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(54) **Title**: PEPTIDE LIGANDS FOR TARGETING TO THE BLOOD-BRAIN BARRIER

(57) **Abstract**: The present invention relates to peptides comprising defined amino acid sequences, compositions comprising said peptides and a pharmaceutically acceptable substance, use of said peptides as a central nervous system targeting principle in the treatment, prophylaxis or diagnosis of a central nervous system disorders, combinations of said peptides with pharmaceutically acceptable substances, use of said combinations for the treatment, prophylaxis or diagnosis of a central nervous system disorders, a method of delivery of said peptide and of a pharmaceutically acceptable substance and a nucleic acid sequence encoding for said peptide sequence.

Title: **Peptide ligands for targeting to the blood-brain barrier**

Field of the Invention

The present invention relates to peptides that can be used for the targeting of drug delivery systems or diagnostic agent delivery systems to the brain.

Background of the Invention

The brain is shielded against potentially harmful substances by the blood-brain barrier (BBB). The microvascular barrier between blood and brain is made up of a capillary endothelial layer surrounded by a basement membrane and tightly associated accessory cells (pericytes, astrocytes). The blood-brain barrier regulates homeostasis of the brain and is selectively permeable for the uptake and efflux of ions, nutrients and metabolites. The brain capillary endothelium is much less than other capillary endothelia due to an apical band of tight association between the membranes of adjoining cells, referred to as tight junctions.

The blood-brain barrier also impedes access to beneficial pharmaceutically acceptable substances (e.g. active pharmaceutical ingredients, diagnostic agents) to the central nervous system (CNS) tissues, necessitating the use of carriers for their transit. Blood-brain barrier permeability is frequently a rate-limiting factor for the penetration of pharmaceutically acceptable substances into the central nervous system. This limits the treatment of many central nervous system disorders, such as e.g. Alzheimer's disease and Parkinson's disease.

Several documents disclose an overview of several methods for the administration of pharmaceutically acceptable substances to the central nervous system (e.g. US 6,399,575 B1, WO 2005/002515) but many of them are associated with disadvantages. For example, direct administration of

pharmaceutically acceptable substances to the central nervous system is associated with high safety risks (infections, tissue damage).

A beneficial method for administration of pharmaceutically acceptable substances to the central nervous system is the central nervous system targeting method, i.e. the targeting of pharmaceutically acceptable substances (e.g. active pharmaceutical ingredients, diagnostic agents, proteins) across the blood-brain barrier to the central nervous system.

US 6,399,575 B1 discloses compositions and methods for targeting genes, proteins, pharmaceuticals, or other compounds to the central nervous system. The composition comprises peptide sequences which bind central nervous system tissue with high specifity. Said peptide sequences contain seven amino acids only.

WO 2005/002515 discloses that megalin ligands can be used as carriers or vectors for the delivery of active agents via transcytosis.

US 2002/0025313 discloses compositions and methods for targeting drugs to and across the blood-brain barrier. The compositions are comprised of a liposome, an active pharmaceutical ingredient intended to be transported to the brain, and an antibody molecule or binding fragment thereof which binds to a receptor present on vascular endothelium cells of the brain.

It is desired to provide novel peptide sequences that can be used for targeting of pharmaceutically acceptable substances (such as drugs, diagnostic agents or delivery systems of drugs or diagnostic agents) to the central nervous system (central nervous system targeting). Preferably, such peptides should have selectivity for brain endothelium.

Summary of the Invention

In order to better address one or more of the foregoing desires the invention, in one aspect, is a peptide comprising an amino acid sequence which is in at least 9, preferably at least 11, most preferably at least 13 amino acids identical to

the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:2. As used herein, the term "SEQ ID NO:1" means the amino acid sequence GLAHSFSDFARDFVA (also abbreviated as "GLA"). As used herein, the term "SEQ ID NO:2" means the amino acid sequence GYRPVHNIRGHWAPG (also abbreviated as "GYR").

In another aspect, the invention presents a composition of matter for the delivery of a pharmaceutically acceptable substance across the blood-brain barrier, the composition comprising

(a) a peptide comprising an amino acid sequence which is in at least 9, preferably at least 11, most preferably at least 13 amino acids identical to the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:2, and

(b) a pharmaceutically acceptable substance

In yet another aspect, the invention is in the use of the aforementioned peptide or the aforementioned composition in the manufacture of a medicament for the treatment, prophylaxis of a central nervous system disorder or for the manufacture of a diagnostic agent of a central nervous system disorder.

In a further aspect, the invention provides a peptide or a composition as mentioned above for use as a central nervous system targeting principle in the treatment, prophylaxis or diagnosis of a central nervous system disorder.

In a still further aspect, the invention resides in a composition comprising (a) and (b) as defined above, for use as a delivery system in delivering a pharmaceutically acceptable substance (such as a drug, a diagnostic agent, or a delivery system for a drug or a diagnostic agent) across the blood-brain barrier to the central nervous system.

Detailed description of embodiments

In a broad sense, the invention is based on the fact that, peptides have been found comprising an amino acid sequence which is in at least 9, preferably at least 11, most preferably at least 13 amino acids identical to the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:2.

As used herein, the term "SEQ ID NO:1" means the amino acid sequence GLAHSFSDFARDFVA (also abbreviated as "GLA"). As used herein, the term "SEQ ID NO:2" means the amino acid sequence GYRPVHNIRGHWAPG (also abbreviated as "GYR").

The peptides according to the invention are able to dock onto the blood-brain barrier and/or cross the blood brain barrier and bind to central nervous system cells. Preferably, the peptides specifically dock onto the blood-brain barrier. Most preferably, the peptides bind to brain endothelial cells.

Preferably, the peptide according to the invention comprise an amino acid sequence which is in at least 10, more preferably 11, 12, 13, 14 amino acids identical to the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:2. In a preferred embodiment, the peptide according to the invention is an isolated peptide.

Most preferably, the peptide according to the invention comprise an amino acid sequence which is in at least 10 amino acids identical to the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:2.

Most preferably, the peptide according to the invention comprise an amino acid sequence which is in at least 11 amino acids identical to the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:2.

Most preferably, the peptide according to the invention comprise an amino acid sequence which is in at least 12 amino acids identical to the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:2.

Most preferably, the peptide according to the invention comprise an amino acid sequence which is in at least 13 amino acids identical to the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:2.

Most preferably, the peptide according to the invention comprise an amino acid sequence which is in at least 14 amino acids identical to the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:2.

Most preferably, the peptide according to the invention comprise an amino acid sequence which is in at least 15 amino acids identical to the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:2.

Most preferably, the peptide according to the invention consists of an amino acid sequence according to SEQ ID NO:1 or SEQ ID NO:2.

In a particular embodiment of the present invention, the peptide according to the invention comprises the amino acid sequence set forth in SEQ ID NO:1 or SEQ ID NO:2.

In the following, these peptides, i.e. peptides which comprise an amino acid sequence which is in at least 10, more preferably 11, 12, 13, 14, 15 amino acids identical to the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:2 or which consists of an amino acid sequence according to SEQ ID NO:1 or SEQ ID NO:2 are called “peptides according to the invention”.

The present invention also relates to a composition of matter for the delivery of a pharmaceutically acceptable substance across the blood-brain barrier, the composition comprising:

(a) a peptide according to the invention

(b) a pharmaceutically acceptable substance.

In a preferred embodiment, said pharmaceutically acceptable substance is selected from the group of active pharmaceutical ingredients, proteins, nucleic

acids, siRNA molecules, antisense molecules, expression constructs that comprises a nucleic acid that encodes a therapeutic protein of interest, liposomes, nanoparticles, diagnostic agents, markers of a disease of the central nervous system (CNS), antibodies, monoclonal antibodies, labelled monoclonal antibodies which bind a marker of a CNS disorder, and/or a fragment of an antibody or of a monoclonal antibody.

Most preferably said pharmaceutically acceptable substance is an active pharmaceutical ingredient.

Most preferably said pharmaceutically acceptable substance is a protein.

Most preferably said pharmaceutically acceptable substance is a nucleic acid.

Most preferably said pharmaceutically acceptable substance is a siRNA molecule.

Most preferably said pharmaceutically acceptable substance is an antisence molecule.

Most preferably said pharmaceutically acceptable substance is an expression constructs that comprises a nucleic acid that encodes a therapeutic protein of interest.

Most preferably said pharmaceutically acceptable substance is a liposome.

Most preferably said pharmaceutically acceptable substance is a nanoparticle.

Most preferably said pharmaceutically acceptable substance is a diagnostic agent.

Most preferably said pharmaceutically acceptable substance is a marker of a disease of the central nervous system (CNS).

Most preferably said pharmaceutically acceptable substance is an antibody.

Most preferably said pharmaceutically acceptable substance is a monoclonal antibody.

Most preferably said pharmaceutically acceptable substance is a labelled monoclonal antibody which binds a marker of a CNS disorder.

Most preferably said pharmaceutically acceptable substance is a fragment if an antibody or of a monoclonal antibody.

In a preferred embodiment, the peptide according to the invention has a carrier function for the pharmaceutically acceptable substance. In a more preferred embodiment, the peptide-pharmaceutically acceptable substance-structure is able to cross the blood-brain barrier, i.e. with the help of the peptide according to the invention the pharmaceutically acceptable substance is able to cross the blood-brain barrier and to enter the central nervous system. In a more preferred embodiment, the peptide-pharmaceutically acceptable substance-structure is able to dock onto the blood-brain barrier, preferably to brain endothelial cells, and releases the pharmaceutical acceptable substance, preferably the active pharmaceutical ingredient, more preferably the protein, the nucleic acid, the siRNA molecule, the antisense molecule, the expression construct that comprises a nucleic acid that encodes a therapeutic protein of interest, the diagnostic agent, the marker of a disease of the central nervous system, the antibody, the monoclonal antibody which bind a marker of a CNS disorder, and/or the fragment of an antibody or of a monoclonal antibody which penetrates across the blood-brain barrier to the central nervous system subsequently.

The composition of the present invention can take the form of separate components, such as a kit of parts. Preferably, the composition takes the form of a physical combination of the peptide and the pharmaceutically acceptable substance, and more preferably the peptide and the pharmaceutically acceptable substance are chemically linked to each other.

In a particular embodiment, the present invention relates to a combination of a peptide according to the invention with a pharmaceutically acceptable substance, wherein said peptide is coupled to said pharmaceutically acceptable

substance by covalent or non-covalent bonds. In a preferred embodiment, the peptide according to the invention has a carrier function for the pharmaceutically acceptable substance.

In a preferred embodiment, the composition of the invention is a delivery system wherein a structure comprising the peptide and the pharmaceutically acceptable substance is able to cross the blood-brain barrier, i.e. with the help of the peptide according to the invention the pharmaceutically acceptable substance is able to cross the blood-brain barrier and to enter the central nervous system.

In a further preferred embodiment, the delivery system is able to dock onto the blood-brain barrier preferably to brain endothelial cells, and releases the pharmaceutical acceptable substance, preferably the active pharmaceutical ingredient, more preferably the protein, the nucleic acid, the siRNA molecule, the antisense molecule, the expression construct that comprises a nucleic acid that encodes a therapeutic protein of interest, the diagnostic agent, the marker of a disease of the central nervous system, the antibody, the monoclonal antibody which bind a marker of a CNS disorder, and/or the fragment of an antibody or of a monoclonal antibody which penetrates across the blood-brain barrier to the central nervous system subsequently. In another preferred embodiment of the present invention, more than one peptide according to the invention is coupled to a pharmaceutically acceptable substance by covalent or non-covalent bonds, whereas the peptide has a carrier function for the pharmaceutically acceptable substance. In a further preferred embodiment, said pharmaceutically acceptable substance is selected from the group of active pharmaceutical ingredients, proteins, nucleic acids, siRNA molecules, antisense molecules, expression constructs that comprises a nucleic acid that encodes a therapeutic protein of interest, liposomes, nanoparticles, diagnostic agents, markers of a disease of the central nervous system (CNS), antibodies,

monoclonal antibodies, labelled monoclonal antibodies which bind a marker of a CNS disorder, and/or a fragment of an antibody or a monoclonal antibody.

In a particular embodiment of the present invention, the peptide according to the invention is coupled to a liposome by covalent or non-covalent bonds to carry a active pharmaceutical ingredient, a protein, a nucleic acid, a siRNA molecule, an antisense molecule, an expression construct that comprise a nucleic acid that encodes a therapeutic protein of interest, a diagnostic agent, a marker of a disease of the central nervous system, an antibody, a monoclonal antibody which bind a marker of a CNS disorder, and/or a fragment of an antibody or of a monoclonal antibody.

In the liposome preparations of the present invention (i.e. when the pharmaceutically acceptable substance is a liposome), the active pharmaceutical ingredient, the protein, the nucleic acid, the siRNA molecule, the antisense molecule, the expression construct that comprises a nucleic acid that encodes a therapeutic protein of interest, the diagnostic agent, the marker of a disease of the central nervous system, the antibody, the monoclonal antibody which bind a marker of a CNS disorder, and/or the fragment of an antibody or of a monoclonal antibody to be delivered across the blood-brain barrier is either (i). incorporated into the volume of the liposome, (ii). incorporated into the lipid bilayer and/or (iii). attached to the surface of the liposome by covalent or non-covalent bonds. The liposome preparation can cross the blood-brain barrier and transport the content of the liposome across the blood-brain barrier to the central nervous system and/or dock onto the blood-brain barrier and release the incorporated and/or attached active pharmaceutical ingredient, the protein, the nucleic acid, the siRNA molecule, the antisense molecule, the expression construct that comprise a nucleic acid that encodes a therapeutic protein of interest, the diagnostic agent, the marker of a disease of the central nervous system, the antibody, the monoclonal

antibody which bind a marker of a CNS disorder, and/or the fragment of an antibody or of a monoclonal antibody which penetrates across the blood-brain barrier to the central nervous system subsequently. The foregoing can be generally referred to as a preferred embodiment in which the pharmaceutically acceptable substance comprises an active substance (such as an active pharmaceutical ingredient or a diagnostic agent), and a carrier (such as a nanoparticle), preferably a liposome.

In a particular embodiment, the present invention relates to a combination of a peptide according to the invention with a pharmaceutically acceptable substance, wherein said peptide and said pharmaceutically acceptable substance are covalently linked by a linking structure. In a preferred embodiment, said peptide and said pharmaceutically acceptable substance are directly linked to each other. In a more preferred embodiment, said pharmaceutically acceptable substance is selected from the group of active pharmaceutical ingredients, proteins, nucleic acids, siRNA molecules, antisense molecules, expression constructs that comprises a nucleic acid that encodes a therapeutic protein of interest, liposomes, nanoparticles, diagnostic agents, markers of a disease of the central nervous system (CNS), antibodies, monoclonal antibodies, labelled monoclonal antibodies which bind a marker of a CNS disorder, and/or a fragment of an antibody or a monoclonal antibody.

In a particular embodiment, the present invention relates to a combination of a peptide according to the invention with a pharmaceutically acceptable substance, wherein said peptide and said pharmaceutically acceptable substance are each separately covalently coupled to a linking structure and wherein the covalently coupled linking structures are coupled to each other by non-covalent bonds. In a preferred embodiment, the peptide according to the invention is covalently coupled to biotin and the pharmaceutically acceptable substance is covalently coupled to streptavidin. Biotin and streptavidin are

known to interact via non-covalently binding forces, i.e. the biotin is non-covalently coupled to streptavidin, which is known to the person skilled in the art. In another preferred embodiment, said pharmaceutically acceptable substance is selected from the group of active pharmaceutical ingredients, proteins, nucleic acids, siRNA molecules, antisense molecules, expression constructs that comprises a nucleic acid that encodes a therapeutic protein of interest, liposomes, nanoparticles, diagnostic agents, markers of a disease of the central nervous system (CNS), antibodies, monoclonal antibodies, labelled monoclonal antibodies which bind a marker of a CNS disorder, and/or a fragment of an antibody or a monoclonal antibody.

In general such peptide-pharmaceutically acceptable substance-conjugates can be prepared using techniques known in the art. Preferably, the linking structure is selected from the group of a linker peptide, a carbo-hydrogen bond, streptavidin-biotin, polyethylene glycol (PEG), a disulfide bridge.

In a more particular embodiment, the present invention relates to a combination of a peptide according to the invention with a pharmaceutically acceptable substance, wherein said pharmaceutically acceptable substance is selected from the group of active pharmaceutical ingredients, proteins, nucleic acids, siRNA molecules, antisense molecules, expression constructs that comprises a nucleic acid that encodes a therapeutic protein of interest, liposomes, nanoparticles, diagnostic agents, markers of a disease of the central nervous system (CNS), antibodies, monoclonal antibodies, labelled monoclonal antibodies which bind a marker of a CNS disorder, and/or a fragment of an antibody or a monoclonal antibody. In a particular embodiment of the present invention, the peptide according to the invention has the function of a carrier for the pharmaceutical acceptable substance to transport the pharmaceutical acceptable substance across the blood-brain barrier to the central nervous system (central nervous system targeting principle). The peptide can also dock

onto the blood-brain barrier and release the pharmaceutically acceptable substance which penetrates across the blood-brain barrier to the central nervous system subsequently.

In another particular embodiment the active pharmaceutical ingredient, the protein, the nucleic acids, the siRNA molecules, the antisense molecules, the expression constructs that comprises a nucleic acid that encodes a therapeutic protein of interest, the diagnostic agents, the markers of a disease of the central nervous system (CNS), the antibodies, the erythrocytes, erythrocytes ghosts, the spheroplasts, the monoclonal antibodies, the labelled monoclonal antibodies which bind a marker of a CNS disorder, and/or the fragment of an antibody or a monoclonal antibody can be incorporated into a nanoparticle (e.g. a liposome), whereas the peptide according to the invention is coupled to said nanoparticle (e.g. a liposome) by covalent or non-covalent bonds and whereas the nanoparticle (e.g. a liposome) acts as a carrier for the incorporated compounds. In this structure, the complex (comprising the peptide according to the invention, the nanoparticle and the incorporated compound) is able to cross the blood-brain barrier or to dock onto to blood-brain barrier. In another embodiment, the active pharmaceutical ingredient is an antibody directed toward a constituent of the central nervous system.

The present invention also relates to the use of a peptide according to the invention as a central nervous system targeting principle in the treatment, prophylaxis or diagnosis of a central nervous system disorder. In a preferred embodiment, said central nervous system disorder is selected from the group consisting of depression, dementia, Prion Disease, Alzheimer's Disease, Parkinson's Disease, Multiple Sclerosis, Amylotrophic Lateral Sclerosis, Schizophrenia, Lyme Disease, Poliomyelitis, stroke, traumatic brain injury, psychosis, Chorea Huntington Disease, encephalopathy, epilepsy, cerebrovascular diseases and neurodegenerative disorders and a central nervous system cancer.

Most preferably said central nervous system disorder is depression.
Most preferably said central nervous system disorder is dementia.
Most preferably said central nervous system disorder is Prion Disease.
Most preferably said central nervous system disorder is Alzheimer's Disease.
Most preferably said central nervous system disorder is Parkinson's Disease.
Most preferably said central nervous system disorder is Multiple Sclerosis.
Most preferably said central nervous system disorder is Amylotrophic Lateral Sclerosis.
Most preferably said central nervous system disorder is Schizophrenia.
Most preferably said central nervous system disorder is Lyme Disease.
Most preferably said central nervous system disorder is Poliomyelitis.
Most preferably said central nervous system disorder is stroke.
Most preferably said central nervous system disorder is traumatic brain injury.
Most preferably said central nervous system disorder is psychosis.
Most preferably said central nervous system disorder is Chorea Huntington Disease.
Most preferably said central nervous system disorder is encephalopathy.
Most preferably said central nervous system disorder is epilepsy.
Most preferably said central nervous system disorder is a cerebrovascular disease.
Most preferably said central nervous system disorder is a neurodegenerative disorder.
Most preferably said central nervous system disorder is a central nervous system cancer.

The present invention also relates to the use of a composition comprising a peptide according to the invention with a pharmaceutically acceptable substance in the treatment, prophylaxis or diagnosis of a central nervous system disorder. In a preferred embodiment, said central nervous system

disorder is selected from the group consisting of depression, dementia, Prion Disease, Alzheimer's Disease, Parkinson's Disease, Multiple Sclerosis, Amylotrophic Lateral Sclerosis, Schizophrenia, Lyme Disease, Poliomyelitis, stroke, traumatic brain injury, psychosis, Chorea Huntington Disease, encephalopathy, epilepsy, cerebrovascular diseases and neurodegenerative disorders and a central nervous system cancer.

The present invention also relates to the use of a peptide according to the invention in the manufacture of a medicament for the treatment, prophylaxis of a central nervous system disorder or for the manufacture of a diagnostic agent of a central nervous system disorder. In a particular embodiment, said medicament is a combination of a peptide according to the invention with a pharmaceutically acceptable substance. The present invention also relates to the use of a combination of a peptide according to the invention with a pharmaceutically acceptable substance for the manufacture of a medicament for the treatment, prophylaxis of a central nervous system disorder or for the manufacture of a diagnostic agent of a central nervous system disorder. In a preferred embodiment, said central nervous system disorder is selected from the group consisting of depression, dementia, Prion Disease, Alzheimer's Disease, Parkinson's Disease, Multiple Sclerosis, Amylotrophic Lateral Sclerosis, Schizophrenia, Lyme Disease, Poliomyelitis, stroke, traumatic brain injury, psychosis, Chorea Huntington Disease, encephalopathy, epilepsy, cerebrovascular diseases and neurodegenerative disorders and a central nervous system cancer.

The present invention also relates to a peptide according to the invention for use as a central nervous system targeting principle in the treatment, prophylaxis or diagnosis of a central nervous system disorder. In a preferred embodiment, said central nervous system disorder is selected from the group consisting of depression, dementia, Prion Disease, Alzheimer's Disease,

Parkinson's Disease, Multiple Sclerosis, Amylotrophic Lateral Sclerosis, Schizophrenia, Lyme Disease, Poliomyelitis, stroke, traumatic brain injury, psychosis, Chorea Huntington Disease, encephalopathy, epilepsy, cerebrovascular diseases and neurodegenerative disorders and a central nervous system cancer.

The present invention also relates to a combination of a peptide according to the invention with a pharmaceutically acceptable substance, in any of the embodiments described hereinbefore, for use in the treatment, prophylaxis or diagnosis of a central nervous system disorder. In a preferred embodiment, said central nervous system disorder is selected from the group consisting of depression, dementia, Prion Disease, Alzheimer's Disease, Parkinson's Disease, Multiple Sclerosis, Amylotrophic Lateral Sclerosis, Schizophrenia, Lyme Disease, Poliomyelitis, stroke, traumatic brain injury, psychosis, Chorea Huntington Disease, encephalopathy, epilepsy, cerebrovascular diseases and neurodegenerative disorders and a central nervous system cancer.

The present invention also relates to a method of delivering a pharmaceutically acceptable substance across the blood-brain barrier to the central nervous system, comprising

(a) a peptide according to the invention

(b) a pharmaceutically acceptable substance.

In a preferred embodiment, the peptide according to the invention can cross the blood-brain barrier. In a further preferred embodiment, the peptide according to the invention can dock onto the blood-brain barrier, preferably to the brain endothelial cells, and releases the pharmaceutically acceptable substance which penetrates across the blood-brain barrier to the central nervous system subsequently..

In a preferred embodiment, said central nervous system disorder is selected from the group consisting of depression, dementia, Prion Disease, Alzheimer's

Disease, Parkinson's Disease, Multiple Sclerosis, Amylotrophic Lateral Sclerosis, Schizophrenia, Lyme Disease, Poliomyelitis, stroke, traumatic brain injury, psychosis, Chorea Huntington Disease, encephalopathy, epilepsy, cerebrovascular diseases and neurodegenerative disorders and a central nervous system cancer.

The present invention also relates to a pharmaceutical formulation comprising a peptide according to the invention or a combination of a peptide according to the invention with a pharmaceutically acceptable substance and one or more pharmaceutically acceptable excipients. Said pharmaceutical formulation may be provided as suitable for parenteral (e.g. subcutaneous, intradermal, intramuscular, intravenous, intraspinal and intraarticular), oral or inhalation administration. Alternatively, pharmaceutical formulations of the present invention may be suitable for administration to the mucous membranes of the subject (e.g. intranasal administration). It is recognized that intranasal administration could be used to target pharmaceutically acceptable substances to olfactory neurons, which are otherwise difficult to access. In the same manner, intraocular administration could be used to target pharmaceutically acceptable substances to the optic nerve. Accordingly, the peptides of the present invention could also be used to target pharmaceutically acceptable substances to the optic nerve to treat optic nerve disorders, e.g. retinipathics. Where needed, the pharmaceutical formulations of the present invention may be administered by direct delivery to the central nervous system by intrathecal injection to cerebrospinal fluid or directly into the brain. The pharmaceutical formulations may be conveniently prepared in unit dosage form and may be prepared by any of the methods well-known in the art. Any inert pharmaceutically-acceptable carrier may be used, such as saline, or phosphate-buffered saline, or in any other such well-known carriers (e.g. nanoparticles) suitable for the mentioned application area in which the pharmaceutical formulations of the present invention have suitable solubility properties for

use in the methods of the present invention. The pharmaceutical formulation according to the invention also includes, but is not limited to pharmaceutical formulations like liposomes or other vesicular systems, microemulsions, micellar systems, unilamelar or multilamellar vesicular systems, erythrocytes, erythrocytes ghosts, or spheroplasts, any other nanoparticulate system in this filed to mention some of already used devices. The pharmaceutical formulation according to the invention can also be used in lyophilized form.

The present invention also relates to a nucleic acid sequence encoding for a peptide sequence according to the present invention. The nucleic acid sequences of the invention include SEQ ID No. 3 and SEQ ID No. 4.

SEQ ID No. 3 is a nucleic acid sequence encoding for the peptide GLA (which is the amino acid sequence of SEQ ID No. 1), viz.:
GGTCTTGCTCATTCTTTTTCTGATTTTGCTCGTGATTTTGTTGCT

SEQ ID No. 4 is a nucleic acid sequence encoding for the peptide GYR (which is the amino acid sequence of SEQ ID No. 2), viz.:
GGTTATCGTCCTGTTCATAATATTCGTGGTCATTGGGCTCCTGGT

The present invention also relates to a method of treatment, prevention and/or diagnosis of a central nervous system disorder comprising the use of a peptide according to the invention as a central nervous system targeting principle.

The present invention also relates to a method of treatment, prevention and/or diagnosis of a central nervous system disorder comprising the use of a composition comprising a peptide according to the invention with a pharmaceutically acceptable substance, in any of the embodiments described hereinbefore.

In a more particular embodiment, the present invention relates to a method of treatment, prevention and/or diagnosis of a central nervous system disorder comprising

(a) the use of a peptide according to the invention as a central nervous system targeting principle or

(b) the use of a combination of a peptide according to the invention with a pharmaceutically acceptable substance,

wherein said central nervous system disorder is selected from the group consisting of depression, dementia, Prion Disease, Alzheimer's Disease, Parkinson's Disease, Multiple Sclerosis, Amylotrophic Lateral Sclerosis, Schizophrenia, Lyme Disease, Poliomyelitis, stroke, traumatic brain injury, psychosis, Chorea Huntington Disease, encephalopathy, epilepsy, cerebrovascular diseases and neurodegenerative disorders and a central nervous system cancer.

The present invention also relates to a process of preparing a peptide according to the present invention. Methods of preparing peptides are known in the art, e.g. solid phase peptide synthesis (SPPS) as disclosed by B. Merrifield, Science 232: 341-347 (1986).

The invention described and claimed herein is not to be limited in scope by the specific embodiments herein disclosed, since these embodiments are intended as illustrations of several aspects of the invention. Any equivalent embodiments are intended to be within the scope of this invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims. In the case of conflict, the present disclosure including definitions will control.

Definitions

As used herein, the singular forms "a", "an", and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to a fill material containing "a carrier" includes one or more carriers, reference to "an additive" includes reference to one or more of such additives.

As used herein, unless specifically indicated otherwise, the conjunction "or" is used in the inclusive sense of "and/or" and not the exclusive sense of "either/or".

As used herein, the term "active pharmaceutical ingredient" (API) includes any pharmaceutically active chemical or biological compound and any pharmaceutically acceptable salt thereof and any mixture thereof, that provides some pharmacologic effect and is used for treating or preventing a condition. Exemplary pharmaceutically acceptable salts include hydrochloric, sulfuric, nitric, phosphoric, hydrobromic, maleric, malic, ascorbic, citric, tartaric, pamoic, lauric, stearic, palmitic, oleic, myristic, lauryl sulfuric, naphthalinesulfonic, linoleic, linolenic acid, and the like. Exemplary pharmaceutically acceptable salts also includes salts whereas the active pharmaceutical ingredient acts as the acidic component (i.e. in the pharmaceutically acceptable salt the active pharmaceutical ingredient is charged negatively) and the counter ion is a basic component (i.e. in the pharmaceutically acceptable salt the counter ion is positively charged). The active pharmaceutical ingredient can be present in the composition in an amount of about 0.1 μg to about 2000 mg, in an amount of about 1 μg to about 1000 mg, in an amount of 5 μg to about 500 mg, in an amount of about 10μg to about 250 mg, in an amount of about 50 μg to about 200mg, in an amount of about 100μg to about 150mg, or in an amount of about 1mg to 100mg once daily, twice daily, or more than twice daily. As used herein, the terms "drug",

"active agent", "active ingredient", "active substance" and "drug" are meant to be synonyms, i.e., have identical meaning. In the present invention the active pharmaceutical ingredient is preferably chosen from one or more of the following categories/groups: abortifacient/interceptive agents, ace-inhibitors, alpha-adrenergic agonists, beta-adrenergic agonists, alpha-adrenergic blockers, beta-adrenergic blockers, adrenocortical steroids, adrenocortical suppressants, adrenocorticotropic hormones, alcohol deterrents, aldose reductase inhibitors, aldosterone antagonists, 5-alpha reductase inhibitors, anabolic agents, analeptic agents, analgesic, androgens, angiotensin converting enzyme inhibitors, angiotensin II receptor antagonists, anorexic agents, antacid agents, anthelmintic agents, antiacne agents, antiallergic agents, antialopecias, antiamebic agents, antiandrogens, antianginal agents, antiarrhythmic agents, antiarteriosclerotic agents, antiarthritic/antirheumatic agents, antiasthmatic agents, antibacterial agents, antibacterial adjuncts, antibiotics, anticancer agents, anticholelithogenic agents, anticholesteremic agents, anticholinergic agents, anticoagulants, anticonvulsants, antidepressants, antidiabetic agents, antidiarrheal agents, antidiuretic agents, antidotes, antidyskinetic agents, antieczematic agents, antiemetic agents, antiepileptic agents, antiestrogens, antifibrotic agents, antiflatulents, antifungal agents, antiglaucoma agents, antigonadotropins, antigouts, antihemorrhagic agents, antihistaminic agents, antihypercholesterolemic agents, antihyperlipidemic agents, antihyperlipoproteinemic agents, antihyperphosphatemic agents, antihypertensives, antihyperthyroid agents, antihypotensive agents, antihypothyroid agents, anti-infective agents, anti-inflammatory agents, antileprotic agents, antileukemic agents, antilipemic agents, antimalarial agents, antimanic agents, antimethemoglobinemic agents, antimigraine agents, antimycotic agents, antinauseants, antineoplastic agents, antineoplastic adjuncts, antineutropenic agents, antiosteoporotic agents, antipagetic agents, antiparkinsonians, antiperistaltic agents, antipheochromocytomas, antipneumocystis agents, antiprostatic hypertrophy

agents, antiprotozoals, antipruritic agents, antipsoriatic agents, antipsychotic agents, antipyretic agents, antirheumatic agents, antirickettsials, antiseborrheic agents, antiseptic/disinfectant agents, antispasmodic agents, antisyphilitic agents, antithrombocythemic agents, antithrombotic agents, antitubercular agents, antitumor agents, antitussive agents, antiulcerative agents, antiurolithic agents, antivenins, antivertigo agents, antiviral agents, anxiolytic agents, aromatase inhibitors, astringent agents, benzodiazepine antagonists, beta-blockers, bone resorption inhibitors, bradycardic agents, bradykinin antagonists, bronchodilators, calcium channel blockers, calcium regulators, calcium supplements, cancer chemotherapys, capillary protectants, carbonic anhydrase inhibitors, cardiac depressants, cardiotonic agents, cathartic agents, CCK antagonists; central stimulants, central nervous depressants, cerebral vasodilators, chelating agents, cholecystokinin antagonists, cholelitholytic agents, choleretic agents, cholinergic agents, cholinesterase inhibitors, cholinesterase reactivators, CNS stimulants, cognition activators, contraceptive agents, control of intraocular pressures, converting enzyme inhibitors, coronary vasodilators, cytoprotectants, cytotoxic chemotherapeutic agents (e.g. adriamycin, cisplatin, 5-fluorouracil, camptothecin, paclitaxel), debriding agents, decongestants, depigmentors, dermatitis herpetiformis suppressants, digestive aids, diuretic agents, dopamine receptor agonists, dopamine receptor antagonists, ectoparasiticides, emetic agents, enkephalinase inhibitors, enzymes, enzyme cofactors, enzyme inducers, estrogens, estrogen antagonists, expectorants, fibrinogen receptor antagonists, gastric and pancreatic secretion stimulants, gastric proton pump inhibitors, gastric secretion inhibitors, gastroprokinetic agents, glucocorticoids, alpha-glucosidase inhibitors, gonad-stimulating principles, gout suppressants, growth hormone inhibitors, growth hormone releasing factors, growth stimulants, hematinic agents, hematopoietic agents, hemolytic agents, hemostatic agents, heparin antagonists, hepatoprotectants, histamine H1 -receptor antagonists, histamine H2 -receptor antagonists, HIV proteinase

inhibitors, HMG CoA reductase inhibitors, hypnotic agents, hypocholesteremic agents, hypolipidemic agents, hopotensives, immunomodulators, immunosuppressants, intropic agents, insulin sensitizers, ion exchange resins, keratolytic agents, lactation stimulating hormones, laxative/cathartic agents, leukotriene antagonists, LH-RH agonists, lipotropic agents, 5-lipoxygenase inhibitors, lupus erythematosus suppressants, major tranquilizers, mineralocorticoids, minor tranquilizers, miotic agents, monoamine oxidase inhibitors, mucolytic agents, muscle relaxants, mydriatic agents, narcotic analgesic agents, narcotic antagonists, nasal decongestants, neuroleptic agents, neuromuscular blocking agents, neuroprotective agents, nootropic agents, NSAIDs, opioid analgesic agents, oral contraceptives, ovarian hormones, oxytocic agents, parasympathomimetic agents, peptides (e.g. nerve growth factos, peptide hormones, growth factors, peptide neurotransmitters), proteins (e.g. nerve growth factos, protein hormones, growth factors, protein neurotransmitters), pepsin inhibitors, peripheral vasodilators, peristaltic stimulants, pigmentation agents, plasma volume expanders, potassium channel activators/openers, pressor agents, progestogens, prolactin inhibitors, prostaglandin/prostaglandin analogs, protease inhibitors, proton pump inhibitors, pulmonary surfactants, 5-alpha-reductase inhibitors, replenishers/supplements, respiratory stimulants, retroviral protease inhibitors, reverse transcriptase inhibitors, scabicides, sclerosing agents, sedatives/hypnotics, serenic agents, serotonin noradrenaline reuptake inhibitors, serotonin receptor agonists, seratonin receptor antagonists, serotonin uptake inhibitors, skeletal muscle relaxants, somatostatin analogs, spasmolytic agents, stool softeners, succinylcholine synergists, sympathomimetic agents, thrombolytic agents, thromboxane A2-receptor antagonists, thromboxane A2-sythetase inhibitors, thyroid hormones, thyroid inhibitors, thyrotropic hormones, tocolytic agents, topical protectants, topoisomerase I inhibitors, topoisomerase II inhibitors, tranquilizers, ultraviolet screens, uricosuric agents, vaccines, vasodilators, vasopressors,

vasoprotectants, vitamins/vitamin sources, vulnerary agents, Wilson's disease treatment agents, xanthine oxidase inhibitors.

More preferably the active pharmaceutical ingredient is chosen from one or more of the following categories/groups: psychopharmacological agents, such as (1) central nervous system depressants, e.g. general anesthetics (barbiturates, benzodiazepines, steroids, cyclohexanone derivatives, and miscellaneous agents), sedative-hypnotics (benzodiazepines, barbiturates, piperidinediones and triones, quinazoline derivatives, carbamates, aldehydes and derivatives, amides, acyclic ureides, benzazepines and related drugs, phenothiazines, etc.), central voluntary muscle tone modifying drugs (anticonvulsants, such as hydantoins, barbiturates, oxazolidinediones, succinimides, acylureides, glutarimides, benzodiazepines, secondary and tertiary alcohols, dibenzazepine derivatives, valproic acid and derivatives, GABA analogs, etc.), analgesics (morphine and derivatives, oripavine derivatives, morphinan derivatives, phenylpiperidines, 2,6-methane-3-benzazocaine derivatives, diphenylpropylamines and isosteres, salicylates, p-aminophenol derivatives, 5-pyrazolone derivatives, arylacetic acid derivatives, fenamates and isosteres, etc.) and antiemetics (anticholinergics, antihistamines, antidopaminergics, etc.), (2) central nervous system stimulants, e.g. analeptics (respiratory stimulants, convulsant stimulants, psychomotor stimulants), narcotic antagonists (morphine derivatives, oripavine derivatives, 2,6-methane-3-benzoxacine derivatives, morphinan derivatives) nootropics, (3) psychopharmacologicals, e.g. anxiolytic sedatives (benzodiazepines, propanediol carbamates) antipsychotics (phenothiazine derivatives, thioxanthine derivatives, other tricyclic compounds, butyrophenone derivatives and isosteres, diphenylbutylamine derivatives, substituted benzamides, arylpiperazine derivatives, indole derivatives, etc.), antidepressants (tricyclic compounds, MAO inhibitors, etc.), (4) respiratory tract drugs, e. g. , central antitussives (opium alkaloids and their derivatives); pharmacodynamic agents, such as (1) peripheral nervous system drugs, e.g.

local anesthetics (ester derivatives, amide derivatives), (2) drugs acting at synaptic or neuroeffector junctional sites, e.g. cholinergic agents, cholinergic blocking agents, neuromuscular blocking agents, adrenergic agents, antiadrenergic agents, (3) smooth muscle active drugs, e.g. spasmolytics (anticholinergics, musculotropic spasmolytics), vasodilators, smooth muscle stimulants, (4) histamines and antihistamines, e. g. , histamine and derivative thereof (betazole), antihistamines (Hl-antagonists, H2-antagonists), histamine metabolism drugs, (5) cardiovascular drugs, e.g. cardiotonics (plant extracts, butenolides, pentadienolids, alkaloids from erythrophleum species, ionophores, adrenoceptor stimulants, etc), antiarrhythmic drugs, antihypertensive agents, antilipidemic agents (clofibric acid derivatives, nicotinic acid derivatives, hormones and analogs, antibiotics, salicylic acid and derivatives), antivaricose drugs, hemostyptics, (6) blood and hemopoietic system drugs, e.g. antianemia drugs, blood coagulation drugs (hemostatics, anticoagulants, antithrombotics, thrombolytics, blood proteins and their fractions), (7) gastrointestinal tract drugs, e.g. digestants (stomachics, choleretics), antiulcer drugs, antidiarrheal agents, (8) locally acting drugs; chemotherapeutic agents, such as (1) anti-infective agents, e. g., ectoparasiticides (chlorinated hydrocarbons, pyrethins, sulfurated compounds), anthelmintics, antiprotozoal agents, antimalarial agents, antiamebic agents, antileiscmanial drugs, antitrichomonal agents, antitrypanosomal agents, sulfonamides, antimycobacterial drugs, antiviral chemotherapeutics, etc., and (2) cytostatics, i.e., antineoplastic agents or cytotoxic drugs, such as alkylating agents, e. g., Mechlorethamine hydrochloride (Nitrogen Mustard, Mustargen, HN2), Cyclophosphamide (Cytovan, Endoxana), Ifosfamide (IFEX), Chlorambucil (Leukeran), Melphalan (Phenylalanine Mustard, L-sarcolysin, Alkeran, L-PAM), Busulfan (Myleran), Thiotepa (Triethylenethiophosphoramide), Carmustine (BiCNU, BCNU), Lomustine (CeeNU, CCNU), Streptozocin (Zanosar) and the like; plant alkaloids, e. g. , Vincristine (Oncovin), Vinblastine (Velban, Velbe), Paclitaxel (Taxol), and the like; antimetabolites, e. g. , Methotrexate (MTX),

Mercaptopurine (Purinethol, 6-MP), Thioguanine (6-TG), Fluorouracil (5-FU), Cytarabine (Cytosar-U, Ara-C), Azacitidine (Mylosar, 5-AZA) and the like; antibiotics, e. g. , Dactinomycin (Actinomycin D, Cosmegen), Doxorubicin (Adriamycin), Daunorubicin (duanomycin, Cerubidine), Idarubicin (Idamycin), Bleomycin (Blenoxane), Picamycin (Mithramycin, Mithracin), Mitomycin (Mutamycin) and the like, and other anticellular proliferative agents, e. g. , Hydroxyurea (Hydrea), Procarbazine (Mutalane), Dacarbazine (DTIC-Dome), Cisplatin (Platinol) Carboplatin (Paraplatin), Asparaginase (Elspar) Etoposide (VePesid, VP-16-213), Amsarcrine (AMSA, m- AMSA), Mitotane (Lysodren), Mitoxantrone (Novatrone), and the like.

Active pharmaceutical ingredients which especially benefit from this invention are those which are typically poorly transported across the blood-brain barrier, such as hydrophilic peptides, and which are often highly potent in small quantities in the brain. These include, for example, peptide neurotrophic factors, neurotransmitters and neuromodulators, e.g. beta-endorphins and enkephalins or especially other hydrophilic, amphiphilic small molecules.

As used herein, the term "administration" and variants thereof (e.g., "administering" a compound) in reference to a compound of the invention means introducing the compound or a prodrug of the compound into the system of the animal in need of treatment. When a compound of the invention or prodrug thereof is provided in combination with one or more other active agents (e.g., a cytotoxic agent, etc.), "administration" and its variants are each understood to include concurrent and sequential introduction of the compound or prodrug thereof and other agents.

As used herein, the term "amino acid" means an organic compound containing both a basic amino group and an acidic carboxyl group. Included within this term are natural amino acids, modified and unusual amino acids, as well as amino acids which are known to occur biologically in free or combined form but

usually do not occur in proteins. In this invention, amino acids were abbreviated using the One-Letter-Symbols (e.g. S, P, I, R, etc.) and/or the Three-Letter-Symbols (e.g. Ser, Pro, Ile, Arg, etc.) as listed e.g. in Voet & Voet, Biochemistry, 3rd Edition, John Wiley & Sons Inc.

As used herein, the term "antibody" means immunoglobulin (Ig) molecules and immunologically active portions of immunoglobulin molecules, i.e., molecules that contain an antigen binding site which specifically binds (immunoreacts with) an antigen. The term “antibody” includes, but is not limited to: IgA, IgG, IgD, IgE, IgM.

As used herein, the term “antibody fragment” means any derivative of an antibody which is less than full-length. Preferably, the antibody fragment retains at least a significant portion of the full-length antibody's specific binding ability. The antibody fragment may be produced by any means, e.g. the antibody fragment may be enzymatically or chemically produced by fragmentation of an intact antibody or it may be recombinantly produced from a gene encoding the partial antibody sequence. Alternatively, the antibody fragment may be wholly or partially synthetically produced. The antibody fragment may optionally be a single chain antibody fragment. Alternatively, the fragment may comprise multiple chains which are linked together, for instance, by disulfide linkages. The fragment may also optionally be a multimolecular complex.

As used herein, the term “antisense molecule” and/or “antisence sequence” is to be understood as follows: a first sequence is an “antisense molecule” and/or “antisence sequence” with respect to a second sequence if a polynucleotide whose sequence is the first sequence specifically hybridizes with a polynucleotide whose sequence is the second sequence.

As used, herein the term "composition" refers to a product comprising specified ingredients in predetermined amounts or proportions, as well as any product that results, directly or indirectly, from combining specified ingredients in specified amounts. In the context of the present invention, the term "combination" can be used as a synonym for "composition."

The term "pharmaceutical formulation" encompasses a product comprising one or more active pharmaceutical ingredients, and an optional carrier comprising inert ingredients, as well as any product that results, directly or indirectly, from combination, complexation or aggregation of any two or more of the ingredients, or from dissociation of one or more of the ingredients, or from other types of reactions or interactions of one or more of the ingredients.

The terms "composition", "combination," and "pharmaceutical formulation" also encompass a product comprising one or more diagnostic agents, and an optional carrier comprising inert ingredients, as well as any product that results, directly or indirectly, from combination, complexation or aggregation of any two or more of the ingredients, or from dissociation of one or more of the ingredients, or from other types of reactions or interactions of one or more of the ingredients. In general, pharmaceutical formulations are prepared by uniformly and intimately bringing the active ingredient into association with a liquid carrier or a finely divided solid carrier or both, and then, if necessary, shaping the product into the desired formulation. The pharmaceutical formulation includes enough of the active object compound to produce the desired effect upon the progress or condition of diseases. Accordingly, the pharmaceutical formulations of the present invention encompass any composition made by mixing a compound of the present invention and a pharmaceutically acceptable carrier wherein the carrier has to be compatible with the other ingredients of the composition. As used herein, the terms "composition" and "formulation" are meant to be synonyms, i.e., have identical

meaning. The pharmaceutical formulations are made following conventional techniques of pharmaceutical technology involving mixing, granulating, and compressing, when necessary, for tablet forms, or mixing, filling and dissolving the ingredients, as appropriate, to give the desired oral, parenteral, rectal, transdermal, or topical products.

As used throughout the description and the claims of this specification the word "comprise" and variations of the word, such as "comprising" and "comprises" is not intended to exclude other additives, components, integers or steps.

As used herein, the term "condition" includes any disease or a collection of symptoms that requires treatment with a drug. Exemplary central nervous system diseases include, but are not limited to: Depression, Dementia, Prion Disease, Alzheimer's Disease, Parkinson's Disease, Multiple Sclerosis, Amylotrophic Lateral Sclerosis, Schizophrenia, Lyme Disease, Poliomyelitis, Stroke, Traumatic brain injury, psychosis, Chorea Huntington Disease, encephalopathy, epilepsy, cerebrovascular diseases and neurodegenerative disorders and a central nervous system cancer.

As used herein, the term "covalent bond" means a form of chemical bonding that is characterized by the sharing of pairs of electrons between atoms, or between atoms and other covalent bonds. In short, attraction-to-repulsion stability that forms between atoms when they share electrons is known as covalent bonding. Covalent bonding includes many kinds of interaction, including sigma-bonding, pi-bonding, metal to non-metal bonding, agostic interactions, and three-center two-electron bonds.

As used herein, the terms “diagnostic” and/or “diagnosis” mean identifying the presence or nature of a pathologic condition. Diagnostic methods differ in their specificity and selectivity.

As used herein, the term "diagnostic agent" means a substance or a composition employed to determine the nature or extent of a disease, or employed to confirm the presence of a disorder or a disease, or employed to provide other diagnostic information, e.g. staining of specific tissues, cells, compartments. Examples of diagnostic agents are antibodies or antibody fragments, ligands of receptors, nucleic acids, contrast agent / contrast medium, dyes, radioactive labeled substances, fluorescent radioactive labeled substances or in another way labeled substances.

As used herein, the term “excipient” refers to a component of a pharmaceutical product that does not exhibit any therapeutic activity in or on a human or animal, such as filler, diluent, carrier, and so on. The excipients that are useful in preparing a pharmaceutical formulation are preferably generally safe, non-toxic and neither biologically nor otherwise desirable, and are acceptable for veterinary use as well as human pharmaceutical use. “A pharmaceutically acceptable excipient” as used in the specification and claims includes one or more of such excipients.

As used herein the term “hydrogel” means an inorganic or organic compound capable of absorbing aqueous fluids and retaining them under moderate pressures.

As used herein, the terms “identical” or “identity” in the context of two or more amino acid sequences and/or peptides describes the relatedness between two amino acid sequences or peptides. The terms “identical” or “identity” refers to two or more sequences or subsequences that are the same or have a specified

percentage or a specified number of amino acid residues that are the same, when compared and aligned for maximum correspondence, as by visual inspection. The term "identical" or "identity" means, that an alignment of two sequences within a stretch of a defined number of amino acids (in the present invention: 15 amino acids) comprises the indicated number of identical amino acids, i.e. the term "identical" or "identity" is equal to the number of exact matches in an alignment of an amino acid sequence of the present invention and a different amino acid sequence. An exact match occurs when the amino acid sequence of the present invention and the different amino acid sequence have identical amino acid residues in the same positions of the overlap. The length of a sequence is the number of amino acid residues in the sequence (e.g the length of SEQ ID NO: 1 is 15). In a particular embodiment, the identity of an amino acid sequence of a polypeptide with, or to, amino acids 1-15 of SEQ ID NO: 1 is determined by (i). aligning the two amino acid sequences (e.g. by the Needle program from the EMBOSS package, version 2.8.0, which implements the global alignment algorithm described in Needleman, S.B. Wunsch, C.D., 1970, J. Mol. Biol. 48, 443-453 and which uses the substitution matrix BLOSUM62, gap opening penalty is 10, and gap extension penalty is 0.5) and (ii). counting the number of exact matches in the alignment. In a further preferred embodiment, the identity can also be expressed in percentage by the following steps: (i). dividing the number of exact matches by the length of the shortest of the two amino acid sequences and (ii). converting the result of the division of (i) into percentage.

As used herein, the term "including" means "including but not limited to". "Including" and "including but not limited to" are used interchangeably.

As used herein, the terms "linking structure" and/or "linker" mean a structure that links a peptide according to the invention and a pharmaceutically acceptable substance (the term "pharmaceutically acceptable substance" is to

be understood as defined herein) by covalent or non-covalent bonds. The term "linking structure" includes, but is not limited to: a linker peptide, a carbo-hydrogen bond, streptavidin-biotin, polyethylene glycol (PEG), a disulfide bridge, and/or metal-coordinated linker. As used herein, the terms "linking structure" and "linker" are meant to be synonyms, i.e., have identical meaning.

As used herein, the term "liposome" includes any structure composed of a lipid bilayer that enclose one or more volumes, wherein the volume can be an aqueous compartment. Liposome consist of one, two, three, four, five, six, seven, eight, nine, ten or more lipid bilayers. The term "lipid bilayer" includes, but is not limited to: phospholipid bilayer, bilayer consisting of nonionic surfactants. Liposomes consisting of a phospholipid bilayer can be composed of naturally-derived phospholipids with mixed lipid chains (like e.g. phosphatidylethanolamine), or of pure components like DOPE (dioleolylphosphatidyl-ethanolamine) but are not limited to these components. Liposomes include –but are not limited to- emulsions, foams, micelles, exosomes, vesicles, insoluble monolayers, liquid crystals, phospholipid dispersions, lamellar layers and the like. The term "liposome" also includes so called "stealth liposomes" which consist of water-soluble polymers (e.g. polyethyleneglycol, PEG) attached to the surface of conventional liposomes composed of a lipid mono- or bilayer that enclose a volume (e.g. so called PEGylated liposomes).

A wide variety of methods for preparing liposomes suitable for targeting to the blood-brain barrier in the present invention is available. See, for example, Szoka et al., Ann. Rev. Biophys. Bioeng. 9:467 (1980), Liposome Technology, ed. G. Gregoriadis , CRC Press, Inc., Boca Raton, Fla. (1984), U.S. patents nos. 4,235,871, 4,501,728, 4,837,028, 4,957,735 and 5,019,369, each of which is incorporated herein by reference. Following liposome preparation, the liposomes may be sized to achieve a desired size range and relatively narrow distribution of liposome sizes. For delivery to the brain, liposomes should

preferably be less than about 1.0 μm in size (as for the diameter of the liposomes), more preferably about 50 to 500 nm, more preferably 75 to 400nm, more preferably 100 to 200nm which allows the liposome suspension to be sterilized by filtration. Methods of coupling peptides according to the present invention to liposomes generally involve either covalent cross linking between a liposomal lipid and a peptide. In another approach, a peptide according to the present invention has been covalently derivatized with a hydrophobic anchor, such as fatty acids, is incorporated into a preformed lipid.

As used herein, the term "mammals" include animals of economic importance such as bovine, ovine, and porcine animals, especially those that produce meat, as well as domestic animals / pet animals, sports animals, zoo animals, and humans, the latter being preferred.

As used herein, the method of treatment in accordance with this invention comprises administering stepwise or in physical combination a peptide according to the present invention coupled to a suitable pharmaceutically acceptable substance (e.g. an active pharmaceutical ingredient) in an amount sufficient to treat central nervous system disorders like depression, dementia, Prion Disease, Alzheimer's Disease, Parkinson's Disease, Multiple Sclerosis, Amylotrophic Lateral Sclerosis, Schizophrenia, Lyme Disease, Poliomyelitis, stroke, traumatic brain injury, psychosis, Chorea Huntington Disease, encephalopathy, epilepsy, cerebrovascular diseases and neurodegenerative disorders and a central nervous system cancer. The active medicament preferably will be in an amount of from about 0.1 mg to about 2000 mg, in an amount of about 1 mg to about 1000 mg, in an amount of 5 mg to about 500 mg, in an amount of about 10 mg to about 250 mg, in an amount of about 50mg to about 200mg, or in an amount of about 75mg to about 150mg. Advantageously, equal doses, or dosage units, will be administered from on to 5 times daily. The total daily dosage will be from about 0.1 mg to about 2000

mg. When the administration described above is carried out suitable action is achieved in Alzheimer's Disease, Parkinson's Disease, Multiple Sclerosis, Amyotrophic Lateral Sclerosis, Dementia, Prion disease, Schizophrenia, Lyme Disease, Poliomyelitis, Stroke, Traumatic brain injury, and/or a central nervous system cancer subjects in need thereof.

As used herein, the term "nanoparticle" means a particle with a diameter between 0.1 and 1000 nanometers. Nanoparticles include any particle having a diameter between 1 and 1000 nanometers, e.g. liposomes, polymer micelles, polymer-DNA complexes (polycomplexes), nanospheres, nanofibres, nanotubes, nanocapsules, liquid crystal disperions, dendrimers, fullerenes, calcium phosphate nanoparticles, and/or polymersomes. All these nanoparticles are known in the art. The surface of such nanoparticles is often modified by PEG brush (PEGylation, i.e. polyethylene glycol (PEG) is attached to the surface of the nanoparticles).

As used herein, the term "nanocapsules" means vesicular systems in which the drug is confined to a cavity surrounded by a uniquer polymer membrane.

As used herein, the term "nanosphere" means a matrix system in which the drug is physically and uniformly dispersed.

As used herein, the term "noncovalent bond" means a type of chemical bond that does not involve the sharing of pairs of electrons, but rather involves more dispersed variations of electromagnetic interactions. "Noncovalent bonding" refers to a variety of interactions that are not covalent in nature between molecules or parts of molecules that provide force to hold the molecules or parts of molecules together, usually in a specific orientation or conformation. The term "non-covalent bonds" include, but are not limited to: ionic bonds,

hydrophobic interactions, hydrogen bonds, Van der Waals forces, i.e. ´London dispersion forces´, dipole-dipole bonds and combinations thereof.

As used herein, the term “nucleic acid” is a macromolecule composed of chains of monomeric nucleotides. The most common nucleic acids are deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). The term “nucleic acid” includes, bit is not limited to: siRNA, antisense RNA, a ribonucleic acid enzyme (ribozyme), triplex-forming oligonucleotides, nucleic acids suitable for gene therapy and expression constructs that comprises a nucleic acid that encodes a therapeutic protein of interest.

As used herein, the term "peptide" means a compound that consists of up to 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 amino acids (as defined herein) that are linked by means of a peptide bond (if more than 100, 500, 1000, 1500, 2000, 2500, 3000, 4000, 5000, 7500, 10000, 100000 amino acids are linked by means of a peptide bind the term “protein” is used). The term "peptide" also includes compounds containing both peptide and non-peptide components, such as e.g. pseudopeptide or peptide mimetic residues or other non-amino acid components, such as e.g. lipids, carbohydrates, phosphate groups. Such a compound containing both peptide and non-peptide components may also be referred to as a "peptide analog". Peptides can be linear or cyclic, preferably linear. Peptides can also be modified in any way at the C-terminus and/or the N-terminus.

The amino acid sequence of a peptide and/or a protein can be determined by methods as known in the art (e.g. described in F. Lottspeich (J.W. Engels, ´Bioanalytik´, Elsevier GmbH, 2nd Edition 2006 and Voet & Voet, Biochemistry, 3rd Edition, John Wiley & Sons Inc).

As used herein, the term "peptide bond" means a covalent amide linkage formed by loss of a molecule of water between the carboxyl group of one amino acid and the amino group of a second amino acid.

As used herein, the term “pharmaceutically acceptable” refers to physiologically well tolerated by a mammal or a human.

As used herein, the term "pharmaceutically acceptable salt" refers to those salts that are, within the scope of sound medical judgment, suitable for use in contact with the tissues of humans and lower animals without undue toxicity, irritation, allergic response, etc., and are commensurate with a reasonable benefit/risk ratio. Pharmaceutically acceptable salts are well-known in the art. They can be prepared in situ when finally isolating and purifying the compounds of the invention, or separately by reacting them with pharmaceutically acceptable non-toxic bases or acids, including inorganic or organic bases and inorganic or organic acids (Berge, 1977). Common anions used in pharmaceutically acceptable salts include: chloride, bromide, sulfate, nitrate, phosphate, bicarbonate, mesylate, esylate, isothianate, tosylate, napsylate, besylate, acetate, propionate, maleate, benzoate, salicylate, fumarate, citrate, lactate, maleate, tartrate, pamoate, succinate, glycolate, hexanoate, octanoate, decanoate, stearate, oleate, aspartate and glutamate. Common cations used as counterions in pharmaceutically acceptable salts include: sodium, potassium, calcium, magnesium, lithium, zinc, aluminum, arginine, lysine, histidine, triethylamine, ethanolamine, triethanolamine, ethilenediamine, meglumine, procaine and benzathine.

As used herein, the term “pharmaceutically acceptable substance” includes, but is not limited to an item selected from the group consisting of active pharmaceutical ingredients, proteins, nucleic acids, siRNA molecules, antisense molecules, expression constructs that comprises a nucleic acid that

encodes a therapeutic protein of interest, liposomes, nanoparticles, diagnostic agents, markers of a disease of the central nervous system (CNS), antibodies, erythrocytes, erythrocytes ghosts, spheroplasts, monoclonal antibodies, labeled monoclonal antibodies which bind a marker of a CNS disorder, and/or a fragment of an antibody or a monoclonal antibody.

As used herein the term "polymersomes" means nanoparticles which are formed by the spontaneous self-assembly of amphiphilic block copolymers. They can be loaded with a suitable active pharmaceutical ingredient, preferably a hydrophobic active pharmaceutical ingredient integrated in the polymer membrane (mono-, bi or multiple layers), or a hydrophilic active pharmaceutical ingredient in the interior of the polymersome (lumen).

Block copolymers which form polymersomes exist of one or more hydrophilic polymeric parts and one or more hydrophobic polymeric parts. Polymers which can be used are generally known to experts in the field and can vary in molecular weight from 1kDa to 100 kDa. One would preferably use a water insoluble biocompatible, biodegradable or bio-inert polymer (e.g. polystyrene, polybutadiene, polyolefins) for the hydrophobic polymeric parts (the hydrophobic block). The hydrophilic polymeric parts (the hydrophilic block) consist of a water soluble biodegradable, biocompatible or bio-inert polymer (e.g. poly(ethyleneglycol), peptides containing polar or charged amino acid residues, polyacrylates). Poly(ethylene glycol) is generally preferred as hydrophilic polymer for its biocompatibility and its bio-inert character. The synthesis of block copolymers is well known in the field and can generally be achieved by living polymerization or by a modular approach. In living polymerizations monomers of the polymeric blocks are fed after the previous monomer has been consumed or removed. In a modular approach the polymeric parts of the block copolymer are synthesized separately and covalently connected afterwards.

Bioconjugation of peptides can either be achieved by (i). covalently attaching peptides to the block copolymer or by (ii). non covalent interactions:

(i). For bio-conjugation of block copolymers to the peptides one would preferably use block copolymers containing a functional end group like an alcohol, thiol, carboxylic acid, amine, azide, alkyne or any other functional group. Many bioconjugation techniques are generally known and can be applied. By these techniques one can either introduce the peptides before polymersome formation to the block copolymers or after polymersome formation via bio-conjugation onto the polymersome periphery.

(ii). Non-covalent bioconjugation can be achieved by embedding peptides in the polymersome periphery or by anchoring peptides in the membrane. Anchoring involves a peptide polymer hybrid of which the polymeric part can be embedded in the polymersome membrane.

Polymersome formulation can be divided in four concepts: (i). polymersome formation; (ii). overall size; (iii). polymersome periphery functionalities; (iv). drug loading:

(i). Firstly, polymersomes can be formed either by rehydration methods generally known for liposomes. Secondly, polymersomes can be formed by dissolving a block copolymer or a blend of different block copolymers in a good solvent followed by controlled dilution with water or any buffering solution. Finally polymersomes can be obtained by injecting a block copolymer solution into water or any aqueous buffering solution.

(ii). The overall size and the size distribution of polymersomes can be reduced by extrusion techniques well known to liposomes. One would preferably obtain polymersomes of 50 to1000 nanometers.

(iii). To introduce peptides to the periphery of polymersomes one can either fully or partially cover the periphery with peptides or blends of peptides. To achieve this, polymersomes are formed as described starting off with block copolymers, block copolymer–bioconjugates or blends of any composition.

Anchor peptide conjugates or peptides/enzymes can be embedded in polymersomes by forming vesicles as described in the presence of peptides or anchor peptide conjugates.

(iv). Hydrophobic drugs can be loaded in the hydrophobic part of the polymersome membrane and hydrophilic drugs can be loaded in the aqueous interior of polymersomes (lumen). To load polymersomes with drugs polymersomes are formed as described in the presence of the desired drug.

After the full syntheses and assembly of polymersomes the periphery displays one or more peptides sequences according to the invention on the surface. These peptides sequences according to the invention associate with the blood brain barrier and induce actual transport into the cells by endocytosis and/or over the blood brain barrier by transcytosis. Further on, the polymersomes can also be transported by diffusion. The polymersome exists of a membrane which is non-covalently loaded with any hydrophobic therapeutic agent. The cavity which is an aqueous environment is loaded with any hydrophilic therapeutic agent.

Although circulation times for polymersomes are much longer than for liposomes they will be broken down enzymatically in the cellular environment by natural degradation or biodegradation depending on the choice of polymeric parts. The breakdown will release the content resulting in a local higher concentration of therapeutic agents near the blood brain barrier and/or within the brain.

As used herein, the term "prophylaxis" means a complete prevention of a disease, the prevention of occurrence of symptoms in a diseased subject, the prevention of recurrence of symptoms in a diseased subject, or a decrease in severity or frequency of symptoms of diseases or condition in the subject.

As used herein, the term "protein" is intended to encompass any amino acid sequence and include modified sequences such as glycoproteins. For amino

acid sequences up to 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 amino acids which are linked by means of a peptide bond the term “peptide” is used. For amino acid sequences with more than 100, 500, 1000, 1500, 2000, 2500, 3000, 4000, 5000, 7500, 10000, 100000 amino acids which are linked by means of a peptide bond the term “protein” is used. The term “protein” includes, but is not limited to naturally occurring proteins and peptides as well as those which are recombinantly or synthetically synthesized.

Concentrations, amounts, solubilities, particle size, wavelength, pH values, weight mass, molecular weight, percent and other numerical date my be expressed or presented herein in a range format. It is to be understood that such a range format is used merely for convenience and brevity and thus should be interpreted flexibly to include not only the numerical values explicitly recited as the limits of the range, but also to include all the individual numerical values or sub-ranges encompassed within that range as if each numerical value and sub-range is explicitly recited.

As used herein, the term “spheroplast” means a cell from which the cell wall has been removed

As used herein, the term "subject" means an animal, preferably a mammal, most preferably a human, who has been the object of treatment, diagnosis, observation or experiment.

As used herein, the term "therapeutically effective amount" means an amount of a therapeutic agent to treat a condition treatable by administrating a composition of the invention. That amount is the amount sufficient to exhibit a detectable therapeutic or ameliorative response in a tissue system, animal or human. The effect may include, for example, treating the conditions listed herein. The precise effective amount for a subject will depend upon the

subject's size and health, the nature and extent of the condition being treated, recommendations of the treating physician (researcher, veterinarian, medical doctor or other clinician), and the therapeutics, or combination of therapeutics, selected for administration. Thus, it is not useful to specify an exact effective amount in advance.

As used herein, the term "treatment" means any treatment of a mammalian, for example human condition or disease, and includes: (1) inhibiting the disease or condition, i.e. partial or complete arresting its development, (2) relieving the disease or condition, i.e., causing the condition to regress, or (3) stopping the symptoms of the disease or curing the disease.

Brief Descriptions of the Figures

Figure 1

Binding and uptake of phage in hCMEC/D3 cells, expressed as a percentage of the input. Mean±S.D.,n=3. A. Binding of phage to hCMEC/D3 cells. One way ANOVA after log transformation to correct for non-Gaussian distributions with Newman-Keuls correction for multiple comparisons *$p<0.05$ GLA vs RVR and GYR vs YLR. **$p<0.01$ GYR vs RVR. B. Uptake of phage in hCMEC/D3 cells. One way ANOVA after log transformation with Newman-Keuls correction for multiple comparisons **$p<0.01$ vs all controls. Anterior vs posterior Mann-Whitney test $p<0.01$.

Figure 2

Dose-response curves of phage GLA, GYR and control phage YLR. Mean±S.D., n=2. One way ANOVA after log transformation to correct for non-Gaussian distributions with Dunnet's correction for multiple comparisons against

control. $p<0.01$ vs YLR. AUC is 14x and 26x higher for GLA and GYR, respectively (vs YLR).

Figure 3

Binding (A) and uptake (B) of phage GLA, GYR, GTW and control phage YLR in hCMEC and HUVEC cells. Binding and uptake are expressed as a percentage of the input. Mean±S.D., n=3. Students t-test after log transformation to correct for non-Gaussian distributions. *$p<0.05$, **$p<0.01$ hCMEC vs HUVEC.

Figure 4

Recovery of phage GLA, GYR, and control phage RVR from whole murine brains after phage perfusion and washing. Recovery is expressed as a percentage of the input. Mean±S.D., n=3. One way ANOVA after log transformation to correct for non-Gaussian distributions with Dunnet's correction for multiple comparisons against control. $p<0.01$ vs RVR.

Figure 5

Recovery of phage GLA, GYR, and control phage RVR from pieces of liver after phage incubation and washing. Recovery is expressed as a percentage of the input. Mean±S.D., n=3. One way ANOVA after log transformation to correct for non-Gaussian distributions. $p=0.6$.

Figure 6

Example of an amino acid alignment. Sequence A shows by example a sequence and its identity to SEQ ID NO: 1. "Z" can be any amino acid but not the corresponding amino acid in SEQ ID NO: 1 (i.e. in position 3 "Z" can be any amino acid but not "A"). In this example, 10 amino acids are identical to SEQ ID NO: 1.

Figure 7

Recovery of phage GLA, GYR, and control phage RVR from murine lungs after phage perfusion and washing. Recovery in lungs (300 mg) is expressed as a percentage of the input.

Figure 8

Bar diagram of flow cytometry analysis of liposome binding in accordance with Example 6. Shown is the percentage of cells bound to liposomes. Three different concentrations of liposomes are used for each sample (including RGD and transferring as positive controls).

Figure 9

Extract from Figure 8 (without positive controls so as to more clearly display the differentiation.

Figure 10

Bar diagram showing the perfusion of peptide-coupled liposomes through mouse brain *in situ*. Shown is the percentage of the injected dose that is found in the whole brain.

Figure 11

Bar diagram showing the binding of peptide-coupled liposomes to mouse brain *in vivo*, in different regions of the brain.

EXAMPLE 1

A 15-mer peptide library to identify brain-targeting peptide ligands was used. Ligand screening was performed *in vivo* using a mouse perfusion model, where the library is perfused through the *in situ* brain. As the brain was intact, the endothelial cells kept their polarity and their contact with the surrounding cells. The perfusion of fluid through the brain kept the intraluminal flow going, maintaining the expression of relevant receptors.

After the initial *in situ* screening had been performed, identified peptide ligands were tested for cross-reactivity with human cell-surface receptors expressed by endothelial cells in a human blood-brain barrier model *in vitro*.

Material and Methods

Phage library and bacteria

Filamentous phage fd-tet, which confers tetracycline resistance on the host, was used for this study. Random peptide library f3-15mer, containing 2.5 x 10^8 primary clones (GenBank Accession AF246445) with foreign 15-mer peptide displayed on pIII was used. Kanamycin resistant *Escherichia coli* K91BluKan (K91BK) host bacteria were also used. The *Escherichia coli* K91BluKan (K91BK) sex is Hfr Cavalli, resulting in deployment of the F pilus: the attachment site for filamentous phage infection.

Animals

In situ phage display screening was performed in male 6-8 weeks old C57Bl/6 mice (Charles River, The Netherlands). Perfusion of single clones was done in male 28-32 g Balb/c mice (Harlan, Horst, The Netherlands). Food and water were supplied *ad libitum*. Animal studies were performed according to

national regulations and were approved by the local animal experiments ethical committee.

In situ perfusion of phage

40 units of heparin (Sigma) were injected i.v. into the tail vein to prevent coagulation of blood inside the microvessels. Five minutes after injection mice were sacrificed by CO_2 asphyxiation. Next, thorax was opened and bulldog clamps with 8 x 1.2 mm serrated jaws (WPI, Berlin, Germany) were placed on aorta (left from the carotid arteries) and pulmonary veins and arteries. Inferior caval vein was cut to allow outflow of perfused fluids. A 26G (G = Gauche, a value to specify the needle thickness) needle, connected to a peristaltic pump, was inserted into the left ventricle of the heart. Mice were initially perfused with 1.5 ml Hanks' Balanced Salt Solution (HBSS: 140 mg/L $CaCl_2$ anhydr., 100 mg/L $MgCl_2 \cdot 6\ H_2O$, 100 mg/L $MgSO_4 \cdot 7\ H_2O$, 400 mg/L KCl, 60 mg/L KH_2PO_4, 350 mg/L $NaHCO_3$, 8000 mg/L NaCl, 48 mg/L Na_2HPO_4 anhydr., 1000 mg/L D-glucose, pH 7.0-7.4, Invitrogen, Carlsbad, CA) in all experiments. Directly afterwards, mice were perfused with phage. Upon the three selection rounds, 1 ml of phage library in HBSS (8.9×10^{11} TU, 3.1×10^{11} TU, and 4.8×10^{10} TU for the first, second and third selection rounds, respectively – "TU" means transducing units, see below)) were perfused (input). To wash away non-binders, 3.5 ml HBSS was perfused directly afterwards. For the third selection round, HBSS + 1% FBS was used instead of HBSS. For perfusions of selected phage (single clones), 750 µl of phage (~10^{10} TU) in HBSS + 1% FBS (fetal bovine serum) was perfused, directly followed by 2 ml of HBSS + 1% serum to wash. Peristaltic pump speed was 200 µl/min. Directly after perfusion, brains were taken out. For the phage display screening, phage were isolated from the brain (see below). For the immunohistochemistry experiments, brains were directly snap-frozen in liquid nitrogen, and stored at -80 °C.

Phage isolation from brain

After phage display selection rounds, brain cerebrum was ground in 600 µl 100 mM triethanolamine (Fluka, Munich, Germany) and centrifuged for 10 min at 3300x g. Supernatant was transferred to a new tube, neutralized with 300 µl 1 M Tris (tris(hydroxymethyl)aminomethane) pH 7.4, and centrifuged for 5 min at 3300x g. Late log phase *Escherichia coli* K91BluKan (K91BK) were obtained by inoculation of 10 ml of terrific broth (preparation: 12 g bacto-tryptone, 24 g yeast extract, 5.04g glycerol are dissolved in 900mL water; subsequently the preparation is autoclaved; subsequently –after cooling down- 100 mL of separately autoclaved potassium phosphate buffer is added under aseptic conditions; subsequently the preparation is mixed to have a homogenous solution; the potassium phosphate buffer is prepared as follows: 2.31 g KH_2PO_4 anhydr. and 12.54 g K_2HPO_4 anhydr. and dissolved in 90 mL water, subsequently the volume is added to 100mL) with 100 µl of an overnight culture, and incubation for 4 hours in a shaker incubator (250 rpm, 37 °C). When late log phase was reached (when the OD_{600} of a 10 x dilution reached 0.2 on a spectrophotometer), shaking was slowed down to 75 rpm for 5 minutes to allow sheared F pili to regenerate. Brain supernatant was incubated with late log phase *Escherichia coli* K91BluKan (K91BK) for 10 min to allow phage to infect the bacteria. The infected bacteria were added to 40 ml LB Broth (Sigma; composition: Tryptone (pancreatic digest of casein) 10 g/L, Yeast extract 5 g/L, NaCl 5 g/L), containing 0.2 µg/ml tetracycline and amplified for 30 min in a shaker incubator (250 rpm, 37 °C). Two hundred µl of the infected *Escherichia coli* K91BluKan (K91BK) was spread on LB agar plates (SIGMA; composition: Tryptone (pancreatic digest of casein) 10 g/L, Yeast extract 5 g/L, NaCl 5 g/L, Agar 15 g/L) for titration. Tetracycline concentration was then brought to 20 µg/ml, and phage were amplified overnight in the shaker incubator. Cultures were cleared of bacteria by two 10 min centrifugations at 3000x g and 8000x g, i.e. the cultures were centrifuged

for 10 min at 3000x g, subsequently the supernatant was taken and centrifuged for 10 min at 8000 x g, the resulting supernatant was used for further processing. To the cleared culture 0.15 volume PEG/NaCl (16.7% v/v /3.3 M stock) was added (PEG 8000, Promega, Breda, The Netherlands). Phage were precipitated overnight at 4 °C. Precipitated phage were collected by centrifugation for 15 min at 17500x g at 4 °C. Pellets were dissolved in 1 ml TBS (Tris buffered saline: 50 mM Tris-HCl pH 7.5, 150 mM NaCl) and precipitated again with 0.15 volume PEG/NaCl for at least 1 hour at 4 °C. Phage were collected by centrifugation for 5 min at 16000x g. Pellets were dissolved in 400 µl TBS and stored at 4 °C. Two hundred µl of purified phage + 800 µl HBSS was used for the next selection round using the method as described above. Three successive selection rounds were done in total using this perfusion method. After the third selection round, brain cerebrum was separated into anterior and posterior part. Phage were separately isolated from both parts. Titration of input phage was performed as described below. After perfusion of single phage clones using the same method as described above, whole brains were ground in 1000 µl triethanolamine and centrifuged for 10 min at 3300x g. Two hundred µl supernatant was transferred to a new tube, and neutralized with 100 µl 1 M Tris pH 7.4. Titration of both input and output phage was performed as described below.

Titration of phage

Transducing units (TU) were determined by titration. Phage were serially diluted in 0.1% (w/v) gelatin (SIGMA; type B gelatin from bovine skin) in TBS. Ten µl of phage dilution was incubated with 10 µl of late log phase *Escherichia coli* K91BluKan (K91BK) for 10 min at room temperature. 1 ml LB, containing 0.2 µg/ml tetracycline was added. This was incubated for 40 min in a shaker incubator (250 rpm, 37 °C). Two hundred µl of the infected *Escherichia coli* K91BluKan (K91BK) was spread on LB agar plates containing 40 µg/ml tetracycline and 100 µg/ml kanamycin. Plates were

incubated overnight at 37 °C. Next day, the number of colonies was counted and the number of transducing units was calculated (number of transducing units = number of bacteria colonies x dilution factor). Phage output titers are presented as a percentage of the input titers.

Sequence alignment

Peptide sequences were aligned to check for peptides consensus sequences along the 15 amino acids. Software used was Vector NTI Advance 10 AlignX (Invitrogen).

Cells

Human brain endothelial cells (hCMEC/D3) were obtained from Institut National de la Santé et de la Recherche Médicale (INSERM, Paris, France). Human brain endothelial cells (hCMEC/D3) are described and characterized in Weksler et al. (FASEB Journal 19, 13, pp. 1872-1874). Cells were cultured at 37°C, 5% CO_2, in EGM-2 basal medium supplemented with EGM-2 MV BulletKit (both supplied by Lonza, Basel, Switzerland), containing growth factors and 2.5% FBS. Additionally, 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 1 ng/ml bFGF (human, recombinant Basic Fibroblast Growth Factor; supplied by Invitrogen), and antibiotics (100 units/mL Penicillin and 0.1 mg/mL Streptomycin) were added. hCMEC/D3 cells were grown on surfaces coated with 100 µg/ml Rat tail collagen type 1 (BD Biosciences). Prior to use, cell culture medium was replaced by cell differentiation medium, consisting of EBM-2 basal medium supplemented with 2.5% FBS, 1.4 µM hydrocortisone, 1 ng/ml bFGF, 10 mM HEPES, and antibiotics (100 units/mL Penicillin and 0.1 mg/mL Streptomycin). Cells were grown to a monolayer in differentiation medium for one week. Medium was replaced every 2-3 days.

Human umbilical vein endothelial cells (HUVEC) (Human Umbilical Vein Endothelial Cells, pooled; supplied by Lonza, Basel, Switzerland) were

cultured at 37°C, 5% CO_2, in EBM-2 basal medium supplemented with antibiotics (100 units/mL Penicillin and 0.1 mg/mL Streptomycin) and EGM-2 BulletKit (Lonza, Basel, Switzerland), containing growth factors and 2% FBS.

In vitro binding and uptake of single phage clones

hCMEC cells were seeded at 3.4x 10^5 cells/well in 12-well plates. HUVEC cells were seeded at 4x 10^4 cells/well in 12-well plates. Single phage clones were diluted to ~10^{10} TU/ml in 25mM HEPES in HBSS, and 1 ml phage solution was incubated with either hCMEC/D3 or HUVEC cells (Weksler et al. FASEB Journal 19, 13, pp. 1872-1874) for 1 hour at 37°C. The buffer containing unbound phage was removed and cells were washed 3 times with PBS. Cells were treated 3 times with 1 ml of 0.2 M glycine (pH 2.2) to elute surface-bound phages. Pooled eluated phases were collected and neutralized with 0.45 ml 1 M tris (pH 9.1). Cells were washed again with 1 ml of PBS and were subsequently lysed with 0.25 ml 100 mM triethanolamine for 10 min. Cell lysates were collected and neutralized with 0.0625 ml 1 M tris (pH 7.4). The neutralized eluates (binding fraction) and cell lysates (uptake fraction) were titrated as described above. Input phage were titrated as well at the same time.

Immunohistochemistry hCMEC/D3 cells

hCMEC/D3 cells were grown to a monolayer on Rat tail collagen coated Lab-Tek chamber slides (Nunc, Rochester, NY). Single phage clones were diluted to $5 \cdot 10^{12}$ V/ml in HBSS, and 100 µL phage solution or 100 µL HBSS only was incubated with the cells for 1 hour at 37°C. The buffer containing unbound phage was removed and cells were washed 3 times with PBS. Cells were fixed in 4% formaldehyde for 30 min. Cells were washed 2 times with PBS and incubated with 0.8 µg/mL biotinylated anti-phage antibody for 1 hour. Thereafter, cells were incubated with 5 µg/mL alexa568-conjugated streptavidin for 35 min, and washed 3 times. Cells were incubated with 300 nM DAPI (4′,6-diamidino-2-phenylindole dihydrochloride) (Invitrogen) for 2

minutes and washed 3 times with demineralized water. Cells were mounted in FluorSave and viewed under a Nikon Eclipse TE2000-U epi-fluorescence microscope at a 20x magnification.

Phage incubation on liver

Mouse liver was cut into 9 pieces of equal weight. Pieces were incubated with ~10^{10} TU (GLA-,GYR-, and RVR-phases, triplicate) in 500 µl HBSS + 1% serum + 2 U/ml heparin (HBSSF/heparin) for 10 min at room temperature while shaking at 125 rpm. Liver pieces were washed twice for 10 min in HBSS/heparin. After the incubations, 0.3 ml triethanol amine (TEA) was added to each pieces and each pieces were processed and titrated in exactly the same way as was done for the brains.

In situ lung perfusion of phage

In situ lung perfusion was performed in the same way as was done for brain perfusions of single clones, with the only modification being the placement of the bulldog clamps. The ascending aorta was clamped and phage were perfused through the left ventricle of the heart. Inferior caval vein was cut to allow outflow of perfused fluids. Phage isolation and titration were also performed in the same was as was done for the brain perfusions.

Statistical analysis

Data were analyzed using Graphpad Prism 4 for Windows software (Graphpad Software, San Diego, CA).

Method

In situ phage display screening was used as a tool to search for blood-brain barrier-binding peptides. Screening was performed by perfusion of a 15 amino acid random peptide phage display library through the murine brain *in situ*. Phage perfusion was chosen over intravenous injection, because clearance of

phage occurs rapidly via the major organs of the reticuloendothelial system (mainly liver and spleen), resulting in a half-life of fd-tet of only 12 minutes. Therefore, phage were directly perfused through the brain, preventing loss to other organs, and ensuring a high fraction of individual peptide-displaying phage to reach the target tissue.

Various *in situ* brain perfusion methods have been developed. Most models have been established in rats, but murine models have been used as well. Most perfusion models have been used to study brain uptake of substrates and were performed under anesthesia. Since filamentous phage are approximately 1 µm in size, they are not likely to be taken up into the brain, and the perfusion allows screening for binding rather than for internalizing peptides. Therefore, body temperature maintenance was not required, and perfusion was performed at room temperature, postmortem. The large majority of brain proteins are stable during a postmortem interval of up to 4 hours at 25 °C. As perfusions were completed within 30 min after sacrifice, the postmortem state was not expected to cause significant changes in brain micro vessel morphology.

Phage were infused through the heart. The major advantage compared to direct infusion into the brain is that the phage have to travel a longer distance before they reach the brain. On their way, they already encounter endothelial cells: the endothelium of the aorta and carotid artery. Phage with affinity to ubiquitous endothelial receptors are given the opportunity to bind to these cells and will not reach the brain. This negative selection filters out unspecific binders, and allows for the identification of peptides that bind more specifically to brain endothelium, e.g. the blood-brain barrier-binding peptides.

Subsequently, the specificity of the blood-brain barrier-binding peptides was confirmed subjecting these phages to primary liver tissue.

Phage display screening was performed by perfusion of a mouse brain with 8.9 x 10^{11} Transducing Units (TU) in Hanks' Balanced Salt Solution (HBSS), containing 1 g/L glucose, to keep the brain in a viable state. Phage were perfused for 5 minutes. Phage incubation times of 5-15 minutes are common and have been successful *in vivo*. Non-binders were washed away by perfusion of HBSS. The brain was removed from the body and phage were isolated from the cerebrum, and submitted to the next selection round. During the third selection round, 1% FBS was added to the HBSS, to limit unspecific interactions. Because the infused fluid was not evenly perfused throughout the brain, cerebrum was divided into its anterior (weakly perfused) and posterior (strongly perfused) side after the third selection round, to investigate whether different ligands could be identified in the different brain parts.

After three successive selection rounds, 17 clones isolated from the anterior part, and 17 clones isolated from the posterior part of the brain were sequenced (table 1). Two of the sequences were found twice, once in the anterior part and once in the posterior part. However, most of the sequences were found only once. There was no clear discrimination in amino acid sequence between clones isolated from the different brain parts. Alignment analysis revealed no consensus sequences, which is not surprising, considering the complexity of the brain endothelium surface, and the many possible targets it may contain.

Brief Description of Table 1

Sequenced phage clones. Clones after 3 selection rounds, isolated from anterior (A) and posterior (B) cerebrum. Control clones (C), randomly taken from naive library. Isoelectric point (pI) and charge at pH 7 were calculated by Vector NTI Advance 10 AlignX software. Clones tested in vitro (y = yes) are shown in figure 1.

Table 1

A. Clones isolated from anterior cerebrum

Clone	*Name*	*Sequence*	*Frequency*	*pI*	*charge at pH 7*	*Tested in vitro*
A1	RFL	RFLEFPRFFPAIILP	1	9.60	+0.76	no
A2	PGR	PGRLLPGVIQRHFFI	1	12.50	+1.85	no
A3	GAF	GAFSSPRSLTVPLRR	1	12.80	+2.76	no
A4	PFA	PFARAPVEHHDVVGL	2	5.99	-1.06	yes
A5	CGG	CGGLFAGCAALIDVF	1	3.80	-1.26	yes
A6	EFP	EFPTFSWSYINDSLL	1	3.67	-2.24	no
A7	ADW	ADWPHARGKFALGNA	1	8.75	+0.85	yes
A8	GFT	GFTDVHLHLPGNSHR	1	7.02	+0.02	yes
A9	GLD	GLDLLGDVRIPVVRR	2	9.51	+0.76	yes
A10	PVA	PVAGMPLFPTAWFAH	1	6.74	-0.15	no
A11	SAY	SAYAATVRGPLSSAS	1	8.75	+0.76	yes
A12	RYA	RYASQLSDQILFTLP	1	5.84	-0.24	yes
A13	RDG	RDGAFSPPVRWWSFS	1	9.60	+0.76	no
A14	TGA	TGAQAGLHEWRPWGV	1	6.75	-0.15	no
A15	EDW	EDWFSASIRRVPTFA	1	6.07	-0.24	yes
A16	GTW	GTWSSTCPLCSATAV	1	5.51	-0.26	yes
A17	PWL	PWLPSNLGSRPGLMR	1	12.50	+1.76	no

B. Clones isolated from posterior cerebrum

Clone	*Name*	*Sequence*	*Frequency*	*pI*	*charge at pH 7*	*Tested in vitro*
P1	GVV	GVVNYARAFNVGAAV	1	8.75	+0.76	yes
P2	GLD	GLDLLGDVRIPVVRR	2	9.51	+0.76	yes
P3	HAA	HAAFEPRGDVRHTLL	1	6.92	-0.06	yes
P4	GDG	GDGRFHFLRGFFDSD	1	5.30	-1.15	yes
P5	VRS	VRSIALFPPEWSATS	1	6.00	-0.24	yes
P6	GLA	GLAHSFSDFARDFVA	1	5.21	-1.15	yes
P7	VTG	VTGTQIRLPAYLRFD	1	8.75	+0.76	no
P8	GYR	GYRPVHNIRGHWAPG	1	10.84	+1.93	yes
P9	ITR	ITRGGYVIYHDALLA	1	6.74	-0.15	no
P10	GAY	GAYFLSNHAVVRGVG	1	8.75	+0.85	no
P11	PFA	PFARAPVEHHDVVGL	2	5.99	-1.06	yes
P12	VGR	VGRPGGLVGGFASSL	1	9.75	+0.76	no
P13	LGR	LGRAGQSYPSFARGL	1	10.83	+1.76	yes
P14	VVS	VVSSRSVLSSQYRGH	1	10.84	+1.85	yes
P15	ALP	ALPCNGAGCSRVTAR	1	9.02	+1.74	yes
P16	VPM	VPMGLGFLGRGLAPL	1	9.75	+0.76	no
P17	RSS	RSSHHPSFAVSLEPL	1	6.92	-0.06	yes

C. Random control clones

Clone	*Name*	*Sequence*	*Frequency*	*pI*	*charge at pH 7*	*Tested in vitro*
C1	SVE	SVEVALVPGRQSRHF	-	9.61	+0.85	yes
C2	YLR	YLRWAALCSIGSSCW	-	8.06	+0.74	yes
C3	RVR	RVREPYPGMLERYRA	-	9.97	+1.76	yes

After selection, the enriched library was tested for its ability to bind to the brain by immunohistochemistry. Frozen untreated murine brains were sectioned and both the unselected (naive) and enriched library were incubated on the brain sections. Bound phage were stained with a labelled anti-phage antibody. Fluorescence microscopy revealed that the enriched library showed enhanced binding to the brain sections, compared to the naive library. The staining intensity was comparable in the anterior and posterior side.

The peptides were selected for affinity to the murine brain endothelium. However, for clinical applications, affinity for human brain endothelium is desired. Cross-reactivity between mouse and human has been shown for phage display selected peptides. Therefore, a number of selected and sequenced phage clones were tested for their ability to bind to and to be taken up by human brain endothelial cells. Additionally, three clones were randomly selected from the naive library as a negative control. The hCMEC/D3 cell line was used to represent the human blood-brain barrier endothelial cells. hCMEC/D3 is a stable, fully characterized, well-differentiated human brain endothelial cell line. Phage were added to the cells and incubated for 1 hour at 37°C. Surface-bound and internalized phage were recovered. The percentages of recovered phage are shown in figure 1. Figure 1A shows that only seven out of nineteen selected clones showed higher binding to the cells compared to the highest random control clone (SVE). This may be due to non-specific electrostatic interactions of the peptides with the cell membranes, limiting discrimination between specific and non-specific binders. Uptake, however, can only occur through specific interactions (e.g. receptor-ligand interactions). As shown in figure 1B, seventeen out of nineteen selected clones showed a higher uptake compared to the highest random control clone (YLR), indicating that peptides were identified that can be specifically internalized by brain endothelial cells. When the anterior and posterior sides of the cerebrum are compared, phage isolated from the posterior side showed significantly higher

uptake than phage isolated from the anterior side. This can be correlated to the perfusion pattern seen upon Evans blue perfusion, which showed better perfusion through the posterior side of the brain. Likely, more individual phage reached the posterior side, increasing the selection strength, and resulting in higher affinity phage. Although the *in situ* perfusion was not set up to select for internalizing phage, surprisingly, *in vitro* testing showed that most of the phage were taken up into the hCMEC cells. Of all clones tested *in vitro*, phage GLA and GYR showed the best brain endothelium binding, and were among the phage that were best taken up. Therefore GLA and GYR were chosen for further testing.

Binding of GLA, GYR, and negative control YLR to the hCMEC cells was determined for different concentrations of input phage. Figure 2 shows that the phage bound to the cells in a dose dependent manner. Significantly more GLA and GYR were bound to the cells compared to control phage YLR.

Phage GLA and GYR showed affinity for the hCMEC/D3 brain endothelium. We tested *in vitro* whether this affinity was brain-specific, or whether these phage have affinity for other endothelial cells as well. Therefore, phage binding and uptake was tested on non-brain endothelium: human umbilical vein endothelial cells (HUVEC). Best hCMEC binding phage GLA and GYR were tested, as well as poor hCMEC binding phage GTW, and random control phage YLR. Phage GLA and GYR show significantly less affinity to HUVECs compared to hCMECs (figure 3), indicating that these phage do not have affinity to non-blood-brain barrier endothelial cells, and are to be blood-brain barrier endothelium specific.

To confirm their ability to bind to blood-brain barrier endothelium *in situ*, GLA, GYR, and random control phage RVR were individually perfused through the brain again in the *in situ* perfusion model. After perfusion and

washing, phage were recovered from the brain (figure 4). Phage GYR showed a 5.0 times higher affinity to the brain than random control phage RVR. Phage GLA showed a 5.9 times higher affinity.

During phage display screening, GLA and GYR have been isolated from the brain after a series of processing steps (i.e. grinding, TEA incubation, neutralization, and centrifugation). One of these steps may favour the selection of a certain phage clone over another, since phage display selections are based on survival of the fittest. To check for this, GLA, GYR, and control phage RVR were incubated with a piece of control tissue (liver), and were processed in exactly the same way as was done after brain perfusion. As is shown in figure 5, recovery of phage from the liver pieces was comparable between selected and unselected phage clones, indicating that GLA and GYR had not been selected due to enhanced survival in the processing method, but have genuine brain endothelium affinity. This is further supported by the observation that the difference in affinity between selected and unselected phage can be seen upon brain but not upon liver incubation.

To establish whether this affinity was brain endothelium specific, lung endothelium was used as a control. Just like the brain, lungs comprise microvascular endothelium, and affinity of the phage for this endothelium was tested. GLA, GYR, and control phage RVR were perfused rough murine lungs, and were processed in exactly the same way as was done for the brain perfusion. As is shown in figure 7, GLA and GYR do not have a higher affinity for the lungs compared to random control phage RVR.

Using the powerful technique of phage display we identified two peptides that show significant binding to the brain compared to a random control peptide. It is interesting to see that two peptides with opposite net charges both bind to the blood-brain barrier. Cell membranes are negatively charged. Therefore,

positively charged molecules may bind to the cell surface non-specifically by electrostatic interactions. Selected peptide GYR and control peptide RVR are both positively charged (table 1), yet GYR shows a significantly higher binding than RVR. Furthermore, also the negatively charged GLA shows significant binding to the brain. These results indicate that the binding of the selected peptides is dependent on more than electrostatic interactions with the endothelium.

Identification of the peptide binding site can be an important step in the ligand discovery process. This can give insight into the ligand-target interaction and the uptake mechanism. The binding site should preferably be a receptor, which can specifically internalize a ligand, and the attached drug delivery vehicle, into the cells.

GLA and GYR may bind to a brain endothelium receptor. Because of the negative selection that was performed by perfusion through the heart, this receptor is likely to be brain endothelium specific. Therefore it could be a new, yet unidentified receptor, suitable for specific targeting to the blood-brain barrier.

To visualize binding of phage GLA, GYR, and control phage RVR to human brain endothelial cells, hCMEC/D3 cells were incubated with single phage clones for 1 hour at 37°C. Bound phage were stained with a labelled anti-phage antibody. Fluorescence microscopy showed phage staining which was evenly distributed throughout the cell layer. This can be seen from color pictures generated, which clearly show enhanced binding of selected phage GLA and GYR compared to the control phage RVR. For the control phage RVR only a few fluorescent spots can be seen, indicating only a few phage had bound to the cell layer. For both selected phage GLA and GYR a much more extensive

pattern of spots can be seen, indicating a much higher amount of phage binding.

EXAMPLE 2

An appropriate pharmaceutical formulation comprising both inventive peptide sequences include liposomes by using the film building method as example for their preparation.

The composition of liposomes (30 µmol/ml lipid) comprises the following ingredients (MW = molecular weight, c = concentration):

Lipid composition	Ratio	Name	c [µmol/mL]	MW	c´ [mg/ml]
DPPC (Lipoid GmbH, D)	1.85	DiPalmitoyl-PhosphoCholine	18.5	734.05	13.580
Cholesterol (Sigma)	1.0		10	386.65	3.867
PEG-2000-DSPE (Lipoid GmbH, D))	0.09	PolyEthyleneGlycol-2000 DiStearoylPhospho-Ethanolamine	0.9	2731.39	2.458
Maleimide-PEG-2000-DSPE (Avanti polar lipids Inc, US	0.06		0.6	2941.64	1.765

All ingredients are given into a suitable round bottom flask. Chloroform as solvent is added to dissolve all excipients. The round bottom flask is attached to a rotavopor for evaporation of the solvent and building a film of the phospholipids. The residue solvent is evaporated using nitrogen. The received lipid film is rehydrated using HEPES buffered saline pH 7.4. To disperse the lipid film from the glass vial glass beads are added and shaking is used. Afterwards the built liposomes are sonicated (ultrasound) and/or stepwise extruded through several polycarbonate membranes with a pore size of 400, 200 and/or 100 nm.

The peptide sequences GLA and GYR are be coupled to prepared liposomes.

Peptides have been synthesized with a N-terminal cysteine. The disulfide bonds of the peptides are reduced using Immobilized TCEP Disulfide Reducing

Gel (supplied by Pierce; containing 8mM TCEP; TCEP = Tris(2-carboxyethyl)phosphine hydrochloride) to make it react with the maleimide, separeated by centrifugtion from the gel and added to the previous prepared liposomes immediately.

The ratio peptide:maleimide is 1:1. The composition is incubated overnight at 4°C. In the next step the free, not reacted maleimide groups are blocked by incubation for 2h at 4°C with 2mM 2-mercaptoethanol to prevent non-specific reactions with other components like proteins/molecules during the preparation or after application in the body..

Afterwards the resulted liposomes are purified using PD-10Sephadex G-25 columns or ultracentrifugation is used alternatively. The free peptide and 2-mercaptoethanol are removed.

The final lipid content of liposomes and API will be determined due to loss according to the production procedure. The final lipid will be calculated after determination of the phosphate content and the API concentration using a validated HPLC-UV method.

The liposomes will have a size preferably in the range 100-200nm and about 1000 to 4000 peptides are present per liposome (depends on their size).

This composition includes Pegylated phospholipids for sterical hindrance of build liposomes to enlarge distribution and reduce liver and spleen uptake.

The according this procedure produced drug-peptide-liposomes show until now a short time stability of 2 weeks if stored at 4°C.

EXAMPLE 3

Synthesis of block copolymers

(1) alpha- azido omega-methoxy poly(ethylene glycol)

5 g of poly(ethylene glycol) monomethyl ether (Mw 1kD) was coevaporated with benzene three times and dissolved in 250 mL dry and under argon distilled THF. The flask was cooled on an ice bath and air was replaced by

argon as 5 mL freshly distilled triethyl amide was added. The mixture was stirred for 3 hours, after which 1.14 gram (2 eq.) of mesyl chloride in 10 mL THF was added. The mixture was allowed to warm to room temperature and was stirred for 6 hours. All THF was removed and 100 mL methanol containing 3.25 gram (10 eq.) sodium azide was added. The mixture was refluxed overnight after which methanol was removed and 100 mL brine was added. The product was extracted with 5x 100 mL dichloromethane. The combined organic layers were dried over magnesium sulphate and dichloromethane was removed, yielding 4.5 gram (90%) product.

1HNMR** : δ 3.380 (s, 3H, $C\underline{H}_3O$), δ 3.658 (bs, 90H, $C\underline{H}_2C\underline{H}_2O$), δ 3.640 (t, 2H, $C\underline{H}_2N_3$)

IR : 2100 cm-1, azide

(2) alpha-azido omega-carboxy poly(ethylene oxide)

10 g of poly(ethylene glycol), Mw 1kD, was coevaporated with benzene three times and dissolved in 500 mL dry and under argon distilled THF. The flask was cooled on an ice bath and air was replaced by argon as 10 mL freshly distilled triethyl amide was added. The mixture was stirred for 3 hours, after which 1.25 gram (1.1 eq.) of mesyl chloride in 10 mL THF was added. The mixture was allowed to warm to room temperature and was stirred for 6 hours. All THF was removed and 200 mL methanol containing 6.5 gram (10 eq.) sodium azide was added. The mixture was refluxed overnight after which methanol was removed and 200 mL brine was added. The product was extracted with 5x 200 mL dichloromethane. The combined organic layers were dried over magnesium sulphate and dichloromethane was removed, yielding 9.5 gram (95%) intermediate I. (azide in IR at 2100 cm-1)

Intermediate I was coevaporated with benzene three times and dissolved in 500 mL freshly distilled THF. 700 mg sodium hydride (60% on mineral oil, 1.75 eq.) was added under an argon atmosphere. While stirring, hydrogen gas was allowed to escape for three hours. The temperature was

raised to 60 °C and 5.85 gram of tert-butyl bromoacetate (3 eq.) was added via a syringe. The reaction was allowed to proceed for 12 hours after which all THF was removed and the products were dissolved in 50 mL dichloromethane. The solution was poured over a plunge of silica gel and flushed with 250 mL dichloromethane. Intermediate II was eluted with 10 percent methanol in dichloromethane, yielding 4.5 gram (90%).

Intermediate II was dissolved in 100 mL 2M HCl solution and refluxed overnight. The product was extracted with 5x 100 mL dichloromethane and the combined organic layer was dried over $MgSO_4$. Dichloromethane was evaporated and the product was obtained after silica column purification (eluent: dichloromethane : methanol : concentrated ammonia as 88:12:4). The desired product with R_f 0.52 was obtained in a 35% overall yield (70% of theoretical yield).

1HNMR** : δ 3.380 (s, 3H, $C\underline{H}_3O$), δ 3.658 (bs, 90H, $C\underline{H}_2C\underline{H}_2O$), δ 4.120 (s, 2H, $OC\underline{H}_2COO$)

IR : 2100 cm-1, azide, 3400 cm-1 carboxylic acid, 1680 cm-1, carboxylic acid

(3) Alkyne terminated polybutadiene

All glassware was extensively cleaned, flushed with 1.7 M butyllithium, rinsed again and flame dried. 2.6 gram 1,3 butadiene was condensed into a Schlenk tube and 5 mL freshly distilled THF was added. The solution was stirred and cooled to -35 °C as 0.42 mL 1.4 M sec-butyllithium was added at ones. The deep orange reaction mixture was allowed to warm to -10 °C and stirred until the color changed to pale yellow. Another aliquot of 10 mL THF was added and the temperature was lowered to -35 °C. The reaction was quenched by the addition of 170 mg (2eq) of trimethylsilyl propargyl chloride added via a syringe as the reaction was allowed to warm to room temperature. After the yellow color had disappeared, 1 mL of a 1M tetrabutyl ammonium fluoride solution in THF was added and stirred for one hour. THF was evaporated. The

product was extracted by adding 50 mL dichloromethane and washed with two times 25 mL water. The organic layer was reduced and the product was purified over a silica column, eluting with dichloromethane (R_f = 0.95). After removal of all DCM, the product was vacuum dried overnight to yield 2.5 gram of product (95%).

1HNMR** : 1.0-1.4 (C<u>H</u>$_2$ backbone), 1.8-2.2 (C<u>H</u> backbone), 4.8-5.0 (C<u>H</u>$_2$ vinyl), 5.2-5.6 (C<u>H</u> vinyl)

Mw : 4.5 kD PDI : 1.04

(4) Polybutadiene-b-poly(ethylene oxide) methyl ether

80 mg **1** and 720 mg **3** (2 eq.) was dissolved in 10 mL dry THF under an argon atmosphere. The temperature was raised to 55 °C and 30 mg CuBr (2.5 eq.) and 70 mg pentamethyldiethylenetriamine (5 eq) was added. The reaction was allowed to proceed for 12 hours after which all solvents were removed. The products were dissolved in 30 mL dichloromethane and washed with three times 25 mL 0.33 M EDTA. The organic layer was dried over over $MgSO_4$ and poured over on a short silica column eluting with dichloromethane. After all non reacted **3** was flushed off the product was eluted with 8 percent methanol in dichloromethane. After removal of all solvents 300 mg of the product was obtained in 60 % yield.

1HNMR** : Combination of **1** and **3**

Mw : 5.5 kD PDI : 1.07

(5) Polybutadiene-b-poly(ethylene oxide) carboxylic acid

80 mg **2** and 720 mg **3** (2 eq.) was dissolved in 10 mL dry THF under an argon atmosphere. The temperature was raised to 55 °C and 30 mg CuBr (2.5 eq.) and 70 mg pentamethyldiethylenetriamine (5 eq.) was added. The reaction was allowed to proceed for 12 hours after which all solvents were removed. The products were dissolved in 30 mL dichloromethane and washed with three times 25 mL 0.33 M EDTA. The organic layer was dried over over $MgSO_4$ and

poured over on a short silica column eluting with dichloromethane. After all non reacted **3** was flushed off the product was eluted with 8 percent methanol in dichloromethane. After removal of all solvents 300 mg of the product was obtained in 60 % yield.

1HNMR** : Combination of **2** and **3**

Mw : 5.5 kD PDI : 1.18

EXAMPLE 4

Polymer end functionalization

(6) maleimide introduction

While gently stirring, 200 mg **5** was dissolved in 6 mL DCM. 800 µL 1M DIPCDI in DMF, 900 µL 1M HoBt in DMF and 40 mg 2-(2,5-dioxo-2,5-dihydro-pyrrol-1-yl)-ethylamine was added. The solution was stirred overnight after which all DCM was removed. 20 mL ice cold methanol was added at ones and the precipitated was gently shaken to dissolve all components except the product. After 1 hour the suspension was centrifuged at 5000 rpm for 30 minutes. Methanol was decanted and a new aliquot ice cold methanol was added to repeat the centrifuge step. The pellet was dried under vacuum to yield 120 mg product (60%).

1HNMR** : Combined **2** and **3** plus δ 6.88 (HC=CH , maleimide)

Mw : 5.5 kD PDI : 1.08

(7) Amine introduction

Same procedure as **6** with 2-(2,5-dioxo-2,5-dihydro-pyrrol-1-yl)-ethylamine replaced by 40 mg ethanediamine.

1HNMR** : Combined **2** and **3**

Mw : 5.5 kD PDI : 1.12

(8) Fluorescein introduction

100 mg **7** was dissolved in 3 mL THF. A suspension of 15 mg fluorescein isothiocyanate in 1 mL THF was added together with a drop of triethylamine. After 3 hours all solvents were removed and the product was purified by silica column chromatography eluting with 8% methanol in chloroform. Yield: 40%
R_f: 0.3

EXAMPLE 5

Assembly of polymersomes

(9) Peptides

Peptides GLA and GYR where synthesized with a C-terminal cysteine on the solid phase resin ("Wang resin") using standard Fmoc SPPS coupling conditions. The peptides where obtained with a free amine.

(10) polymersomes

8 mg **4**, 1 mg **6** and 1 mg **8** was dissolved in 1 mL THF. While gently stirring, 3 mL of PBS* buffer was added dropwise over 15 minutes. The opaque solution was transferred to a syringe and extruded five times over a 200 nm membrane to obtain polymersomes of ~200 nm. One mL of the extruded solution was transferred to a vial and 9 mL PBS was added. The polymersome solution was transferred to a dialysis bag with a molecular cut-off of 12-14.000 Da. and dialyzed against one litre PBS for 24 hours.

3 mL of the dialyzed polymersome solution was transferred in a vial. The solution was gently stirred while 100 µL of TCEP solution (10 mg/mL in PBS) and 100 µL peptide (**9**) solution(10 mg/mL in PBS) was premixed and added to the polymersomes. The coupling was allowed to proceed for 2 hours after which the solution was transferred to a dialysis bag with molecular weight cut-off of 12-14.000 Da. The solution was dialyzed for 24 hours against one litre PBS. The particle size was measured with differential light scattering and the presence of polymersomes was confirmed by TEM.

* 8.00 g NaCl, 0.20 g KCl, 1.44 g Na2HPO4 and 0.24 g KH2PO4 per liter MilliQ ;pH7.4

** 1HNMR 400 MHz in deuterated chloroform

EXAMPLE 6

Materials and Methods

Abbreviations:

DPPC	dipalmitoyl phosphatidylcholine
DSPE	distearoyl phosphatidylethanolamine
PEG_{2000}	polyethyleneglycol with molecular weight of 2000 Da
EPG	egg phosphotidylglycerol
EPC	egg phosphatidylcholine
MPB-PE	maleimidophenyl butyryl phosphatidylethanolamine
UPLC	ultra performance liquid chromatography

Liposome preparation

Liposomes were prepared by the film hydration and membrane extrusion method. For *in vitro* flow cytometry and in situ perfusion studies, fluorescently labeled 200 nm liposomes with a DPPC:cholesterol:DSPE-PEG_{2000}:DSPE-PEG_{2000}-maleimide composition were used. For *in vivo* biodistribution studies, 100 nm liposomes with a EPG:EPC:cholesterol:MPB-PE composition were used, radioactively labeled with [^{3}H]cholesteryl hexadecyl ether.

Coupling of synthetic peptides to liposomes

GLA and GYR peptides were synthesized (by company Caslo, Denmark). with a C-terminal cysteine. The thiol group of the cysteine was reacted with the maleimide group on the liposomes. Binding of the peptides to the liposomes was confirmed by UPLC.

Binding of liposomes to hCMEC/D3 cells

Human brain endothelial (hCMEC/D3) cells were grown to a monolayer and then brought to suspension by trypsinization. Targeted labeled liposomes were incubated for 1 hour at 4°C with the cells. Cells were incubated with 3 different liposome concentrations, 100, 500, and 1500 nmol/ml lipid. After washing, binding was investigated by flow cytometry.

Biodistribution of liposomes

Targeted radioactive liposomes were injected iv into the tail vein of mice (n=5). After 12 hours, mice were sacrificed and organs were collected and counted for radioactivity. Brains were submitted to capillary depletion to discriminate between liposomes that had bound to brain capillaries and liposomes that had transcytosed into the brain parenchyma. Capillary depletion was performed by the method described by Triguero et al., J Neurochem. 1990;54(6):1882-1888.and Gutierrez et al., J Neuroimmunol. 1993;47(2):169-176.

Results

When GLA and GYR-coupled liposomes were incubated with human hCMEC/D3 cells, an increased binding of GYR-liposomes was observed. While 0.9% of the cells were positive for control RVR and GLA-liposome binding, 1.6% of cells were positive for GYR-liposome binding. This was an 1.8x increase (figure 8 and 9).

The peptide-coupled liposomes were perfused through the mouse brain by *in situ* perfusion. The fraction of liposomes that had bound to the mouse brain was determined. No significant difference was observed between the peptide-coupled and uncoupled liposomes (figure 10).

GLA-coupled liposomes were injected iv in mice. Brain capillaries were separated from brain parenchyma. In the brain capillary fraction, a 2.4x

increased binding of GLA-liposomes was found compared to uncoupled liposomes (figure 11).

Claims

1. A peptide comprising an amino acid sequence which is in at least 9, preferably at least 11, most preferably at least 13 amino acids identical to the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:2.

2. The peptide according to claim 1, wherein the peptide comprises the amino acid sequence set forth in SEQ ID NO:1 or SEQ ID NO:2.

3. A composition of matter for the delivery of a pharmaceutically acceptable substance across the blood-brain barrier, the composition comprising

(a) at least one peptide according to claim 1 or 2

(b) at least one pharmaceutically acceptable substance.

4. A composition according to claim 3, wherein said peptide is coupled to said pharmaceutically acceptable substance by covalent or non-covalent bonds.

5. A composition according to claim 4, wherein said peptide and said pharmaceutically acceptable substance are covalently linked by a linking structure.

6. A composition according to claim 4, wherein said peptide and said pharmaceutically substance are each separately covalently coupled to a linking structure and wherein the covalently coupled linking structures are coupled to each other by non-covalent bonds.

7. A composition according to any one of the claims 3-6, wherein said pharmaceutically acceptable substance is selected from the group of active pharmaceutical ingredients, proteins, nucleic acids, siRNA molecules, antisense molecules, expression constructs that comprises a nucleic acid that encodes a therapeutic protein of interest, liposomes, nanoparticles, diagnostic agents, markers of a disease of the central nervous system (CNS), antibodies, erythrocytes, erythrocytes ghosts,

spheroplasts, monoclonal antibodies, labeled monoclonal antibodies which bind a marker of a CNS disorder, and/or a fragment of an antibody or a monoclonal antibody.

8. A composition according to any one of the claims 3-7, wherein said pharmaceutically acceptable substance comprises an active substance such as an active pharmaceutical ingredient or a diagnostic agent, and a carrier, the carrier preferably being a nanoparticle enclosing said active substance.

9. A composition according to claim 8, wherein the nanoparticle is a liposome.

10. The use of a peptide according to claim 1 or 2 for the manufacture of a medicament for the treatment, prophylaxis of a central nervous system disorder or for the manufacture of a diagnostic agent of a central nervous system disorder, said central nervous system disorder preferably being selected from the group consisting of depression, dementia, Prion Disease, Alzheimer's Disease, Parkinson's Disease, Multiple Sclerosis, Amylotrophic Lateral Sclerosis, Schizophrenia, Lyme Disease, Poliomyelitis, stroke, traumatic brain injury, psychosis, Chorea Huntington Disease, encephalopathy, epilepsy, cerebrovascular diseases and neurodegenerative disorders and a central nervous system cancer, wherein the peptide serves as a central nervous system targeting principle.

11. A peptide according to claim 1 or 2 for use as a central nervous system targeting principle in the treatment, prophylaxis or diagnosis of a central nervous system disorder, said central nervous system disorder preferably being selected from the group consisting of depression, dementia, Prion Disease, Alzheimer's Disease, Parkinson's Disease, Multiple Sclerosis, Amylotrophic Lateral Sclerosis, Schizophrenia, Lyme Disease, Poliomyelitis, stroke, traumatic brain injury, psychosis, Chorea

Huntington Disease, encephalopathy, epilepsy, cerebrovascular diseases and neurodegenerative disorders and a central nervous system cancer.

12. A composition according to any one of the claims 3-9 for use as a medicament.

13. A composition according to claim 12, for use in the treatment, prophylaxis or diagnosis of a central nervous system disorder.

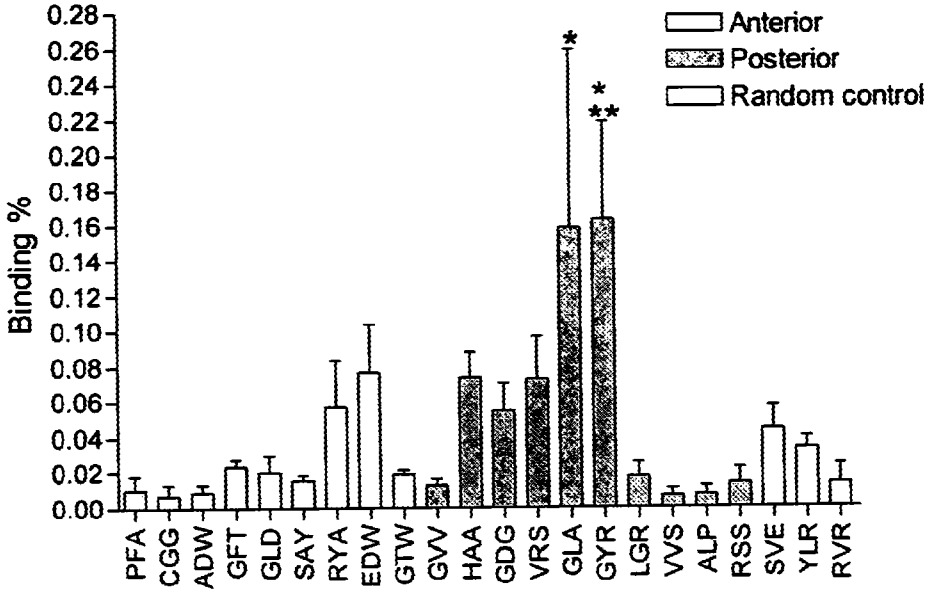
14. A composition according to claim 13, wherein said central nervous system disorder is selected from the group consisting of depression, dementia, Prion Disease, Alzheimer's Disease, Parkinson's Disease, Multiple Sclerosis, Amylotrophic Lateral Sclerosis, Schizophrenia, Lyme Disease, Poliomyelitis, stroke, traumatic brain injury, psychosis, Chorea Huntington Disease, encephalopathy, epilepsy, cerebrovascular diseases and neurodegenerative disorders and a central nervous system cancer.

15. A composition according to any one of the claims 3-9 for use as a delivery system in delivering a pharmaceutically acceptable substance across the blood-brain barrier to the central nervous system.

16. A pharmaceutical formulation comprising a peptide according to claims 1 or 2 or a composition according to any one of claims 3-7, and one or more pharmaceutically acceptable excipients.

17. A nucleic acid sequence encoding for a peptide sequence according to claim 1 or 2.

A. binding of phage *in vitro*



B. uptake of phage *in vitro*

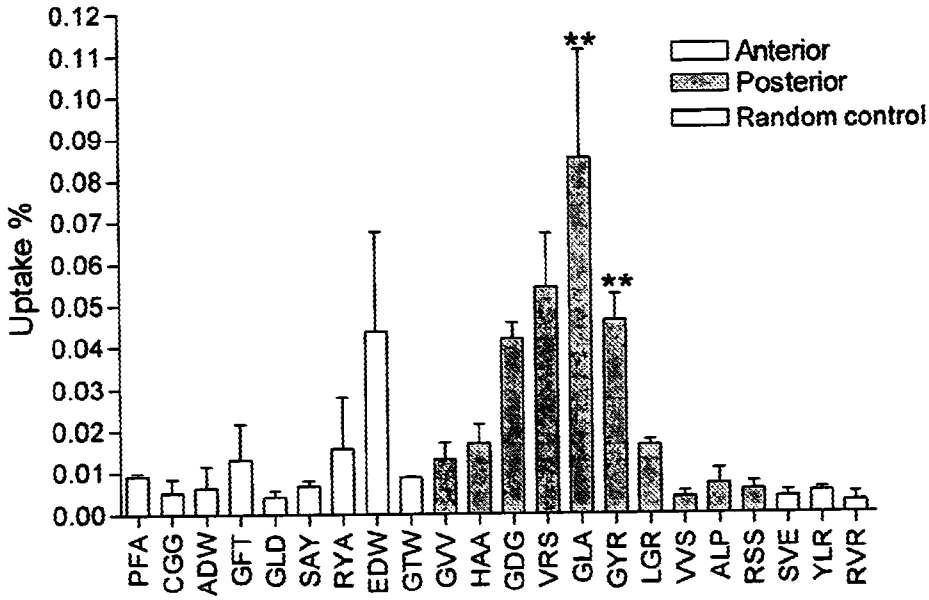


Fig. 1

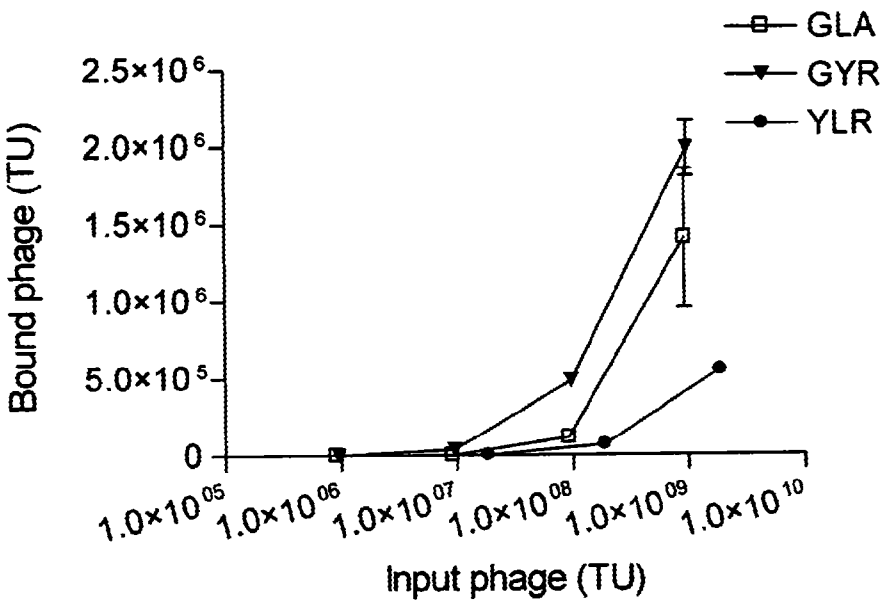

GLA
GYR
YLR
Bound phage (TU)
2.5×10^6
2.0×10^6
1.5×10^6
1.0×10^6
5.0×10^5
0
1.0×10^05
1.0×10^06
1.0×10^07
1.0×10^08
1.0×10^09
1.0×10^10
Input phage (TU)

Fig. 2

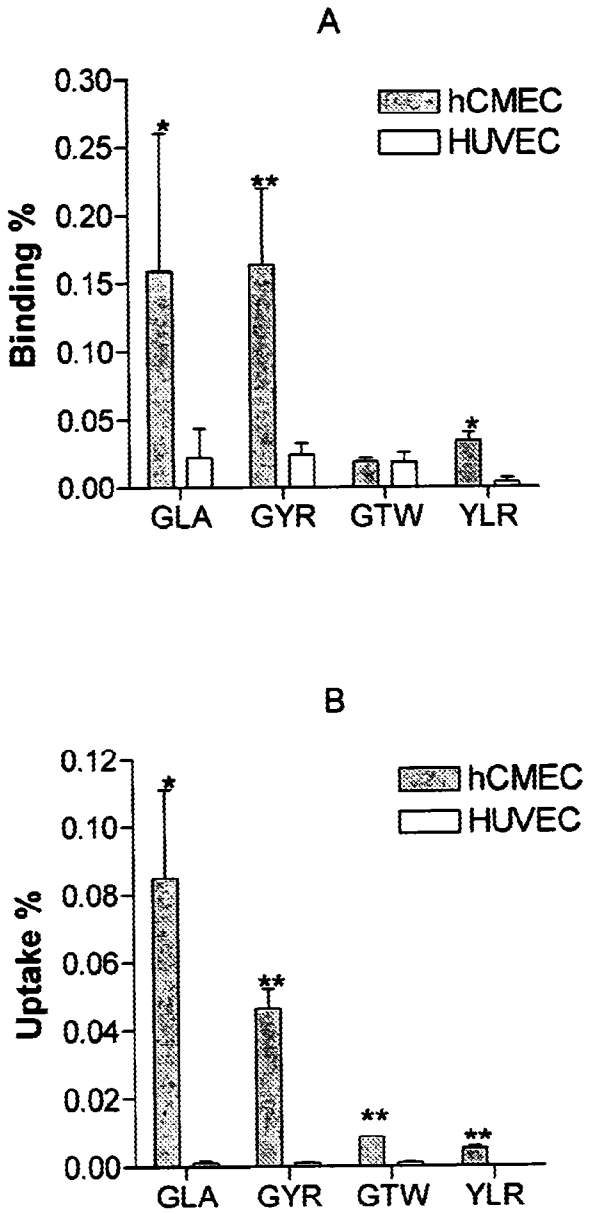

A
Binding %
0.30
0.25
0.20
0.15
0.10
0.05
0.00
hCMEC
HUVEC
*
**
*
GLA
GYR
GTW
YLR
B
Uptake %
0.12
0.10
0.08
0.06
0.04
0.02
0.00
hCMEC
HUVEC
*
**
**
**
GLA
GYR
GTW
YLR

Fig. 3

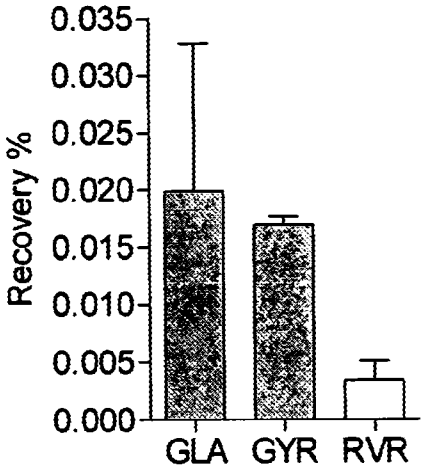

0.035
0.030
0.025
0.020
0.015
0.010
0.005
0.000
Recovery %
GLA
GYR
RVR

Fig. 4

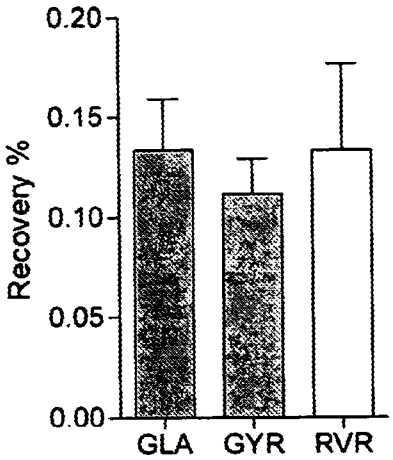

0.20
0.15
0.10
0.05
0.00
Recovery %
GLA
GYR
RVR

Fig. 5

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
SEQ ID NO:1	G	L	A	H	S	F	S	D	F	A	R	D	F	V	A
Identical Amino Acids (marked by "I")	I	I			I	I		I		I	I	I		I	I
Sequence A	G	L	Z	Z	S	F	Z	D	Z	A	R	D	Z	V	A

Fig. 6

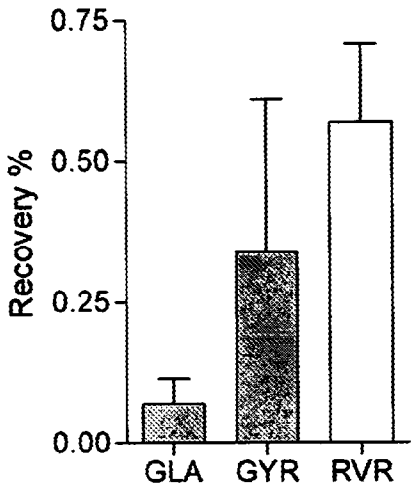


Fig. 7

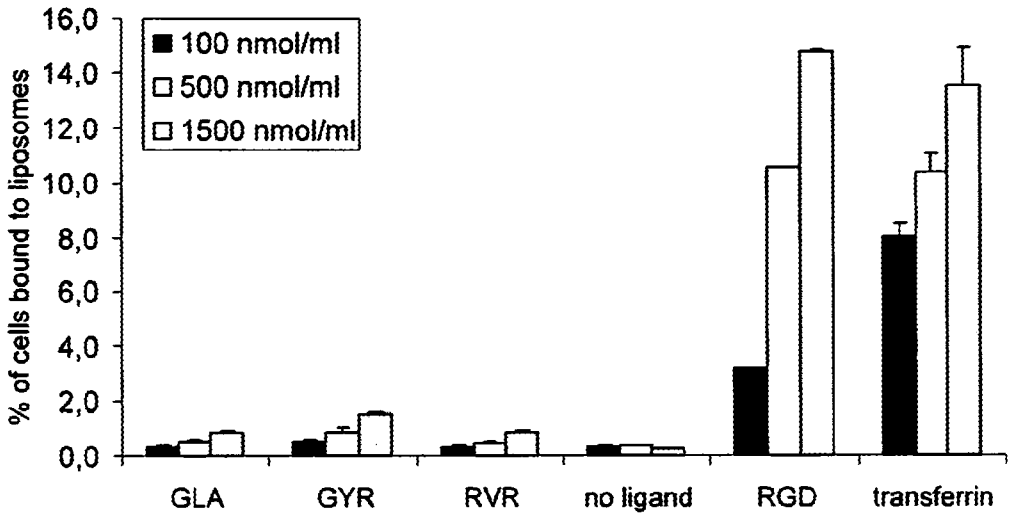

16,0
14,0
12,0
10,0
8,0
6,0
4,0
2,0
0,0
% of cells bound to liposomes
100 nmol/ml
500 nmol/ml
1500 nmol/ml
GLA
GYR
RVR
no ligand
RGD
transferrin

Fig. 8

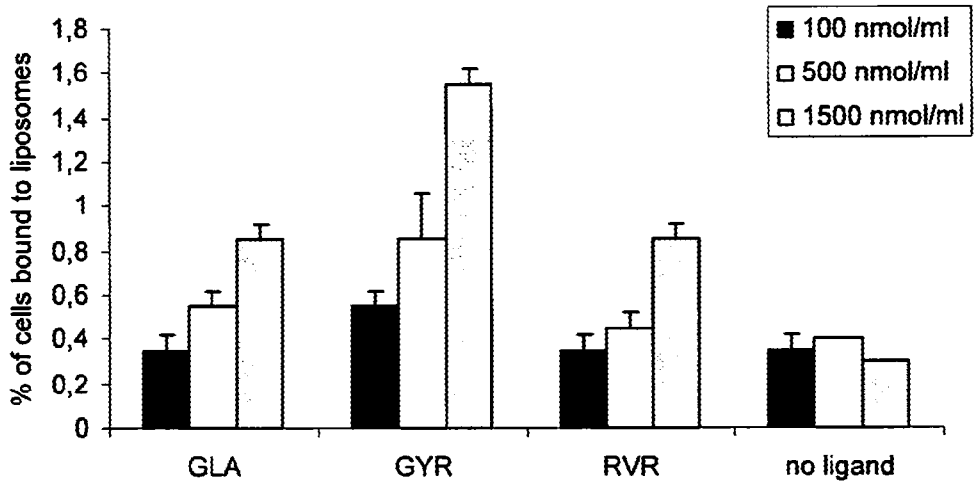

1,8
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
% of cells bound to liposomes
100 nmol/ml
500 nmol/ml
1500 nmol/ml
GLA
GYR
RVR
no ligand

Fig. 9

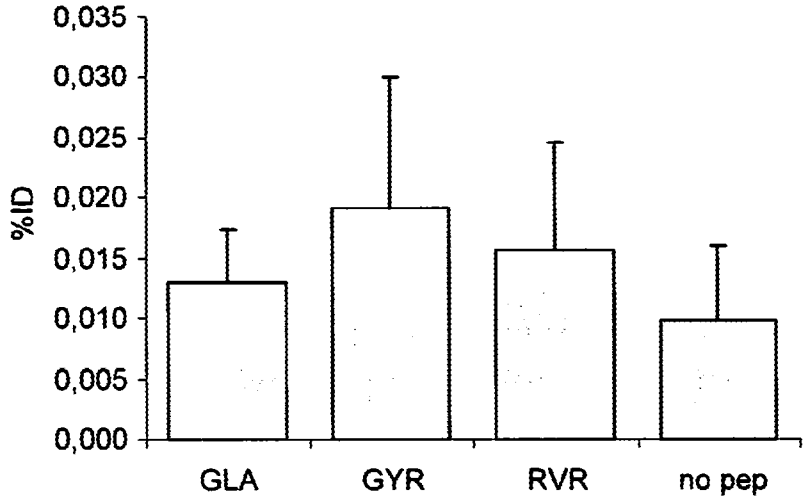

0,035
0,030
0,025
0,020
0,015
0,010
0,005
0,000
%ID
GLA
GYR
RVR
no pep

Fig. 10

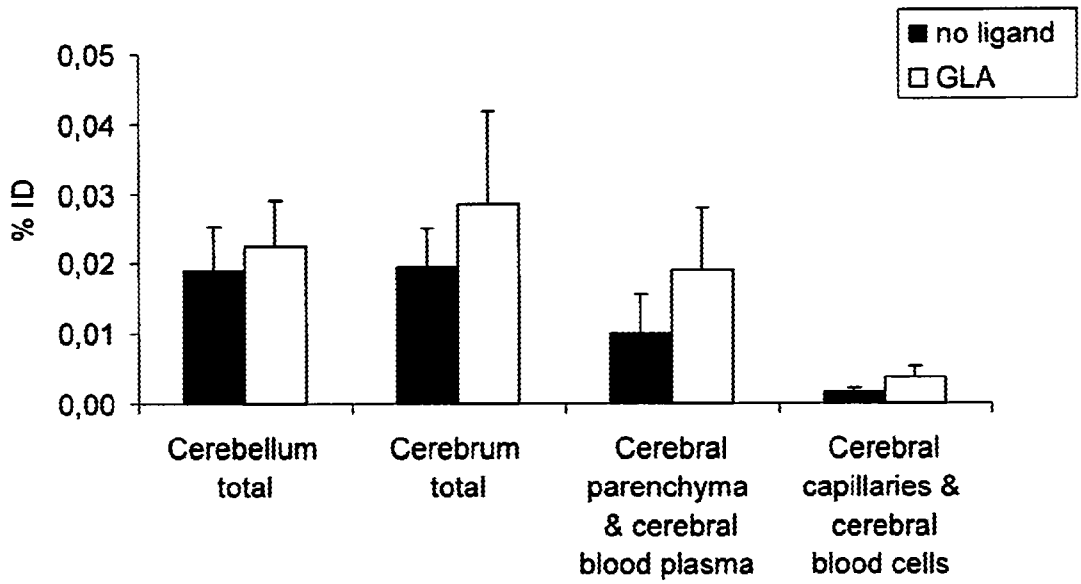

no ligand
GLA
0,05
0,04
0,03
0,02
0,01
0,00
% ID
Cerebellum total
Cerebrum total
Cerebral parenchyma & cerebral blood plasma
Cerebral capillaries & cerebral blood cells

Fig. 11

INTERNATIONAL SEARCH REPORT

International application No
PCT/NL2010/050448

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K7/08 A61K47/48
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, CHEM ABS Data, PAJ, WPI Data, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE UniProt [Online] 5 September 2006 (2006-09-05), Normand e.a.: "Genome characteristics of facultatively symbiotic Frankia sp. strains reflect host range and host plant biogeography" XP002608484 retrieved from EBI Database accession no. Q0RKN1 See positions 217-232 in sequence -----	1
X	US 6 399 575 B1 (SMITH BRUCE F [US] ET AL) 4 June 2002 (2002-06-04) cited in the application the whole document -----	1,3-17
	-/--	

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 November 2010	19/11/2010

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Groenendijk, Matti

INTERNATIONAL SEARCH REPORT

International application No
PCT/NL2010/050448

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	VAN ROOY INGE ET AL: "Identification of Peptide Ligands for Targeting to the Blood-Brain Barrier" PHARMACEUTICAL RESEARCH (DORDRECHT), vol. 27, no. 4, April 2010 (2010-04), pages 673-682, XP002608909 ISSN: 0724-8741 the whole document	1-17

INTERNATIONAL SEARCH REPORT

International application No.
PCT/NL2010/050448

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☒ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-17(partially)

Peptide defined in claim 1 based on SEQ ID NO. 1, its compositions and use, DNA encoding said peptide

---

2. claims: 1-17(partially)

Peptide defined in claim 1 based on SEQ ID NO. 2, its compositions and use, DNA encoding said peptide

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/NL2010/050448

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 6399575 B1	04-06-2002	NONE	