 325 330 335

Thr Ser Thr Val Gln Val Phe Thr Asp Ser Glu Tyr Gln Leu Pro Tyr
 340 345 350

Val Leu Gly Ser Ala His Gln Gly Cys Leu Pro Pro Phe Pro Ala Asp
 355 360 365

Val Phe Met Val Pro Gln Tyr Gly Tyr Leu Thr Leu Asn Asn Gly Ser
 370 375 380

Gln Ala Val Gly Arg Ser Ser Phe Tyr Cys Leu Glu Tyr Phe Pro Ser
 385 390 395 400

Gln Met Leu Arg Thr Gly Asn Asn Phe Thr Phe Ser Tyr Thr Phe Glu
 405 410 415

Asp Val Pro Phe His Ser Ser Tyr Ala His Ser Gln Ser Leu Asp Arg
 420 425 430

Leu Met Asn Pro Leu Ile Asp Gln Tyr Leu Tyr Tyr Leu Ser Arg Thr
 435 440 445

Asn Thr Pro Ser Gly Thr Thr Thr Gln Ser Arg Leu Gln Phe Ser Gln
 450 455 460

Ala Gly Ala Ser Asp Ile Arg Asp Gln Ser Arg Asn Trp Leu Pro Gly
 465 470 475 480

Pro Cys Tyr Arg Gln Gln Arg Val Ser Lys Thr Ser Ala Asp Asn Asn
 485 490 495

Asn Ser Glu Tyr Ser Trp Thr Gly Ala Thr Lys Tyr His Leu Asn Gly
 500 505 510

Arg Asp Ser Leu Val Asn Pro Gly Pro Ala Met Ala Ser His Lys Asp
 515 520 525

Asp Glu Glu Lys Phe Phe Pro Gln Ser Gly Val Leu Ile Phe Gly Lys
 530 535 540

Gln Gly Ser Glu Lys Thr Asn Val Asp Ile Glu Lys Val Met Ile Thr
 545 550 555 560

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Asp Glu Glu Glu Ile Arg Thr Thr Asn Pro Val Ala Thr Glu Gln Tyr
 565 570 575

Gly Ser Val Ser Thr Asn Leu Gln Arg Gly Asn Arg Gln Ala Ala Thr
 580 585 590

Ala Asp Val Asn Thr Gln Gly Val Leu Pro Gly Met Val Trp Gln Asp
 595 600 605

Arg Asp Val Tyr Leu Gln Gly Pro Ile Trp Ala Lys Ile Pro His Thr
 610 615 620

Asp Gly His Phe His Pro Ser Pro Leu Met Gly Gly Phe Gly Leu Lys
 625 630 635 640

His Pro Pro Pro Gln Ile Leu Ile Lys Asn Thr Pro Val Pro Ala Asn
 645 650 655

Pro Ser Thr Thr Phe Ser Ala Ala Lys Phe Ala Ser Phe Ile Thr Gln
 660 665 670

Tyr Ser Thr Gly Gln Val Ser Val Glu Ile Glu Trp Glu Leu Gln Lys
 675 680 685

Glu Asn Ser Lys Arg Trp Asn Pro Glu Ile Gln Tyr Thr Ser Asn Tyr
 690 695 700

Asn Lys Ser Val Asn Val Asp Phe Thr Val Asp Thr Asn Gly Val Tyr
 705 710 715 720

Ser Glu Pro Arg Pro Ile Gly Thr Arg Tyr Leu Thr Arg Asn Leu
 725 730 735

<210> 11
 <211> 598
 <212> PRT
 <213> adeno-associated virus 2 VP1 Capsid protein

<400> 11

Met Ala Pro Gly Lys Lys Arg Pro Val Glu His Ser Pro Val Glu Pro
 1 5 10 15

Asp Ser Ser Ser Gly Thr Gly Lys Ala Gly Gln Gln Pro Ala Arg Lys
 20 25 30

Arg Leu Asn Phe Gly Gln Thr Gly Asp Ala Asp Ser Val Pro Asp Pro
 35 40 45

Gln Pro Leu Gly Gln Pro Pro Ala Ala Pro Ser Gly Leu Gly Thr Asn
 50 55 60

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Thr Met Ala Thr Gly Ser Gly Ala Pro Met Ala Asp Asn Asn Glu Gly
65 70 75 80

Ala Asp Gly Val Gly Asn Ser Ser Gly Asn Trp His Cys Asp Ser Thr
85 90 95

Trp Met Gly Asp Arg Val Ile Thr Thr Ser Thr Arg Thr Trp Ala Leu
100 105 110

Pro Thr Tyr Asn Asn His Leu Tyr Lys Gln Ile Ser Ser Gln Ser Gly
115 120 125

Ala Ser Asn Asp Asn His Tyr Phe Gly Tyr Ser Thr Pro Trp Gly Tyr
130 135 140

Phe Asp Phe Asn Arg Phe His Cys His Phe Ser Pro Arg Asp Trp Gln
145 150 155 160

Arg Leu Ile Asn Asn Asn Trp Gly Phe Arg Pro Lys Arg Leu Asn Phe
165 170 175

Lys Leu Phe Asn Ile Gln Val Lys Glu Val Thr Gln Asn Asp Gly Thr
180 185 190

Thr Thr Ile Ala Asn Asn Leu Thr Ser Thr Val Gln Val Phe Thr Asp
195 200 205

Ser Glu Tyr Gln Leu Pro Tyr Val Leu Gly Ser Ala His Gln Gly Cys
210 215 220

Leu Pro Pro Phe Pro Ala Asp Val Phe Met Val Pro Gln Tyr Gly Tyr
225 230 235 240

Leu Thr Leu Asn Asn Gly Ser Gln Ala Val Gly Arg Ser Ser Phe Tyr
245 250 255

Cys Leu Glu Tyr Phe Pro Ser Gln Met Leu Arg Thr Gly Asn Asn Phe
260 265 270

Thr Phe Ser Tyr Thr Phe Glu Asp Val Pro Phe His Ser Ser Tyr Ala
275 280 285

His Ser Gln Ser Leu Asp Arg Leu Met Asn Pro Leu Ile Asp Gln Tyr
290 295 300

Leu Tyr Tyr Leu Ser Arg Thr Asn Thr Pro Ser Gly Thr Thr Thr Gln
305 310 315 320

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Ser Arg Leu Gln Phe Ser Gln Ala Gly Ala Ser Asp Ile Arg Asp Gln
 325 330 335

Ser Arg Asn Trp Leu Pro Gly Pro Cys Tyr Arg Gln Gln Arg Val Ser
 340 345 350

Lys Thr Ser Ala Asp Asn Asn Asn Ser Glu Tyr Ser Trp Thr Gly Ala
 355 360 365

Thr Lys Tyr His Leu Asn Gly Arg Asp Ser Leu Val Asn Pro Gly Pro
 370 375 380

Ala Met Ala Ser His Lys Asp Asp Glu Glu Lys Phe Phe Pro Gln Ser
 385 390 395 400

Gly Val Leu Ile Phe Gly Lys Gln Gly Ser Glu Lys Thr Asn Val Asp
 405 410 415

Ile Glu Lys Val Met Ile Thr Asp Glu Glu Glu Ile Arg Thr Thr Asn
 420 425 430

Pro Val Ala Thr Glu Gln Tyr Gly Ser Val Ser Thr Asn Leu Gln Arg
 435 440 445

Gly Asn Arg Gln Ala Ala Thr Ala Asp Val Asn Thr Gln Gly Val Leu
 450 455 460

Pro Gly Met Val Trp Gln Asp Arg Asp Val Tyr Leu Gln Gly Pro Ile
 465 470 475 480

Trp Ala Lys Ile Pro His Thr Asp Gly His Phe His Pro Ser Pro Leu
 485 490 495

Met Gly Gly Phe Gly Leu Lys His Pro Pro Pro Gln Ile Leu Ile Lys
 500 505 510

Asn Thr Pro Val Pro Ala Asn Pro Ser Thr Thr Phe Ser Ala Ala Lys
 515 520 525

Phe Ala Ser Phe Ile Thr Gln Tyr Ser Thr Gly Gln Val Ser Val Glu
 530 535 540

Ile Glu Trp Glu Leu Gln Lys Glu Asn Ser Lys Arg Trp Asn Pro Glu
 545 550 555 560

Ile Gln Tyr Thr Ser Asn Tyr Asn Lys Ser Val Asn Val Asp Phe Thr
 565 570 575

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<210> 12
<211> 533
<212> PRT
<213> adeno-associated virus 2 VP3 capsid protein
<400> 12
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Asp Gly Val Gly Asn Ser Ser Gly Asn Trp His Cys Asp Ser Thr Trp
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Met Gly Asp Arg Val Ile Thr Thr Ser Thr Arg Thr Trp Ala Leu Pro
35 40 45

Thr Tyr Asn Asn His Leu Tyr Lys Gln Ile Ser Ser Gln Ser Gly Ala
50 55 60

Ser Asn Asp Asn His Tyr Phe Gly Tyr Ser Thr Pro Trp Gly Tyr Phe
65 70 75 80

Asp Phe Asn Arg Phe His Cys His Phe Ser Pro Arg Asp Trp Gln Arg
85 90 95

Leu Ile Asn Asn Asn Trp Gly Phe Arg Pro Lys Arg Leu Asn Phe Lys
100 105 110

Leu Phe Asn Ile Gln Val Lys Glu Val Thr Gln Asn Asp Gly Thr Thr
115 120 125

Thr Ile Ala Asn Asn Leu Thr Ser Thr Val Gln Val Phe Thr Asp Ser
130 135 140

Glu Tyr Gln Leu Pro Tyr Val Leu Gly Ser Ala His Gln Gly Cys Leu
145 150 155 160

Pro Pro Phe Pro Ala Asp Val Phe Met Val Pro Gln Tyr Gly Tyr Leu
165 170 175

Thr Leu Asn Asn Gly Ser Gln Ala Val Gly Arg Ser Ser Phe Tyr Cys
180 185 190

Leu Glu Tyr Phe Pro Ser Gln Met Leu Arg Thr Gly Asn Asn Phe Thr
195 200 205

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Phe Ser Tyr Thr Phe Glu Asp Val Pro Phe His Ser Ser Tyr Ala His
 210 215 220

Ser Gln Ser Leu Asp Arg Leu Met Asn Pro Leu Ile Asp Gln Tyr Leu
 225 230 235 240

Tyr Tyr Leu Ser Arg Thr Asn Thr Pro Ser Gly Thr Thr Thr Gln Ser
 245 250 255

Arg Leu Gln Phe Ser Gln Ala Gly Ala Ser Asp Ile Arg Asp Gln Ser
 260 265 270

Arg Asn Trp Leu Pro Gly Pro Cys Tyr Arg Gln Gln Arg Val Ser Lys
 275 280 285

Thr Ser Ala Asp Asn Asn Asn Ser Glu Tyr Ser Trp Thr Gly Ala Thr
 290 295 300

Lys Tyr His Leu Asn Gly Arg Asp Ser Leu Val Asn Pro Gly Pro Ala
 305 310 315 320

Met Ala Ser His Lys Asp Asp Glu Glu Lys Phe Phe Pro Gln Ser Gly
 325 330 335

Val Leu Ile Phe Gly Lys Gln Gly Ser Glu Lys Thr Asn Val Asp Ile
 340 345 350

Glu Lys Val Met Ile Thr Asp Glu Glu Glu Ile Arg Thr Thr Asn Pro
 355 360 365

Val Ala Thr Glu Gln Tyr Gly Ser Val Ser Thr Asn Leu Gln Arg Gly
 370 375 380

Asn Arg Gln Ala Ala Thr Ala Asp Val Asn Thr Gln Gly Val Leu Pro
 385 390 395 400

Gly Met Val Trp Gln Asp Arg Asp Val Tyr Leu Gln Gly Pro Ile Trp
 405 410 415

Ala Lys Ile Pro His Thr Asp Gly His Phe His Pro Ser Pro Leu Met
 420 425 430

Gly Gly Phe Gly Leu Lys His Pro Pro Pro Gln Ile Leu Ile Lys Asn
 435 440 445

Thr Pro Val Pro Ala Asn Pro Ser Thr Thr Phe Ser Ala Ala Lys Phe
 450 455 460

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Ala Ser Phe Ile Thr Gln Tyr Ser Thr Gly Gln Val Ser Val Glu Ile
 465 470 475 480

Glu Trp Glu Leu Gln Lys Glu Asn Ser Lys Arg Trp Asn Pro Glu Ile
 485 490 495

Gln Tyr Thr Ser Asn Tyr Asn Lys Ser Val Asn Val Asp Phe Thr Val
 500 505 510

Asp Thr Asn Gly Val Tyr Ser Glu Pro Arg Pro Ile Gly Thr Arg Tyr
 515 520 525

Leu Thr Arg Asn Leu
 530

<210> 13
 <211> 12
 <212> PRT
 <213> Artificial

<220>
 <223> Phage display library

<400> 13

Thr Thr Val Tyr Pro Ala Arg Trp Gly Ala His Pro
 1 5 10

<210> 14
 <211> 12
 <212> PRT
 <213> Artificial

<220>
 <223> Phage display library

<400> 14

Asn His Val His Arg Met His Ala Thr Pro Ala Tyr
 1 5 10