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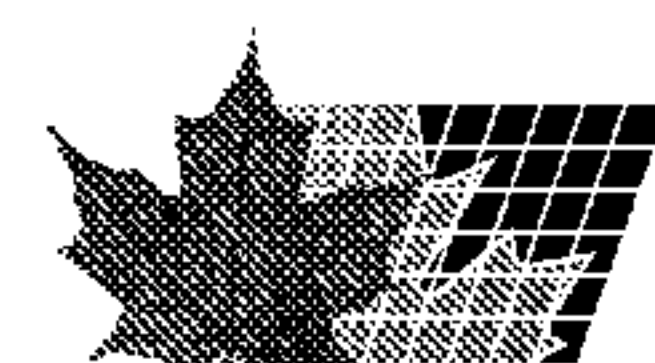
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(57) **Abrégé/Abstract:**

The present invention provides means for treating and preventing amyloid-related pathology in young to middle-aged subjects with Down's syndrome (DS). In particular, the present invention provides antigenic peptide fragments derived from amyloid protein or amyloid-like protein for use in the preventive treatment of amyloid-related pathology in young to middle-aged subjects with Down's syndrome.



ABSTRACT

The present invention provides means for treating and preventing amyloid-related pathology in young to middle-aged subjects with Down's syndrome (DS). In particular, the present invention provides antigenic peptide fragments derived from amyloid protein or amyloid-like protein for use in the preventive treatment of amyloid-related pathology in young to middle-aged subjects with Down's syndrome.

VACCINE THERAPY

Technical Field

[0001] The present invention provides means for treating amyloid-related pathology in young to middle aged subjects with Down's syndrome (DS). In particular, the present invention provides antigenic peptide fragments derived from amyloid protein or amyloid-like protein for use in the treatment of Alzheimer's disease (AD)-like dementia in young to middle aged subjects with Down's syndrome (DS).

Background Art

[0002] The genetic disorder Down's syndrome (DS) is the most common trisomy and it occurs in one out of 700-1000 newborns. Estimates suggest that 25% or more of individuals with Down syndrome over age 35 show the signs and symptoms of Alzheimer's-like dementia (Stanton L.R and Coetzee R.H , 2004). The percentage increases with age. In DS, the entire or at least a part of the chromosome 21 is present in triplicate (Antonarakis *et al.*, 2004; Moncaster *et al.*, 2010). Consequently, the three copies of the gene of the amyloid precursor protein (APP) lead to the generation of an excess of Amyloid- β (A β), one of the main abnormal proteins well known to be responsible for Alzheimer's disease (AD) (Ballard *et al.*, 2011). Therefore, A β is suggested to have a possible role for the AD-like dementia in most DS people (Lee *et al.*, 2005; Liu *et al.*, 2005; Gilman *et al.*, 2005).

[0003] In people with Down syndrome, AD-like memory defects can be related to several pathological proteins. A β increase begins already in the embryonal stage, progresses at birth and continues to build up with increasing age (Stoltzner *et al.*, 2000). In addition, Cerebrospinal fluid (CSF) levels of A β 42 were lower and tau levels were higher in older (>40 years) than in younger DS people (Tapiola *et al.*, 2001). Once A β is deposited in plaques, Apo E can be detected in many plaques at age 12 and augments steadily with age (Lemere *et al.*, 1996). Neurofibrillary tangles (NFTs) are detected in the brain of DS during the fourth decade of life. These NFT are believed to result from tau accumulation. It has been shown that hyperphosphorylation might be caused by overexpression of a kinase named Dyrk1A (dual-specificity tyrosine-phosphorylated and regulated kinase 1A) (Lemere *et al.*,

1996;Liu *et al.*, 2008). Therefore, people with DS can develop amyloid-related pathology by age 40 and most have the clinical symptoms similar to Alzheimer's, such as cognitive decline and memory impairment, by age 60 (Stanton 2004).

[0004] While individuals with DS receive medical care mainly for their various health problems (such as heart defect, infections and hypothyroidism), specific-treatment for neuropathological traits, i.e. mental disability and memory deficiency, is rarely considered. Currently, only few clinical trials are ongoing, all aimed to enhance mental abilities or to reduce nerve damage. The drugs for treating DS are also used in AD and all act on the cholinergic system or the glutamatergic neurotransmission such as Rivastigmine or Donepezil, a cholinesterase inhibitors and Memantine, a NMDA receptor antagonist (Prasher, 1993; Prasher, 2004). However, evidence of efficacy is lacking for people with DS. Currently, there are many amyloid-modifying treatments under review, including targeting-A β -immunotherapy (Rafii, 2010). Several vaccines have reached recently clinical phases (Weiner and Frenkel, 2006) after having shown efficient reduction of cerebral A β burden and reversing cognitive decline in mouse AD models. In contrast to AD, immunotherapies targeting A β are not being addressed in DS.

[0005] There is therefore a need for a treatment of clinical symptoms associated with amyloid-related pathology in subjects with Down's syndrome (DS). In particular, there is a need for a treatment, which leads to an improvement or restoration of memory capacity in subjects with Down's syndrome (DS).

Disclosure of Invention

[0006] Within the scope of the present invention the measures and means are provided which help to meet this need. In particular, the present invention provides antigenic peptides for use in the treatment of AD-like cognitive impairments in young to middle-aged subjects with Down's syndrome.

[0007] In a first embodiment, the present invention provides an antigenic peptide fragment derived from amyloid protein or amyloid-like protein, or a composition comprising said antigenic peptide, for use in the treatment, alleviation and/or prevention of memory and/or cognitive impairments or abnormalities, particularly of AD-like memory and/or cognitive impairments or abnormalities, in young to middle-

aged subjects with Down's syndrome, in particular in young subjects with Down's syndrome, who have not yet developed A β -associated plaques in the brain.

[0008] In certain embodiments, the invention contemplates using the antigenic peptide fragment derived from amyloid protein or amyloid-like protein, or a composition comprising said antigenic peptide according to the invention and as described herein in the treatment, alleviation and/or prevention of memory and/or cognitive impairments or abnormalities, particularly of AD-like memory and/or cognitive impairments or abnormalities, in young to middle-aged subjects with Down's syndrome who have already developed A β -associated plaques in the brain.

[0009] In one embodiment, the patient group to be treated with the antigenic peptide or the composition according to the invention comprises young to middle-aged subjects with Down's Syndrome, particularly subjects, which are below the age of 65, particularly below the age of 60, 55 or 50. In one embodiment, subjects with an age of between 0 and 65, particularly of between 5 and 55, particularly of between 10 and 50, particularly of between 15 and 45, particularly of between 20 and 40, particularly of between 25 and 35, may be treated with the antigenic peptide or the composition according to the invention.

[0010] For the purpose of the present invention, young subjects refer to subjects which are below the age of 35, particularly subjects with an age of between 0 and 35, particularly of between 1 and 30, particularly of between 5 and 25.

[0011] For the purpose of the present invention, middle-aged subjects refer to subjects with an age of between 36 and 65, particularly of between 40 and 60, particularly of between 45 and 55.

[0012] In one embodiment, treatment of a young to middle-aged subject with Down's syndrome and suffering from memory and/or cognitive impairments or abnormalities, particularly from AD-like memory and/or cognitive impairments or abnormalities, using the antigenic peptide or the composition according to the invention and as described herein, leads to an amelioration or restoration of said impairments or abnormalities, particularly to memory amelioration, particularly amelioration or restoration of the recognition memory and/or the contextual associative memory.

[0013] In one embodiment, treatment of a young to middle-aged subject with Down's syndrome and suffering from cognitive impairments or abnormalities, particularly from AD-like memory and/or cognitive impairments or abnormalities, using the antigenic peptide or the composition according to the invention and as described herein, leads to an increase in the retention or a complete restoration of cognitive memory capacity in the treated subject.

[0014] The treatment of AD-like cognitive impairments or abnormalities in young to middle-aged subjects with Down's syndrome with the antigenic peptide according to the invention and as described herein shows the therapeutic effects as disclosed herein without inducing unwanted side effects such as meningoencephalitis and microhemorrhage.

[0015] In one embodiment, the antigenic peptide according to the invention and as described herein is derived from an amyloid protein or amyloid-like protein selected from the group consisting of prion protein, tau protein, alpha-synuclein, huntingtin and amyloid- β or a combination of one or more of the above peptides.

[0016] Said A β antigenic peptide fragment corresponds in one embodiment of the invention to the N-terminal part of the A β peptide, particularly to the N-terminal part comprising at least 5, particularly at least 6, particularly at least 7, particularly at least 8, particularly at least 9, particularly at least 10, particularly at least 11, particularly at least 12, particularly at least 13, particularly at least 14, particularly all, amino acid residues from the A β 1-15 fragment.

[0017] In one embodiment, the A β antigenic peptide fragment corresponds to the N-terminal part of the A β peptide comprising at least 5, particularly at least 6, particularly at least 7, particularly at least 8, particularly at least 9, particularly at least 10, particularly at least 11, particularly at least 12, particularly at least 13, particularly at least 14, particularly at least 15, particularly all, amino acid residues from the A β 1-16 fragment, the A β 1-17 fragment, the A β 1-18 fragment, the A β 1-19 fragment, the A β 1-20 fragment, the A β 1-22 fragment, the A β 1-23 fragment, the A β 1-24 fragment, the A β 1-25 fragment or, the A β 1-26 fragment, or the 3-15 A β fragment.

[0018] In one embodiment, the A β antigenic peptide fragment corresponds to the C-terminal part of the A β peptide comprising at least 5, particularly at least 6,

particularly at least 7, particularly at least 8, particularly at least 9, particularly at least 10, particularly at least 11, particularly at least 12, particularly at least 13, particularly at least 14, particularly at least 15, particularly all amino acid residues from the A β 20-40 or A β 20-42 fragment, the A β 21-40 or A β 21-42 fragment, the A β 22-40 or A β 22-42 fragment, the A β 23-40 or A β 23-42 fragment, the A β 24-40 or A β 24-42 fragment, the A β 25-40 or A β 25-42 fragment, the A β 26-46 or A β 27-42 fragment, or the A β 27-40 or A β 27-42 fragment, or the A β 29-40 .

[0019] In one embodiment, the A β antigenic peptide fragment corresponds to the middle part of the A β peptide comprising at least 5, particularly at least 6, particularly at least 7, particularly at least 8, particularly at least 9, particularly at least 10, particularly at least 11, particularly at least 12, particularly at least 13, particularly at least 14, particularly at least 15, particularly all amino acid residues from the A β 15-35, particularly the A β 20-35 fragment.

[0020] In another embodiments of the invention, the full length A β 1-39, A β 1-40, or A β 1-42 fragment may be used.

[0021] In certain embodiments of the invention, the A β antigenic peptide fragment as disclosed herein may contain one or more modified or non-natural amino acid residues.

[0022] In certain embodiments of the invention, the use of A β antigenic peptide fragments is contemplated, which are not fragments consisting of a single or repetitive stretch of between 13 and 15 contiguous amino acid residues from the N-terminal part of the A β peptide, particularly not fragments, wherein said contiguous stretch of 13 to 15 amino acid residues is obtained from the N-terminal fragment 1-16 or 1-17 of the A β peptide, particularly from the N-terminal part of the A β peptide selected from the group consisting of residues 1-15, 1-14, and 1-13, particularly consisting of A β ₁₋₁₅ peptide antigen as given in SEQ ID NO: 1 and A β ₁₋₁₆(Δ 14) as given in SEQ ID NO: 3 disclosed in WO 2007/068411. In one embodiment, the antigenic peptide according to the invention and as described herein is presented reconstituted in a carrier such as, for example, a vesicle, a particulate body or molecule, but particularly reconstituted in a liposome.

[0023] In one embodiment, the antigenic peptide according to the invention and as described herein is presented in a single or repetitive array on the surface of the carrier or liposome.

[0024] In one embodiment, said highly repetitive array on the surface of the carrier or liposome comprises at least 10 repetitive antigenic units/carrier molecule, particularly at least 50 repetitive antigenic units/carrier molecule, particularly at least 100 repetitive antigenic units/carrier molecule, particularly at least 200 repetitive antigenic units/carrier molecule, particularly at least 300 repetitive antigenic units/carrier molecule; particularly at least 400 repetitive antigenic units/carrier molecule, particularly at least 500 repetitive antigenic units/carrier molecule.

[0025] The antigenic composition of the invention and as described herein may comprise a conformational antigen, particularly an A β antigen as described herein, wherein more than 30%, particularly more than 40%, particularly more than 50%, particularly more than 60%, particularly more than 70%, particularly more than 80%, particularly more than 90%, particularly more than 95% and up to 100% is in a beta-sheet conformation.

[0026] The formation and stabilization of the desired conformation of the antigenic peptide may be achieved by presenting the antigenic peptide attached to, or incorporated or reconstituted, partially or fully, into a carrier such as, for example, a vesicle, a particulate body or molecule or any other means that can suitably serve as a carrier/adjuvant for the antigenic peptide. In a specific embodiment of the invention, the antigenic peptide is attached to, or incorporated or reconstituted in the carrier through weak interactions such as, for example, van der Waal's, hydrophobic or electrostatic interaction, or a combination of two or more of said interactions, such that the peptide is presented on the carrier surface with a specific conformation, which is maintained and stabilized by restricting said antigenic peptide in its three dimensional freedom of movement so that conformational changes are prevented or severely restricted.

[0027] When a vesicle, a particle or a particulate body is used as a carrier/adjuvant such as, for example, a liposome, the composition of the antigenic peptide may be chosen such that its overall net charge is identical to that of the carrier/adjuvant surface to which the peptide is attached. Electrostatic repulsion

forces being effective between the identically charged carrier/adjuvant surface and the antigenic peptide, but particularly the identically charged carrier surface and the amino acid residues constituting the antigenic peptide and more particularly the identically charged carrier surface and the identically charged amino acid residues comprised in the antigenic peptide, may lead to the antigenic peptide taking on a defined, highly specific and stabilized conformation which guarantees a high biological activity. As a result, the antigenic peptide is exposed and presented in a conformation that is highly biologically active in that it allows the immune system of the target organism to freely interact with the antigenic determinants contained in the antigenic construct in the biologically active conformation, which leads to a strong and conformation-specific immune response, resulting in, for example, a high antibody titer in the target organism.

[0028] The immunogenic response may be further increased by using a liposome as a carrier, which liposome may function as an adjuvant to increase or stimulate the immune response within the target animal or human to be treated with the therapeutic vaccine according to the invention. Optionally, the liposome may, in addition, contain a further adjuvant such as, for example, lipid A, alum, calcium phosphate, interleukin 1, and/or microcapsules of polysaccharides and proteins, but particularly a detoxified lipid A, such as monophosphoryl or diphosphoryl lipid A, or any other adjuvant that can be suitably used within the scope of the present invention such as, for example, alum, calcium phosphate, interleukin 1, and/or microcapsules of polysaccharides and proteins, LPS, CpG ODN, Pam2CSK4, Pam3CSK4, dsRNA, ssRNA, muramyl dipeptide, Quil Q, QS-21.

[0029] Liposomes that can be used in the compositions of the present invention include those known to one skilled in the art. Any of the standard lipids useful for making liposomes may be used. Standard bilayer and multi-layer liposomes may be used to make compositions of the present invention. While any method of making liposomes known to one skilled in the art may be used, the most preferred liposomes are made according to the method of Alving et al., *Infect. Immun.* 60:2438-2444, 1992, (Lauer et al., 1992) hereby incorporated by reference. The liposome may have a dual function in that it can be used as a carrier comprising the supramolecular construct as described herein before and, at the same time, function as an adjuvant

to increase or stimulate the immune response within the target animal or human to be treated with the therapeutic vaccine according to the invention. Optionally, the liposome may, in addition, contain a further adjuvant or and immunomodulator or both such as, for example, lipid A, alum, calcium phosphate, interleukin 1, and/or microcapsules of polysaccharides and proteins, but particularly a lipid A, more particularly a detoxified lipid A, such as monophosphoryl or diphosphoryl lipid A, or alum.

[0030] The liposome may be composed of constituents selected from the group consisting of dimyristoyl phosphatidyl choline (DMPC), dimyristoyl phosphatidyl ethanolamine (DMPEA), dimyristoyl phosphatidyl glycerol (DMPG) and cholesterol.

[0031] In one embodiment, the invention contemplates using as a replacement for cationic lipids in the liposomal membrane, anionic lipids selected from the group consisting of:

- a. diacyl-phospholipids with headgroups phosphatidyl glycerol, phosphatidyl serine, phosphatidyl inositol, L- α -phosphatidylinositol-4-phosphate or phosphatidic acid;
- b. lyso-phospholipids with headgroups phosphatidyl glycerol, phosphatidyl serine or phosphatidic acid, and
- c. cardiolipin, dilyso-cardiolipin, monolyso-cardiolipin.

[0032] In one aspect, the invention contemplates using as a replacement for anionic lipids in the liposomal membrane, cationic lipids selected from the group consisting of:

- a. diacyl-phospholipids with headgroups 3-trimethylammonium-propane ,3-dimethylammonium-propane, 3-ethylphosphocholine or 3-phosphatidyl-ethanolamine;
- b. D-erythro-sphingosine, dimethyldioctadecylammonium bromide, N-[1-(2, 3-dimyristyloxy)propyl]-N,N-dimethyl-N-(2-hydroxyethyl) ammonium bromide, N,N,N-trimethyl-2-bis[(1-oxo-9-octadecenyl)oxy]-(Z,Z)-1-propanaminium methyl sulfate or 3 β -[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol hydrochloride.

[0033] In one embodiment of the invention, the lipid chains attached to the above headgroups can

- a. be saturated or unsaturated,
- b. vary in length from $(CH_2)_n$ wherein n is between 3 and 24, and be symmetrically or asymmetrically substituted.

[0034] In one embodiment, the antigenic composition of the invention and as described herein, comprises a liposome preparation and an adjuvant, particularly lipid A, alum, calcium phosphate, interleukin 1, and/or microcapsules of polysaccharides and proteins, but particularly a lipid A, more particularly a detoxified lipid A, such as monophosphoryl or diphosphoryl lipid A, or alum.

[0035] The liposomal composition of the invention comprising an A β peptide coupled with lipophilic moieties embedded into liposomes as described herein may be prepared according to the methodology disclosed herein.

[0036] When liposomes are used as a carrier/adjuvant, the antigenic peptide as provided herein may further be modified by coupling to a lipophilic or hydrophobic moiety that facilitates insertion into the lipid bilayer of the liposome carrier/adjuvant. Said hydrophobic moiety may be a fatty acid, a triglyceride, a diglyceride, a steroid, a sphingolipid, a glycolipid or a phospholipid.

[0037] In a specific embodiment, the the lipophilic or hydrophobic moiety is an alkyl group or a fatty acid with a carbon backbone of at least 1 carbon atom, particularly of at least 2 carbon atoms, particularly of at least 3 carbon atoms, particularly of at least 4 carbon atoms, particularly of at least 6 carbon atoms, particularly of at least 8 carbon atoms, particularly of at least 12 carbon atoms, particularly of at least 16 carbon atoms.

[0038] The lipophilic or hydrophobic moieties may be fatty acids, triglycerides and phospholipids, wherein the fatty acid carbon back bone has at least 4 carbon atoms particularly lipophilic moieties having fatty acids with a carbon backbone of at least approximately 14 carbon atoms and up to approximately 24 carbon atoms, more particularly hydrophobic moieties having a carbon backbone of at least 14 carbon atoms. Examples of hydrophobic moieties include, but are not limited to, palmitic

acid, stearic acid, myristic acid, lauric acid, oleic acid, linoleic acid, linolenic acid and cholesterol or DSPE. In a specific embodiment of the invention the hydrophobic moiety is palmitic acid.

[0039] In a specific embodiment of the invention, the antigenic composition of the invention and as described herein comprises a peptide antigen comprising two palmitic acid moieties, particularly four palmitic acid moieties.

[0040] Palmitoylation, while providing an anchor for the peptide in the liposome bilayer, due to the relative reduced length of the C_{16:0} fatty acid moiety leads to the peptide being presented exposed on or in close proximity to the liposome surface. Therefore, the cells processing the antigen will have to take up the entire liposome with the peptide.

[0041] The antigenic constructs of the present invention may, in one embodiment, comprise peptides modified via pegylation (using polyethylene glycol or modified polyethylene glycol), or modified via other methods such by palmitic acid as described herein before, poly-amino acids (eg poly-glycine, poly-histidine), poly-saccharides (eg polygalacturonic acid, polylactic acid, polyglycolide, chitin, chitosan), synthetic polymers (polyamides, polyurethanes, polyesters) or co-polymers (eg. poly(methacrylic acid) and N-(2-hydroxy) propyl methacrylamide) and the like.

[0042] If PEG is used in the preparation of the antigenic construct, the free PEG terminus may be covalently attached to a molecule of phosphatidylethanolamine (where the fatty acid can be: myristic, palmitic, stearic, oleic etc. or combination thereof). This supramolecular structure may be reconstituted in liposomes consisting of phospholipids and cholesterol (phosphatidylethanol amine, phosphatidyl glycerol, cholesterol in varied molar ratios). Other phospholipids can be used. Lipid A may be used at a concentration of approximately 40 µg/pmole of phospholipids.

[0043] In certain embodiments, the antigenic constructs of the present invention comprise an antigenic peptide sequence as described herein before, covalently attached to pegylated lysine at least one at each terminus, but particularly 1 or 2 at each terminus. The length of the PEG (polyethylenglycol) chain may vary from n = 8 to n = 150.000 or more, particularly from n = 10 to n = 80.000, more particularly from n = 10 to n = 10.000. In a specific embodiment of the invention the length of the

PEG chain is not more than $n = 45$, particularly between $n = 5$ and $n = 40$, more particularly between $n = 10$ and $n = 30$, and even more particularly $n = 10$.

[0044] In certain embodiment, the present invention contemplates post-insertion of different peptide (e.g. antigen) types and/or adjuvant types to the external layer of preformed liposomes in different concentrations as described in EP application no 10 18 8832, the disclosure of which is incorporated herein in its entirety. This post-insertion method comprises pre-forming of liposomes in solution and modification of antigenic peptides through hydrophobic moieties such that the modified antigenic peptide is available in a micellar form. The method further comprises releasing of the antigenic peptides from the micelles by inducing micellar breakdown followed by integration into the pre-formed liposome. This integration process is driven by hydrophobic interactions of the modified antigen and/or the adjuvant with the (phospho)lipid bilayer of the liposomes. In particular, the solubilizing of the modified antigenic peptide and/or adjuvant into the external layer of liposomes is accomplished without the aid of any chemical reaction or additional molecule modification, by diluting the solubilized antigenic peptide or adjuvant (initially presented in micellar form), below the critical micellar concentration of the surfactant. The free form of the antigenic peptide or adjuvant is then integrated in the external layer of the liposomes due to the solubilization of their hydrophobic domains in the acyl moiety of the phospholipids. Thus, the method provides for a stock of “empty liposomes” being disposable for loading according to the respective needs.

[0045] In particular, this post-insertion method for preparing a liposome-based antigenic construct comprising an antigenic peptide of interest modified through hydrophobic moieties reconstituted in a liposome, comprises the steps of i) preparing liposomes in solution; ii) preparing a modified antigenic peptide by adding to the N- and/or C-terminus of the peptide molecule at least one hydrophobic moiety; iii) solubilizing the modified antigenic peptide in the presence of a surfactant; iv) diluting the solubilized peptide and, optionally, an adjuvant below the critical micellar concentration (CMC) of the surfactant; and v) loading the preformed liposomes with the diluted, solubilized antigenic peptide and, optionally, the adjuvant, by adding said antigenic peptide and, optionally, said adjuvant to the preformed liposomal preparation and solubilizing the added peptide and, optionally, the added adjuvant

into the external layer of the liposomes, particularly without the aid of any chemical reaction or additional molecule modification.

[0046] Advantageously, this method leads to high yields of peptide and/or adjuvant incorporation with a unique molecular display on the liposome facing the external layer of the liposome bilayer. In particular, at least 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% and up to 100% of the reconstituted antigenic peptide is present on the surface of the liposome, inserted into the lipid bilayer through its hydrophobic moieties.

[0047] The method further results in liposome preparations which show a homogenous size distribution with a polydispersity index in the range of between 0.4 and 0.6, particularly of 0.45 to 0.55, particularly of 0.5. Further, the method and constructs allow for a high bioavailability of peptide and/or adjuvant for the immune system and, as a consequence, an improved immune response. Adjuvant degradation, e.g. MPLA degradation, is minimized or not present at all and, thus, an increased batch reproducibility is provided. The constructs prepared by the above method are stable, capable of sterile filtration and do not induce side immune responses.

[0048] In certain embodiments, the antigenic peptide is presented on the surface of the carrier molecule in a highly repetitive array, particularly a repetitive array comprising at least 10 repetitive antigenic units/carrier molecule, particularly at least 50 repetitive antigenic units/carrier molecule, particularly at least 100 repetitive antigenic units/carrier molecule, particularly at least 200 repetitive antigenic units/carrier molecule, particularly at least 300 repetitive antigenic units/carrier molecule; particularly at least 400 repetitive antigenic units/carrier molecule, particularly at least 500 repetitive antigenic units/carrier molecule.

[0049] In certain embodiments of the invention, an antibody may be used in the methods according to the invention and as described herein for the treatment, alleviation and/or prevention of memory and/or cognitive impairments or abnormalities, particularly of AD-like memory and/or cognitive impairments or abnormalities, in young to middle-aged subjects with Down's syndrome, which antibody has been generated in response to any one of the antigenic constructs

disclosed herein. This antibody may be a polyclonal antibody, a monoclonal antibody, a humanized antibody, a fully human antibody, a diabody, a camelid antibody or a functional fragment of any of the foregoing antibodies, which has substantially the same

[0050] The terms "antibody" or "antibodies" as used herein is an art recognized term and is understood to refer to molecules or active fragments of molecules that bind to known antigens, particularly to immunoglobulin molecules and to immunologically active portions of immunoglobulin molecules, i.e molecules that contain a binding site that immunospecifically binds an antigen. The immunoglobulin according to the invention can be of any type (IgG, IgM, IgD, IgE, IgA and IgY) or class (IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2) or subclasses of immunoglobulin molecule.

[0051] "Antibodies" are intended within the scope of the present invention to include monoclonal antibodies, polyclonal, chimeric, single chain, bispecific, simianized, human and humanized antibodies as well as active fragments thereof. Examples of active fragments of molecules that bind to antigens include Fab and F(ab')₂ fragments, including the products of an Fab immunoglobulin expression library and epitope-binding fragments of any of the antibodies and fragments mentioned above.

[0052] These active fragments can be derived from an antibody of the present invention by a number of techniques. For example, purified monoclonal antibodies can be cleaved with an enzyme, such as pepsin, and subjected to HPLC gel filtration. The appropriate fraction containing Fab fragments can then be collected and concentrated by membrane filtration and the like. For further description of general techniques for the isolation of active fragments of antibodies, see for example, Khaw, B. A. et al. J. Nucl. Med. 23:1011-1019 (1982); Rousseaux et al. Methods Enzymology, 121:663-69, Academic Press, 1986.

[0053] A "humanized antibody" refers to a type of engineered antibody having its CDRs derived from a non-human donor immunoglobulin, the remaining immunoglobulin-derived parts of the molecule being derived from one (or more) human immunoglobulin(s). In addition, framework support residues may be altered to preserve binding affinity. Methods to obtain "humanized antibodies" are well known

to those skilled in the art. (see, e.g., Queen et al., Proc. Natl Acad Sci USA, 86:10029-10032 (1989), Hodgson et al., Bio/Technology, 9:421 (1991)).

[0054] A "humanized antibody" may also be obtained by a novel genetic engineering approach that enables production of affinity-matured humanlike polyclonal antibodies in large animals such as, for example, rabbits.

[0055] The term "monoclonal antibody" is also well recognized in the art and refers to an antibody that is mass produced in the laboratory from a single clone and that recognizes only one antigen. Monoclonal antibodies are typically made by fusing a normally short-lived, antibody-producing B cell to a fast-growing cell, such as a cancer cell (sometimes referred to as an "immortal" cell). The resulting hybrid cell, or hybridoma, multiplies rapidly, creating a clone that produces large quantities of the antibody.

[0056] The immunogenic composition, which, in one embodiment, may be a therapeutic vaccine, comprises the antigenic construct according to the invention and as described herein before in a therapeutically or prophylactically effective amount and may be prepared as a liquid solution, or as an injectable suspension, or else in a solid form suitable for solubilization prior to injection in the context of, for example, a kit for making use of the present composition, as described below.

[0057] A "therapeutically or prophylactically effective amount" refers to the amount of antibody, peptide, compound or pharmaceutical composition which, when administered to a human or animal, leads to a therapeutic or prophylactic effect in said human or animal. The effective amount is readily determined by one of skill in the art following routine procedures.

[0058] The immunogenic composition of the present invention may be administered to a human or animal, particularly to a human or animal with Down's syndrome suffering from AD-like cognitive impairments or abnormalities, to induce an immune response in said human or animal to alleviate said AD-like symptoms associated with the disease or to restore a condition found in healthy individuals which are unaffected by the disease.

[0059] Since virtually all Down's Syndrome people will eventually suffer from AD-like cognitive impairments at some point in life, it will also be beneficial to administer

the said vaccine to Down's Syndrome people before the manifestation of AD-like impairments. The said vaccine would then act as a preventive treatment.

[0060] The immunogenic composition of the present invention may be administered to a human or animal by any appropriate standard routes of administration. In general, the composition may be administered by topical, oral, rectal, nasal or parenteral (for example, intravenous, subcutaneous, or intramuscular) routes. In addition, the composition may be incorporated into sustained release matrices such as biodegradable polymers, the polymers being implanted in the vicinity of where delivery is desired, for example, at the site of a tumor. The method includes administration of a single dose, administration of repeated doses at predetermined time intervals, and sustained administration for a predetermined period of time.

[0061] In a specific embodiment of the invention the antigenic construct according to the invention, particularly an immunogenic composition comprising said antigenic construct in a therapeutically effective amount, is administered in repeated doses, in particular in 1 to 15 doses, more particularly in 2 to 10 doses, more particularly in 3 to 7 doses and even more particularly in 4 to 6 doses, in time intervals of between 1 and 10 weeks, particularly in time intervals of between 1 and 6 weeks, more particularly in time intervals of between 1 and 4 weeks, and even more particularly in time intervals of between 2 and 3 weeks. The immune response is monitored by taking Sera samples at a suitable time after boosting, particularly 3 to 10 days after boosting, more particularly 4 to 8 days after boosting and more particularly 5 to 6 days after boosting and determining the immunogenicity of the antigenic construct using known methodology, particularly one of the commonly used immunoassays such as, for example, an ELISA assay.

[0062] In particular, the antigenic peptide composition according to the invention is administered by parenteral, particularly by intra-peritoneal, intravenous, subcutaneous and intra-muscular injection.

[0063] The dosage of the composition will depend on the condition being treated, the particular composition used, and other clinical factors such as weight, size and condition of the patient, body surface area, the particular compound or composition

to be administered, other drugs being administered concurrently, and the route of administration.

[0064] The immunogenic composition according to the invention may be administered in combination with other biologically active substances and procedures for the treatment of symptoms associated with Down's syndrome. The other biologically active substances may be part of the same composition already comprising the immunogenic composition according to the invention, in form of a mixture, wherein the immunogenic composition and the other biologically active substance are intermixed in or with the same pharmaceutically acceptable solvent and/or carrier or may be provided separately as part of a separate compositions, which may be offered separately or together in form of a kit of parts.

[0065] The immunogenic composition according to the invention may be administered concomitantly with the other biologically active substance or substances, intermittently or sequentially. For example, the immunogenic composition according to the invention may be administered simultaneously with a first additional biologically active substance or sequentially after or before administration of said composition. If an application scheme is chosen where more than one additional biologically active substance are administered together with the at least one immunogenic composition according to the invention, the compounds or substances may partially be administered simultaneously, partially sequentially in various combinations.

[0066] It is thus another object of the present invention to provide for mixtures of an immunogenic composition according to the invention and, optionally, one or more further biologically active substances in a therapeutically or prophylactically effective amount, as well as to methods of using such a composition according to the invention, or mixtures thereof for the prevention and/or therapeutic treatment and/or alleviation of the effects of amyloid related pathology in young to middle aged subjects with Down's syndrome, particularly for amelioration or restoration of memory impairments or abnormalities, particularly for amelioration or restoration of the recognition memory and/or the contextual associative memory.

[0067] The mixtures according to the invention may comprise, in addition to an immunogenic composition according to the invention, a biologically active substance

such as, for example, known compounds used in the medication of AD-like symptoms in young to middle aged subjects with Down's syndrome.

[0068] The other biologically active substance or compound may exert its biological effect by the same or a similar mechanism as the immunogenic composition according to the invention or by an unrelated mechanism of action or by a multiplicity of related and/or unrelated mechanisms of action.

[0069] Generally, the other biologically active compound may include antibodies raised against and binding to an antigenic peptide as disclosed herein or compounds used in the medication of neurological disorders such as. neutron-transmission enhancers, psychotherapeutic drugs, acetylcholine esterase inhibitors, calcium-channel blockers, biogenic amines, benzodiazepine tranquillizers, acetylcholine synthesis, storage or release enhancers, acetylcholine postsynaptic receptor agonists, monoamine oxidase-A or -B inhibitors, N-methyl-D-aspartate glutamate receptor antagonists, non-steroidal anti-inflammatory drugs, antioxidants, and serotonergic receptor antagonists.

[0070] In particular, the mixture according to the invention may comprise at least one other biologically active compound selected from the group consisting of compounds against oxidative stress, anti-apoptotic compounds, metal chelators, inhibitors of DNA repair such as pirenzepin and metabolites, 3-amino-1-propanesulfonic acid (3APS), 1,3-propanedisulfonate (1,3PDS), secretase activators, β - and γ -secretase inhibitors, β - and γ -secretase modulators, tau proteins, neurotransmitter, β -sheet breakers, anti-inflammatory molecules, or cholinesterase inhibitors (ChEIs) such as tacrine, rivastigmine, donepezil, and/or galantamine and other drugs and nutritive supplements, together with an therapeutic vaccine according to the invention and, optionally, a pharmaceutically acceptable carrier and/or a diluent and/or an excipient.

[0071] The mixtures according to the invention may further comprise niacin or memantine together with an immunogenic composition according to the invention and, optionally, a pharmaceutically acceptable carrier and/or a diluent and/or an excipient.

[0072] In one embodiment of the invention mixtures are provided that comprise “atypical antipsychotics” such as, for example clozapine, ziprasidone, risperidone, aripiprazole or olanzapine for the treatment of positive and negative psychotic symptoms including hallucinations, delusions, thought disorders (manifested by marked incoherence, derailment, tangentiality), and bizarre or disorganized behavior, as well as anhedonia, flattened affect, apathy, and social withdrawal, together with an immunogenic composition and/or a therapeutic vaccine according to the invention and, optionally, a pharmaceutically acceptable carrier and/or a diluent and/or an excipient.

[0073] Other compounds that can be suitably used in mixtures in combination with the immunogenic composition according to the invention are described, for example, in WO 2004/058258 (see especially pages 16 and 17) including therapeutic drug targets (page 36-39), alkanesulfonic acids and alkanolsulfuric acid (pages 39-51), cholinesterase inhibitors (pages 51-56), NMDA receptor antagonists (pages 56-58), estrogens (pages 58-59), non-steroidal anti-inflammatory drugs (pages 60-61), antioxidants (pages 61-62), peroxisome proliferators-activated receptors (PPAR) agonists (pages 63-67), cholesterol-lowering agents (pages 68-75); amyloid inhibitors (pages 75-77), amyloid formation inhibitors (pages 77-78), metal chelators (pages 78-79), anti-psychotics and anti-depressants (pages 80-82), nutritional supplements (pages 83-89) and compounds increasing the availability of biologically active substances in the brain (see pages 89-93) and prodrugs (pages 93 and 94), which document is incorporated herein by reference, but especially the compounds mentioned on the pages indicated above.

[0074] In one embodiment of the invention an antigenic construct is provided which comprises an A β peptide that does not contain a T-cell epitope and thus is free of potential side effects such as neurological complications caused by an over-activated complement system. This can be achieved within the scope of the present invention by administering an A β peptide antigen, particularly a palmitoylated A β peptide antigen, more particularly the palmitoylated A β_{1-15} peptide antigen, but especially the palmitoylated A β_{1-15} peptide antigen, A β_{1-15} in combination with a complement inhibitor.

[0075] The complement inhibitor may be a compound selected from the group consisting of soluble human complement Receptor 1, anti- human complement protein C5 such as, for example, a humanized anti C5 monoclonal antibody or a single-chain fragment of a humanized monoclonal antibody, C1-esterase inhibitor-N and Natural human C1 Inhibitor.

[0076] The modified amyloid peptide antigen such as, for example, the amyloid beta 1-15 peptide antigen may be synthesized following the method reported in (Nicolau *et al.*, 2002). The approach reported in Nicolau *et al* may be modified by first synthesizing the antigenic peptide which is then further modified by an on-resin grafting of a lipophilic or hydrophobic moiety, to the terminal amino acid residues of the pre-formed peptide. In particular, a protected amino acid, particularly a Fmoc-protected amino acid, is attached to a resin using known coupling chemistry. The protecting group is removed and a second protected amino acid residue coupled. Then, standard automated peptide synthesis using known protection chemistry, particularly Fmoc/tBu chemistry, and standard side-chain protecting groups are then used to synthesis the A β antigenic peptide, particularly the A β ₁₋₁₅ antigenic peptide by coupling on amino acids 1 to 15 of amyloid protein A β ₁₋₄₂ to produce the peptide fragment with a given sequence. In a final step two further protected amino acids are coupled to the growing peptide fragment. The Mtt groups on the side chains of first two and last two amino acids can then be selectively cleaved and coupled to palmitic acid. After washing of the resin, the protecting group is removed and the resin simultaneously cleaved, followed by side-chain deprotections using standard methodology. The final product can then be obtained in high purity and its identity confirmed by methods known in the art such as, for example, electrospray mass spectrometry.

[0077] The modified amyloid A β antigenic peptide, particularly the modified A β ₁₋₁₅ antigenic peptide may be reconstituted in a construct consisting of liposomes, particularly liposomes made of dimyristoyl phosphatidyl choline (DMPC), dimyristoyl phosphatidyl ethanolamine (DMPEA), dimyristoyl phosphatidyl glycerol (DMPG) and cholesterol, optionally containing monophosphoryl lipid A.

[0078] In one embodiment, the invention contemplates using as a replacement for cationic lipids in the liposomal membrane, anionic lipids selected from the group consisting of:

- a. diacyl-phospholipids with headgroups phosphatidyl glycerol, phosphatidyl serine, phosphatidyl inositol, L- α -phosphatidylinositol-4-phosphate or phosphatidic acid;
- b. lyso-phospholipids with headgroups phosphatidyl glycerol, phosphatidyl serine or phosphatidic acid, and
- c. cardiolipin, dilyso-cardiolipin, monolyso-cardiolipin.

[0079] In one aspect, the invention contemplates using as a replacement for anionic lipids in the liposomal membrane, cationic lipids selected from the group consisting of:

- a. diacyl-phospholipids with headgroups 3-trimethylammonium-propane ,3-dimethylammonium-propane, 3-ethylphosphocholine or 3-phosphatidyl-ethanolamine;
- b. D-erythro-sphingosine, dimethyldioctadecylammonium bromide, N-[1-(2, 3-dimyristyloxy)propyl]-N,N-dimethyl-N-(2-hydroxyethyl) ammonium bromide, N,N,N-trimethyl-2-bis[(1-oxo-9-octadecenyl)oxy]-(Z,Z)-1-propanaminium methyl sulfate or 3β -[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol hydrochloride.

[0080] In one embodiment of the invention, the lipid chains attached to the above headgroups can

- a. be saturated or unsaturated,
- b. vary in length from $(CH_2)_n$ wherein n is between 3 and 24, and be symmetrically or asymmetrically substituted.

[0081] In a specific embodiment of the invention liposomes with lipid A are used as adjuvant to prepare the anti-amyloid vaccine. Dimyristoylphosphatidyl-choline, -glycerol and cholesterol are mixed, particularly in a molar ratio of 0.9:1.0:0.7. A strong immunomodulator such as, for example, monophosphoryl lipid A is then added

at a suitable concentration, particularly at a concentration of between 30 and 50 mg per mmol, more particularly at 40 mg per mmol of phospholipids. The modified antigenic A β peptide is then added at a molar ratio peptide to phospholipids of between 1:30 and 1:200, particularly at a molar ratio of between 1:50 and 1:120, more particularly of 1:100. Solvents are removed, for example through evaporation, and the resulting film hydrated with sterile buffer solution such as, for example PBS.

[0082] Liposomes may also be prepared by the crossflow injection technique as described, for example, in (Wagner *et al.*, 2002). During the injection of lipid solutions into an aqueous buffer system, lipids tend to form “precipitates”, followed by self arrangement in vesicles. The obtained vesicle size depends on factors such as lipid concentration, stirring rate, injection rate, and the choice of lipids. The preparation system may consist of a crossflow injection module, vessels for the polar phase (e.g. a PBS buffer solution), an ethanol/lipid solution vessel and a pressure device, but particularly a nitrogen pressure device. While the aqueous or polar solution is pumped through the crossflow injection module the ethanol/lipid solution is injected into the polar phase with varying pressures applied.

[0083] In a specific embodiment, the liposomal composition for use in the method according to the invention is prepared to comprise a palmitoylated A β 1-15, particularly a tetrapalmitoylated A β 1-15 together with monophosphoryl lipid A (MPLA) as an adjuvant.

[0084] Four to ten, particularly five to six doses of the liposomal composition may be administered subcutaneously and weekly or bi-weekly to a subject to be vaccinated. Plasma probes may be obtained and analyzed for IgG titers periodically.

[0085] The effectiveness of the liposomal composition of the invention as an immunogen in young to middle aged subjects with Down’s syndrome and the therapeutic potential for treating memory deficits in said subjects could be demonstrated in a relevant animal model for Down’s syndrome. In particular, Ts65Dn mice were used that are widely accepted as an animal model for DS. Ts65Dn mice exert a triplicate of the murine chromosome 16, which hosts the murine APP gene (Davisson *et al.*, 1993; Netzer *et al.*, 2010). These transgenic mice show 1.5 fold increased murine A β (Hunter *et al.*, 2004) and demonstrate behavioral deficits in several memory tasks (Belichenko *et al.*, 2009).

[0086] It could be demonstrated within the present invention that the liposomal composition of the invention induced elevated titers of antibodies and that said antibodies remain elevated even 40 days after the last immunization. Thus, the liposomal composition according to the present invention and as described herein is capable of inducing a robust immune response and of breaking A β self-tolerance in treated subjects with Down's syndrome.

[0087] The liposomal composition of the invention and as described herein was further shown to be capable to induce in young to middle aged mice with Down's syndrome, IgG titers as high as in subjects of a control group without Down's syndrome. The liposomal composition of the invention induces increased titers of antibodies of the IgG2a isotype as compared to the control group, whereas antibody titers of the IgG1 and IgG2b isotype are comparable in the treatment and the control group. The titers of IgM class antibodies are lower in the treatment group as compared to the control group. This slightly lower IgM level may be caused by the altered immune system of mice with Down's syndrome. The liposomal composition is thus capable of overcoming the impaired adaptive immune response to A β described in DS people (Monsonogo *et al.*, 2001).

[0088] The liposomal composition of the invention and as described herein is also safe and does not induce unwanted side effects. In particular, treatment with the liposomal composition does not lead to cell activation the brain, such as activation of astrocytes nor microglia.

[0089] Patients, who suffer from Down's syndrome exhibit cognitive abnormalities such as memory impairment and abnormal activities.

[0090] The liposomal composition of the invention and as described herein was shown to result in a higher discrimination ratio in the animal model indicating that treatment led to a significant improvement of memory in the treated animals.

[0091] The liposomal composition of the invention and as described herein was further shown in a test consisting of training, cued and contextual sessions to result in an enhanced freezing in the animal model reaching levels comparable to that observed in the control animals. This suggests that the immunization was efficient and enhanced the memory capacity of the model animals.

[0092] The liposomal composition of the invention and as described herein is thus capable of rescuing the memory deficits in young to middle aged subjects suffering from Down's syndrome, in particular in young to middle aged subjects with Down's syndrome, who have not yet developed A β -associated plaques in the brain.

[0093] The liposomal composition of the invention and as described herein is further capable of rescuing the memory deficits in young to middle aged subjects suffering from Down's syndrome, in particular in young to middle aged subjects with Down's syndrome, who have already developed A β -associated plaques in the brain.

[0094] In one embodiment, treatment with the liposomal composition can ameliorate or restore the memory deficit in a young to middle aged subject with Down's syndrome, particularly deficits in the recognition memory and/or the contextual associative memory.

BRIEF DESCRIPTION OF DRAWINGS

[0095] Figure 1 shows a schematic presentation of the ACI-DS-01 vaccine, which is a liposome-based vaccine with tetra-palmitoylated mouse A β 1-15 antigen and MPLA as adjuvant. The three amino acid difference between human and mouse A β 1-15 are underlined.

[0096] Figure 2 shows anti-mouse A β antibody levels in Ts65Dn mice immunized with the vaccine ACI-DS-01. A and B) Anti-mouse-A β 40 or 42 IgG titers were detected in the plasma of mice immunized with ACI-DS-01. The induced titers were observed following the second immunization and remained high even 40 days after the 6th injections in comparison to mice immunized with empty-liposome. There was no difference between the measured titers of 2N and Ts65Dn mice. C to F) IgG isotypes were detected following the 4th immunization. G) IgM titers were barely lower in the Ts65Dn mice. H) the same level of anti-MPLA IgG titers were detected in all mice immunized with ACI-DS-01. Graphs represent the mean $\pm$ SD. (n=20 2N-ACI-DS-01, n=15 Ts65Dn-ACI-DS-01, n= 18 2N-Empty and n= 11 Ts65Dn-Empty. Graphs represent the mean $\pm$ SD).

[0097] Figure 3 shows efficacy of immunization on memory performance. A) The difference in the spontaneous locomotor activity between 2N and Ts65Dn mice remained similar following the immunization. B) Mice immunized with ACI-DS-01

showed a significant enhanced recognition index (RI) in the novel object recognition in comparison to the group treated with Empty vaccine. C) In the fear conditioning, immunized mice showed border line significance for a higher level of freezing during the contextual session. Graphs represent the mean $\pm$ SD. (n= 20 2N-ACI-DS-01, n= 13 Ts65Dn ACI-DS-01, n= 18 2N-Empty and n= 11 Ts65Dn-Empty)

[0098] *Figure 4* shows level of A β 40 in mice treated with ACI-DS-01 vaccine. A) In the cortex, the level of A β 40 were barely higher in the Ts65Dn in comparison to the 2N mice. No difference was observed in the hippocampus or the cerebellum. No significant effect of the ACI-DS-01 treatment was observed. Graphs represent the mean $\pm$ SD. n=10 2N-ACI-DS-01, n=10 Ts65Dn ACI-DS-01, n= 8 2N-Empty and n= 5 Ts65Dn-Empty. B and C) Correlation between the ratio A β -40/42 in the cortex or the cerebellum with the RI. D) The level of anti-A β IgG titers correlate weakly with A β 40 level in the plasma of Ts65Dn mice treated with the ACI-DS-01. E) Significant correlation between the level of anti-A β IgG and the RI. (n=19 2N ACI-DS-01, n=13 Ts65Dn ACI-DS-01, n= 18 2N-Empty and n= 11 Ts65Dn-Empty).

[0099] *Figure 5* shows studies of inflammatory reaction. A) Body and brain weight. B) Confocal images of GFAP immunoreactivity (left) and CD45 (right) in treated 2N and Ts65Dn mice. Arrows point individual CD45-positive microglial cells. The optical density of GFAP immunoreactivity was quantified and revealed no difference between groups. n=4 from each group.

EXAMPLES

Example 1 General Methodology

1.1 Animals:

[0100] Ts65Dn mice known as a DS mouse model (Davisson et al., 1993) were used (n =30) and the age matched control 2N (n = 40). At starting date, the used mice were 5 months old. At the end of the study (immunization and behavioral testing), mice were 9 months old. Therefore, all the samples collected at sacrifice are from 9 months old mice.

[0101] Ts65Dn newborn have elevated lethality (around 6%) mainly due to congenital heart malformations (Randall 2006, Moore 2006). However, mice can survive even up to 18 to 24 months. In our study, a death rate of 15% was observed. The survived mice are ~20% smaller in size compared to normal littermates. The most severely affected mice that died at birth, do not survive to be analyzed, resulting in underestimates of the impact of the trisomic genes on some phenotypes (i.e heart defect). However, for our concerns, the surviving mice represent an adequate model of DS for analyzing amyloid and AD-like pathologies.

[0102] In the DS mouse model used for these studies the mice show increased levels of amyloid at the age of 4 months (Hunter 2004). At an age of 9 months, the level of amyloid reaches 3 fold the normal level as is predicted in view of the three copies of the APP gene. Similar to DS people, aging is an important factor for amyloid deposition in Ts65Dn brains. Despite the age-related accumulation of amyloid, the TS65Dn mice do not develop plaques. Nevertheless, they do replicate faithfully the degeneration of neuronal populations that are seen in AD and in people with Down syndrome (Salehi et al., 2009)

[0103] Taken together, the DS-features in the used mouse-model comprise the presence of pathological proteins and phenotypical aspects; i.e amyloid load and cognitive impairment. Therefore, Ts65Dn mice can accurately recapitulate the pathogenic processes of DS. Since these mice start to accumulate amyloid at a comparable level to humans at 6 months of age, the age of the model mice compares to human DS patients at an age range from young to middle age.

1.2 Vaccine preparation - Preparation of a liposome-based antigenic construct

1.2.1 Method 1: The liposome antigenic construct is produced according to the method described in WO2007/068411.

1.2.2 Method 2: The phospholipids dimyristoylphosphatidylcholine (DMPC), dimyristoylphosphatidyl-glycerol (DMPG) and cholesterol (Avanti Polar Lipids, Alabaster, Alabama) were mixed in ethanol (100 ml) at a molar ratio of 9.0; 1.0 and 7.0, respectively. A perfectly clear solution was formed following a continuous agitation at 60°C for 15 min. This lipid mixture was then diluted (17x) by injecting the solution (100ml) in 1600 ml phosphate buffer (PBS) pH 7.4, allowing the formation of multi-layer vesicles (Multilayer Vesicles –stage 1). The resulting prepreparation was then concentrated by ultrafiltration (Vivaflow 200 – 100.000 MWCO Polyethersulphone with a flow of 200 ml/min) and the reaction volume was reduced from 1700ml to 250 ml (Ultrafiltration step). The concentrated solution was further submitted to dialysis in a Vivaflow 200 device where a 10x volume exchange is performed with PBS pH 7.4 (Diafiltration step). The dialysed solution (Multilayer vesicles –stage 2) was then homogenized (7 cycles at 15.000-20.000 Psi) in an Emulsiflex C5 device from Avestin (Homogenization step), followed by 3 cycles of Extrusion (using the same Emulsiflex instrument) through a 0.2 um Polycarbonate membrane with a diameter of 47 mm (Extrusion step). Following those last two sizing steps, unilamellar liposomes with no antigen and adjuvant were formed (Empty liposome preparation).

1.2.2.1 Preparation of Adjuvant Solution:

[0104] A 765 ug/ml solution of MPLA was prepared in 20 ml PBS pH 7.4 with 1.6 % (wt/v) Octyl-Beta-D-Glucopyranoside (B-OG). The resulting solution contained detergent (B-OG) at a concentration above its critical micellar concentration (CMC) of 0.73 % (wt/v). This solution was then heated at 60°C for 30 min and manually injected into the 250 ml preparation of empty liposomes. During this dilution step, the detergent concentration is diluted down 13.5 x, resulting in a final concentration of 0.12 % (wt/v), a concentration of B-OG below its CMC (1st dilution step). The Liposome solution with containing the adjuvant (MPLA) is then diluted (7x) by injecting 1450 ml of PBS pH 7.4 (2nd dilution step).

1.2.2.2 Preparation of Peptide Solution:

[0105] 1.33 mg/ml of the peptide A β -Pal 1-15 was prepared in PBS pH 11.8 (total volume 45 ml) with 2.0 % (wt/v) B-OG. The resulting solution comprised a detergent concentration above its critical micellar concentration (CMC) of 0.73 % (wt/v). This solution was stirred heated at 60°C and stirred for 15 min, until a clear solution was formed. The peptide solution (45ml) was then added to the solution of liposome with adjuvant (1700 ml) and stirred for 1h at 60°C resulting in a solution having a final B-OG concentration of (0.05%), which is far below the detergent's CMC (0.73%) (Dilution step). The resulting solution was then concentrated by ultrafiltration (same condition mentioned above) and the final vaccine volume set to 100 ml. The concentrated solution was dialysed by diafiltration, where a 10x volume exchange was performed with PBS pH 7.4.

[0106] In a final step, the vaccine solution was sterile filtered through a 0.2 μ m Polyethersulfone membrane filter (Sartorius 16541-K). Each filter was used to sterile filter 5 ml of vaccine solution into 15 ml Falcon tubes. This last process step is executed in a sterile environment (laminar flow hood).

1.2.3 Method 3:

[0107] Method 3 only differs from process Method 2 in that the adjuvant (e.g. MPLA) is added together with the lipids in the ethanol solution prior to liposome formation and sizing steps. Therefore, the process of adding adjuvant following liposome formation is suppressed.

1.3 Immunizations.

[0108] All mice received subcutaneous (s.c.) immunizations with 200 μ l of ACI-DS-01 (51.2 μ g per dose of Pal1-15, tetrapalmitoylated A β peptide 1-15) or empty liposome vaccine. A total of six injections were done at day 1, 14, 28, 42, 56, 70 and 110. On the same days, tail bleedings were performed just before the first vaccine injection on day -1 (pre-bleeding), day 56 (14 days after the 4th injection) and at sacrifice, day 110 (40 days after the 6th injection). Mice were sacrificed at the end of the study at the age of 9 months and brains were collected.

1.4 Quantification of mouse A β -specific antibodies.

[0109] A β 1-42-specific IgG responses were determined by ELISA. Briefly, plates were coated with 10 μ g/mL of mouse A β 1-42 (Bachem H5966, lot: 1013710,

1028321 or 1033165) or mouse A β 1-40 (Bachem H5638, lot: 1013645) overnight at 4°C. After washing with PBS-0.05% Tween 20 and blocking with 1% BSA, serial dilutions of plasma were added to the plates and incubated at 37°C for two hours. The antibody anti-A β 4G8 (Covance SIG-39220, lot: 08EC00905, 1 mg/mL diluted 4000) and serum of a C57BL/6JOlaHsd (Harlan) immunized with three injections of the same ACI-DS-01 batch were used as positive control. After washing, plates were incubated with alkaline phosphatase (AP) conjugated anti-mouse IgG antibody (Jackson ImmunoResearch West Grove, PA, USA, Cat. N°115-055-164 Lot 87821, vial diluted at 1/4000) for two hours at 37°C. After final washing, plates were incubated during 2 hours and a half with AP substrate (pNPP) and read at 405 nm using an ELISA plate reader. Results are expressed as optical density (O.D).

1.5 Behavioral testing.

[0110] All mice were exposed to the same series of behavioral tests starting at around 8 months old of age. Each mouse was handled for 10 min, twice a day, during the 7 days that preceded testing and for 3 days in between tests. The tests were performed at the indicated days (according to the last immunization) and in the following order: locomotor activity (starting day 11), novel object recognition task with 24 h delay (starting day 18), T-maze (starting day 25), and contextual fear conditioning test (starting day 34). All behavioral tests took place during the day-light cycle between 7:00 A.M. and 7:00 P.M. and were performed at room temperature (22°C). On the day of testing, mice were kept in their home cages during the day-light phase in the same experimental room for 2 h for habituation. As an indicator of anxiety during each test, the number of fecal pellets and urine drops was also measured. To minimize olfactory cues from the previous trial, each apparatus was thoroughly cleaned with 10% ethanol after each animal occupation. Handling and all behavioral tests were performed in a blinded manner, not revealing the genotype and treatment to the investigator handling the mice.

1.6 Spontaneous locomotor activity test.

[0111] Spontaneous locomotor activity was monitored using Plexiglas activity chambers (model MED-OFA-MS; Med Associates) (27.9 x 27.9 x 20 cm) and activity monitor software (Activity Monitor, version 4.3.6). As described in Belichenko et al., (Belichenko et al., 2009; Belichenko et al., 2007), mice were placed into the center of

the chamber under bright ambient light conditions and activity was monitored for 10 min in three separate trials. Averages were determined for total distance, velocity, total activity time, total activity counts and vertical activity.

1.7 Novel object recognition task.

[0112] We used the Bevins and Besheer protocol (Bevins and Besheer, 2006; O'Doherty *et al.*, 2005): a one-trial non-matching-to-sample learning task to study recognition memory for two sample objects with one environment to study learning and memory with a 24 h delay. Before testing, mice were habituated in a black Plexiglas chamber (31 x 24 x 20 cm) during 10 min for 2 consecutive days under dimmed ambient light conditions. Activity of mice at the age of 8 months during the object recognition task was recorded with a video camera. First, two identical objects were placed in the chamber, as previously described (Bevins and Besheer, 2006; O'Doherty *et al.*, 2005). A mouse was placed at the mid-point of the wall opposite the sample objects. After 10 min exploring the objects, the mouse was returned to the colony for 24 h. To test for object recognition, one familiar object and one novel object were placed in the chamber and the mouse was again placed in the chamber for 3 min to explore the objects. Object recognition was measured in a single trial. Data were determined using direct observations of video recordings. Results were: 1) average total exploration time of the sample objects, both novel and familiar, and 2) discrimination ratio (novel object interaction/total interaction with both objects).

1.8 T-maze testing

[0113] Mice at age 8 months were used. We used a modified Deacon and Rawlins protocol (Deacon and Rawlins, 2006) and a continuous alternation task in a T-maze to evaluate hippocampal function. The maze was made of opaque acrylic glass (Plexiglas) as described (Deacon and Rawlins, 2006) with an additional sliding door at the beginning of the start arm. During the test, a mouse was placed at the beginning of the start arm, with its back to the closed sliding door. After all doors were opened, the mouse ran down the start arm to choose either the right or left goal arm. After all four legs of the mouse had entered one goal arm, the sliding door to another goal arm was closed for 5 s, and then all sliding doors were opened again, allowing the mice to go back to the start arm. T-maze activity was monitored until the

mice finished 10 alternations. This procedure was repeated for 3 consecutive days, for a total of 30 trials. The spontaneous alternation score was defined as the number of left–right and right–left alternations, expressed as a percentage of the total number of possible alternations during the session. Results were averaged for both alternation scores and time spent.

1.9 Contextual fear conditioning test.

[0114] Contextual and cued fear conditioning was conducted for evaluation of fear-dependent learning and retrieval. The test was performed using chambers from Coulbourn Instruments (Whitehall, PA, USA). On the first day animals were placed in a chamber (Context A) for 3 minutes for baseline recording, followed by five tone-shock pairings. The shock (0.5 mA, 2 sec) was delivered following the tone (70 dB, 2 kHz, 20 sec) in each conditional /unconditional stimulus pairing. On the second day, a novel chamber (Context B; new room, new olfactory environment, new texture of floor, blue plastic inserts for walls, extra source of blue light, and visual cues) was used for cued testing. Following a 3-minute pre-tone period, three tones without shocks were presented to animals during a 3-minute testing period. On the last day of the experiment, the mice were placed in Context A for 5 minutes without any conditional or unconditional stimulus (Saxe *et al.*, 2006). Freezing was defined as the complete lack of motion for a minimum of 0.75 seconds as measured by FreezeFrame software (Actimetrics, Evanston, IL). The percentage of freezing in each period was reported.

1.10 Measuring body and brain weights.

[0115] After all behavioral tests, mice were deeply anesthetized with sodium pentobarbital (200 mg/kg i.p.) (Abbott Laboratories), weighed, and transcardially perfused for 1 min with 0.9% sodium chloride (10 ml) and then for 10 min with 4% paraformaldehyde in 0.1 M PBS, pH 7.4 (100 ml). After perfusion, the brain was immediately removed. The weight of the brain (including the olfactory bulbs, cortex, hippocampus, cerebellum, brainstem and the cervical spinal cord through C1–C2) was recorded. The brain was then placed in fixative until further use.

1.11 Immunofluorescence for inflammatory reaction.

[0116] Brain sections were pre-incubated with 5% non-fat milk in 0.1M PBS with 0.3% Triton X-100. Sections were then incubated overnight at 4 °C with rabbit anti-cow glial fibrillary acidic protein (GFAP) antibody (DAKO, Glostrup, Denmark) at a dilution of 1:500, or with polyclonal rat anti-CD45 (Pharmingen) at a dilution of 1:5000. The following incubations (1 h each and at room temperature) were then done; with a biotinylated donkey anti-rabbit secondary antibody (1:200; Jackson ImmunoResearch Labs, West Grove, PA, USA) and with fluorescein isothiocyanate (FITC)-conjugated streptavidin (1:500; Jackson ImmunoResearch Labs). A rinse with PBS (3 times, each 20 min) was done between the incubations described above. Sections were mounted onto microscope glass slides and coverslipped using 90% glycerol in 0.1M phosphate buffer, pH 7.4. To control for specificity of antibody staining, selected sections were submitted to the same protocol but without including the primary antibodies. Immunofluorescence was not observed in control sections. Slices were examined and scanned in a Radiance 2000 (Bio-Rad, Hertfordshire, UK) confocal microscope attached to a Nikon Eclipse E800 fluorescence microscope. The laser was an argon/krypton mixed gas laser with excitation wavelengths for FITC at 488 nm. LaserSharp software (Bio-Rad) was used to establish optimal conditions for collecting images. The optimal conditions for confocal imaging of GFAP- immunoreactivity (IR) or CD45-IR were the following: the lens was a 20x objective (Nikon; Plan Apo 20x/0.75); laser power was 10% or 20%; gain was 34.7; offset was 0.0; the zoom factor was 3; scan speed was 500 lines/s; each optical section was scanned three times and Kalman filtering was then employed to reduce noise; the size of the image was 512x512 pixels and the pixel size was 0.48x0.48 um.

1.12 Statistical analysis

[0117] Data are shown as mean $\pm$ standard deviation (SD) or standard error of mean (SEM). Statistical analysis was done by unpaired T-test, two tailed. A probability of $p < 0.05$ was considered significant.

Example 2 Immunization with ACI-DS-01 produced anti-mouse A β antibodies

[0118] The liposomal vaccine ACI-DS-01 was prepared according to the SupraAntigenTM methodology (WO 2005/081872) with tetrapalmitoylated mouse A β 1-15 peptide (Palm1-15), H-Lys(Pal)-Lys(Pal)-Asp-Ala-Glu-Phe-Gly-His-Asp-Ser-Gly-Phe-Glu-Val-Arg-His-Gln-Lys(Pal)-Lys(Pal)-OH embedded into liposomes along with monophosphoryl lipid A (MPLA), Figure 1. Six doses of ACI-DS-01 were administered subcutaneously and bi-weekly into Ts65Dn male mice and age-matched control mice (2N). The analysis of plasma collected after the 4th dose, showed robust IgG titers against mouse A β 40 or A β 42 in the immunized mice of both Ts65Dn and 2N groups (Figure 2A and 2B). The vaccine-induced antibodies remained elevated even 40 days after the last immunization. No titers were detected in mice immunized with empty liposome without the antigen (Figure 2A and 2B). Thus, ACI-DS-01 was able to break A β self-tolerance in the DS mouse model.

[0119] Subclasses of IgG were analyzed in plasma of mice immunized with ACI-DS-01 following the 4th immunization. The ELISA done with A β 40 showed that the Ts65Dn group had higher IgG2a titers than the 2N group (Figure 2D) while there was no difference between the two groups for IgG1 and IgG2b titers (Figure 2C and 2E) (One-way ANOVA, Tukey posthoc; $P = 0.05$). The IgG3 titers were lower in the Ts65Dn group (Figure 2F) (One-way ANOVA, Tukey posthoc; $P < 0.001$). IgM titers were slightly, but significant, lower in the Ts65Dn mice in comparison to 2N mice (Figure 2G). There (One-way ANOVA, Tukey posthoc; $P = 0.05$). Similar results were obtained in ELISA performed with A β 42 (data not shown). The lower levels of a few of the titers mentioned above in Ts65Dn mice are unlikely to be due to the immune response capacity of the Ts65Dn since the anti-MPLA IgG level was comparable in all mice (Figure 2H).

Example 3 Immunization with ACI-DS-01 restored the memory deficit of Ts65Dn mice

[0120] Ts65Dn mice exhibit many features of DS cognitive abnormalities such as memory impairment and abnormal activities. To investigate the efficacy of the ACI-DS-01 vaccine, a battery of behavioral tests was conducted two weeks after the last immunization. Mice performed the tests in the following order; open field, object recognition, T-maze and fear conditioning.

[0121] The analysis of the open field test showed that Ts65Dn mice have a significant higher spontaneous locomotor activity in comparison to 2N mice (data not shown). Following immunization with ACI-DS-01, Ts65Dn mice continued to be significantly more active than ACI-DS-01 treated 2N mice (Fig.3A, t test unpaired one-tailed; $p = 0.0004$). In the T-maze, 2N and Ts65Dn mice showed no significant difference in the alteration ratio or in the alteration duration, neither before treatment nor post treatment (data not shown).

[0122] The spatial memory capacity was measured in the object recognition (ORT). The Ts65Dn mice exert a weak discrimination ratio and thus recognize poorly the novel object. ACI-DS-01 treated Ts65Dn mice showed a higher discrimination ratio indicating that treatment led to a significant improvement of memory (Fig. 3B, t test unpaired one-tailed; $p = 0.03$. one way ANOVA, genotype, $P = 0.12$, vaccine, $P = 0.002$. Interaction $P = 0.54$). Interestingly, the same results were observed in the 2N group.

[0123] The fear conditioning test consists of training, cued and contextual sessions. The same level of freezing was observed in all groups during the training or the cued session (Figure 3C). During the contextual session, Ts65Dn mice treated with empty vaccine (only the liposome, no antigen) showed a lower percent of freezing than the control 2N mice. In contrast, after immunization Ts65Dn mice demonstrated an enhanced freezing and reached a comparable level to the 2N mice (t-test unpaired one-tailed; $P = 0.04$. (one way ANOVA, genotype, $P = 0.01$, vaccine, $P = 0.16$. Interaction $P = 0.33$). This suggests that the immunization was efficient and enhanced the memory capacity of Ts65Dn mice.

[0124] In summary, these results indicate that ACI-DS-01 treatment can restore the memory deficit of Ts65Dn mice.

Example 4 Mechanism of ACI-DS-01 vaccine for improving the cognitive deficiency

[0125] To address the question about the effect of the ACI-DS-01 induced antibodies on the level of A β , ELISA was performed using protein extracts of the hippocampus, the cortex and the cerebellum. None of these regions showed a difference on A β levels following the treatment with ACI-DS-01; neither for A β 40 nor for A β 42 (Figure 4A). It is worthwhile to note that the ratio A β -40/42 in the cortex and the cerebellum correlated significantly with a higher recognition index (RI) (Figure 4B and 4C. Cortex; Pearson r correlation = -0.3248, P = 0.003. cerebellum; Pearson r correlation = -0.4127, P = 0.009). These results suggest that A β -40/42 might be responsible of the cognitive deficit. Further analysis showed that A β 42 in the plasma was undetectable. In contrast, an increased level of A β 40 in the plasma seemed to be associated with the higher values of anti-A β IgG measured in the group of ACI-DS-01 treated Ts65Dn mice (Figure 4D; Pearson r correlation = 0.51, P = 0.09). Moreover, the RI correlated with anti-A β IgG titers (Figure 4E; Pearson r correlation = 0.3154, P = 0.007).

[0126] These results suggest that ACI-DS-01-induced antibodies may clear A β from the brain to the plasma which then may lead to memory amelioration.

[0127] To further study the effect of ACI-DS-01 vaccination on APP processing, the levels of full-length of APP (FL-APP) and its C-terminal fragment App-CTF (C-83 plus C-99) were analyzed by western blot.

[0128] There is no detectable anti-A β IgG in brain extract.

Example 5 Morphological examination of the brains of Ts65Dn ACI-DS-01-treated mice

[0129] To decipher whether ACI-DS-01 vaccine rescued the memory deficit by restoring morphological abnormalities, immunohistological analysis was conducted. Particularly, the degeneration of neurons in the locus coeruleus and the hippocampus, that has been shown to be associated with contextual learning, was examined. The size and number of cholinergic neuron were identified by immunohistochemical staining for p75NTR, an NT receptor.

[0130] To examine the hippocampal innervations, we measured the intensity of immune-staining for p75NTR and ChAT (the transporter for acetylcholine).

[0131] In addition, the analysis of Rab5b positive endosomes in cholinergic terminals, was visualized by using an antibody against VACht.

Example 6 The safety of the liposomal vaccine ACI-DS-01

[0132] To determine if immunization with ACI-DS-01 may induce safety concerns, body weight, general observation, and inflammation markers in mice were recorded. Before treatment, Ts65Dn mice had a significantly lower body weight than 2N mice (ANOVA, Tukey posthoc, $p = 0.001$). Immunization did not alter significantly the body weight (Figure 5A). Brain weights were similar in all four groups of mice (Figure 5A). The immunohistochemistry results showed no difference in the number of glial fibrillary acidic protein (GFAP)-positive astroglial cells or of the CD45-positive microglial cells. The obtained results were similar in the cortex and hippocampus (Figure 5). These results indicate that the ACI-DS-01 vaccination did not induce an inflammatory response.

[0133] To verify the specificity of ACI-DS-01 induced antibodies, a cross reaction study was conducted. The brain sections of 2N and Ts65Dn were stained with the antiserum of ACI-DS-01-treated 2N mice.

[0134] In summary, these findings indicate that ACI-DS-01 is a safe vaccine.

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CLAIMS

1. An antigenic peptide fragment derived from amyloid protein or amyloid-like protein for use in the treatment, alleviation and/or prevention of memory and/or cognitive impairments or abnormalities in subjects with Down's syndrome.
2. The antigenic peptide of claim 1, wherein said subject is a young to middle-aged subject.
3. The antigenic peptide of claim 1, wherein said subject is below age 65.
4. The antigenic peptide of claim 1, wherein said subject is below age 60.
5. The antigenic peptide of any one of the preceding claims, wherein said impairment or abnormality concerns the recognition memory and/or contextual associative memory.
6. The antigenic peptide of any one of the preceding claims, wherein said treatment leads to
 - a. an amelioration and/or restoration of memory; and/or
 - b. an increase in the retention or a complete restoration of cognitive capacity in the treated subject.
7. The antigenic peptide of any one of the preceding claims, wherein said treatment does not induce unwanted side effects such as meningoencephalitis and microhemorrhage.
8. The antigenic peptide of any of the preceding claims, wherein said subject has not yet developed A β -associated plaques in the brain.
9. The antigenic peptide of any of the preceding claims, wherein said subject has developed A β -associated plaques in the brain.
10. The antigenic peptide of any one of the preceding claims, wherein said amyloid protein or amyloid-like protein is selected from the group consisting of prion protein, tau protein, alpha-synuclein, huntingtin and amyloid beta.

11. The antigenic peptide of any one of the preceding claims, wherein said A β antigenic peptide fragment corresponds to the N-terminal part of the A β peptide, particularly the N-terminal A β 1-15 fragment.
12. The antigenic peptide of any one of the preceding claims, wherein the A β peptide antigen is presented reconstituted in a liposome.
13. The antigenic peptide of any one of the preceding claims, wherein the A β peptide antigen is modified by a lipophilic or hydrophobic moiety that facilitates insertion into the lipid bilayer of the liposome carrier/adjuvant.
14. The antigenic peptide of any one of the preceding claims, wherein the A β peptide antigen is modified by a palmitic acid bound to the N- and/or C-terminus of the peptide molecule.
15. The antigenic peptide of claim 14, wherein the A β peptide antigen is modified by four palmitic acids bound to the N- and/or C-terminus of the peptide molecule.
16. The antigenic peptide of any one of the preceding claims, wherein the antigenic peptide is presented in a repetitive array on the surface of the liposome.
17. The antigenic peptide of claim 16, wherein said highly repetitive array on the surface of the carrier or liposome comprises at least 10 repetitive antigenic units/carrier molecule.
18. The antigenic peptide of any one of the preceding claims, wherein at least 75%, particularly at least 80%, particularly at least 90%, but especially 100% of the reconstituted antigenic peptide is present on the surface of the liposome, inserted into the lipid bilayer through its hydrophobic moieties.
19. A composition comprising the antigenic peptide of any one of claims 1-18 in a therapeutically effective amount together with a pharmaceutically acceptable carrier and/or excipient in the treatment, alleviation and/or prevention of memory and/or cognitive impairments or abnormalities in subjects with Down's syndrome.

20. The composition of claim 19 comprising an adjuvant.
21. A method for treating memory and/or cognitive impairments or abnormalities in a subject with Down's syndrome comprising administering to said subject an antigenic peptide or a composition according to any of the preceding claims.
22. The method of claim 21, wherein said impairment or abnormality concerns the recognition memory and/or contextual associative memory.
23. A method for increasing retention of or completely restoring cognitive capacity in a subject with Down's syndrome comprising administering to said subject an antigenic peptide or a composition according to any of the preceding claims.
24. The method according to any one of the preceding claims, wherein said subject has not yet developed A β -associated plaques in the brain.
25. The method according to any one of the preceding claims, wherein said subject has developed A β -associated plaques in the brain.

Figure 1

Human A β : Asp- Ala- Glu- Phe- Arg- His- Asp- Ser- Gly- Tyr- Glu- Val- His- His- Gln
Mouse A β : Asp- Ala- Glu- Phe- Gly- His- Asp- Ser- Gly- Phe- Glu- Val- Arg- His- Gln

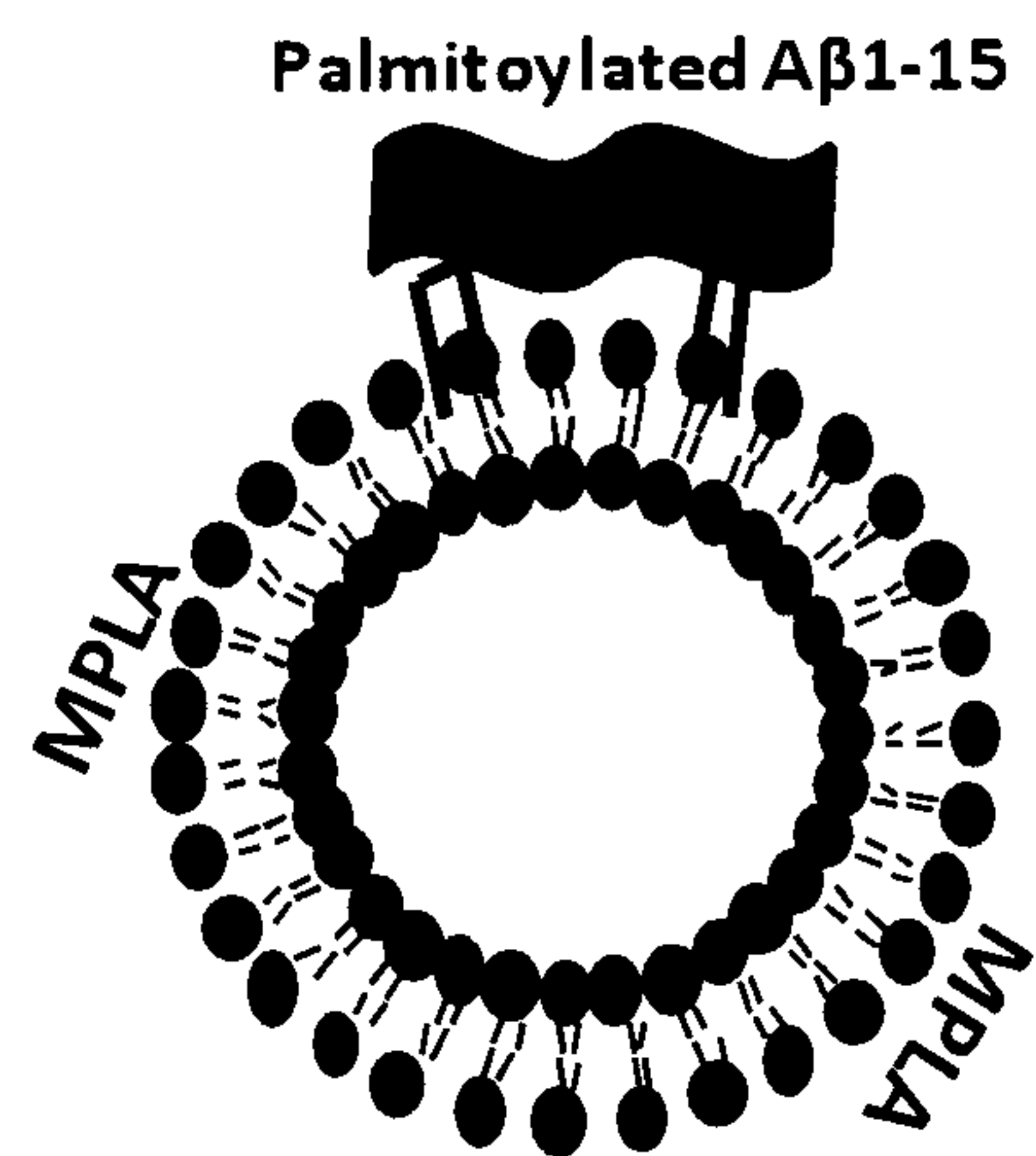


Figure 2

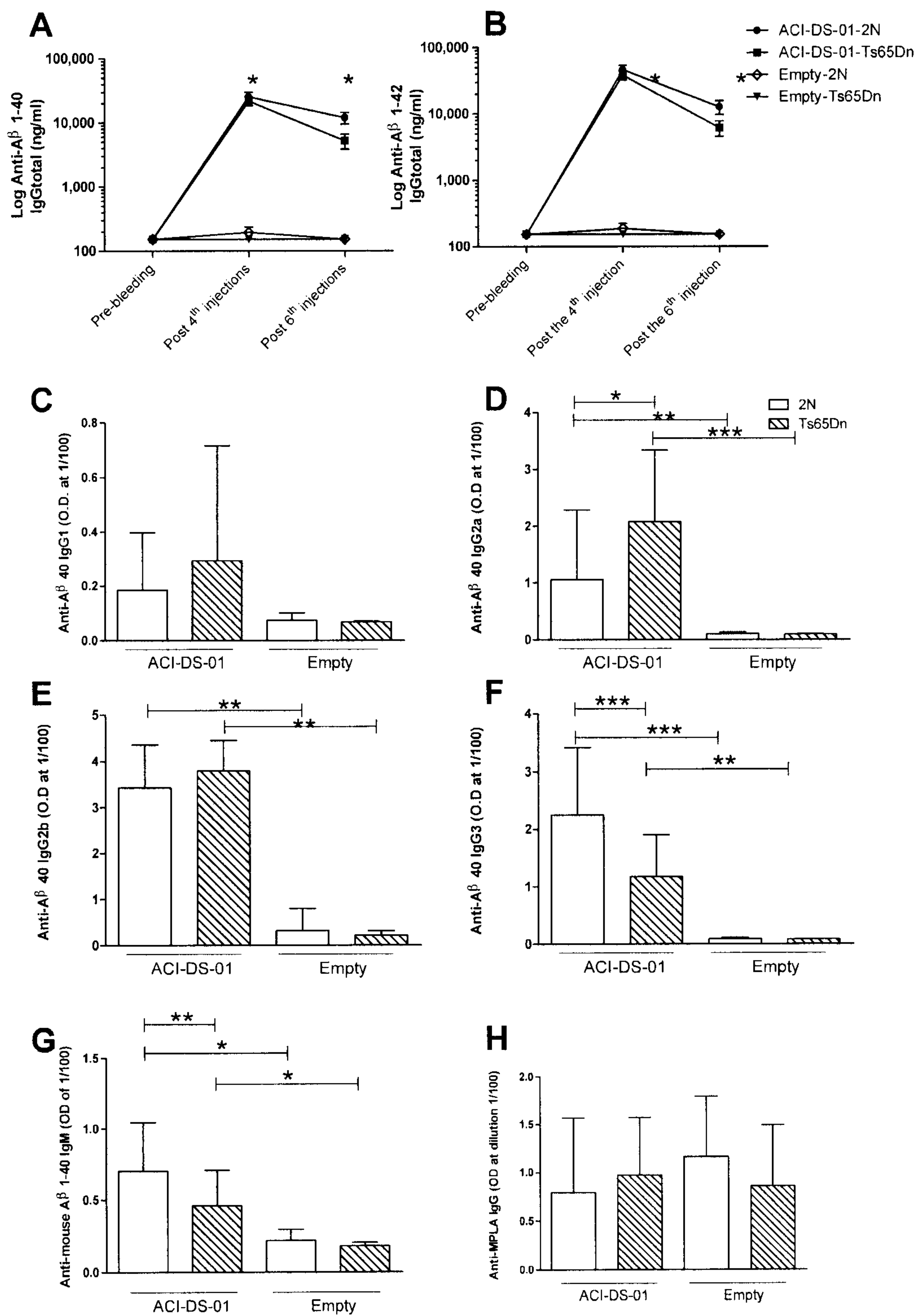


Figure 3

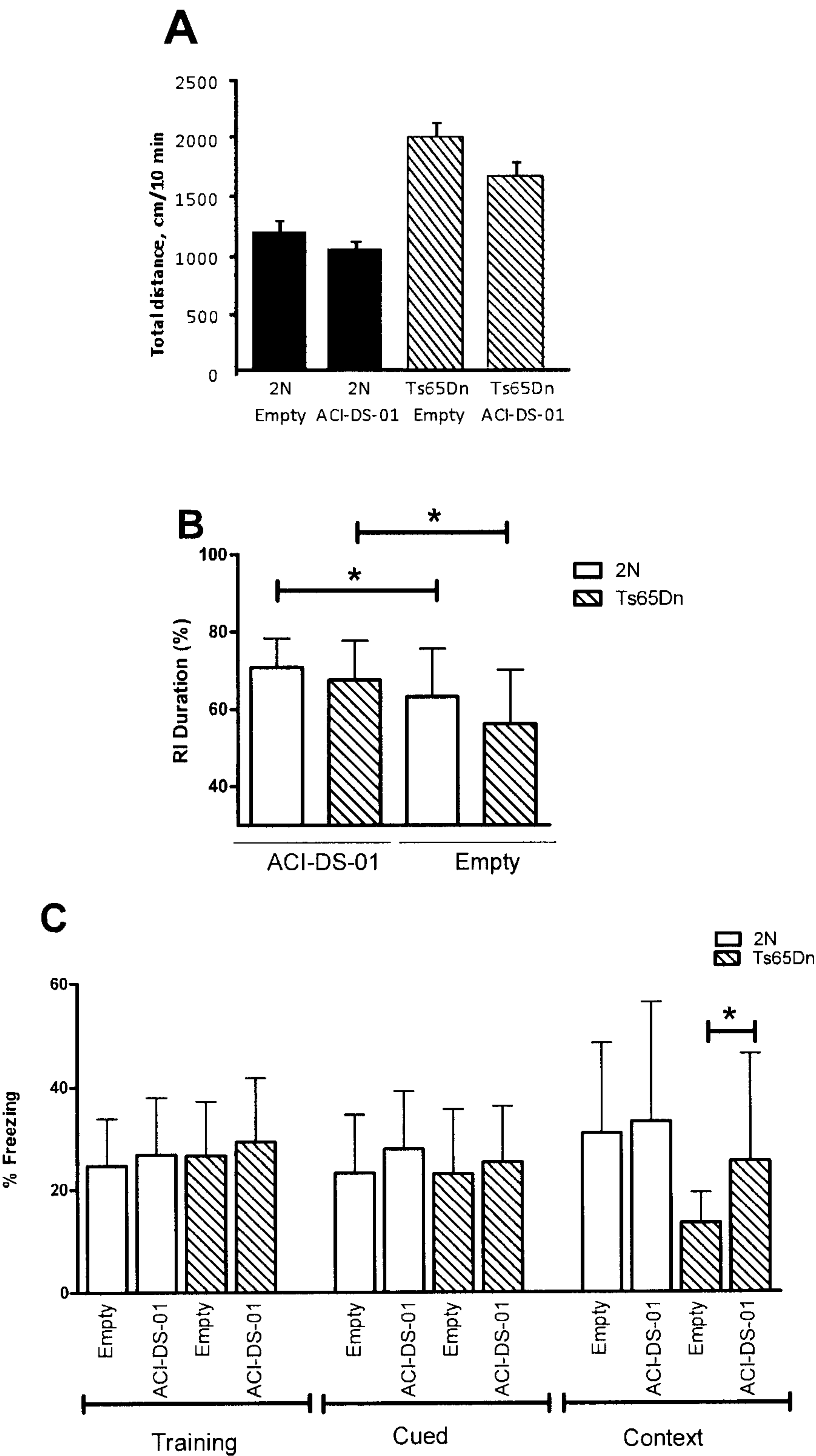


Figure 4

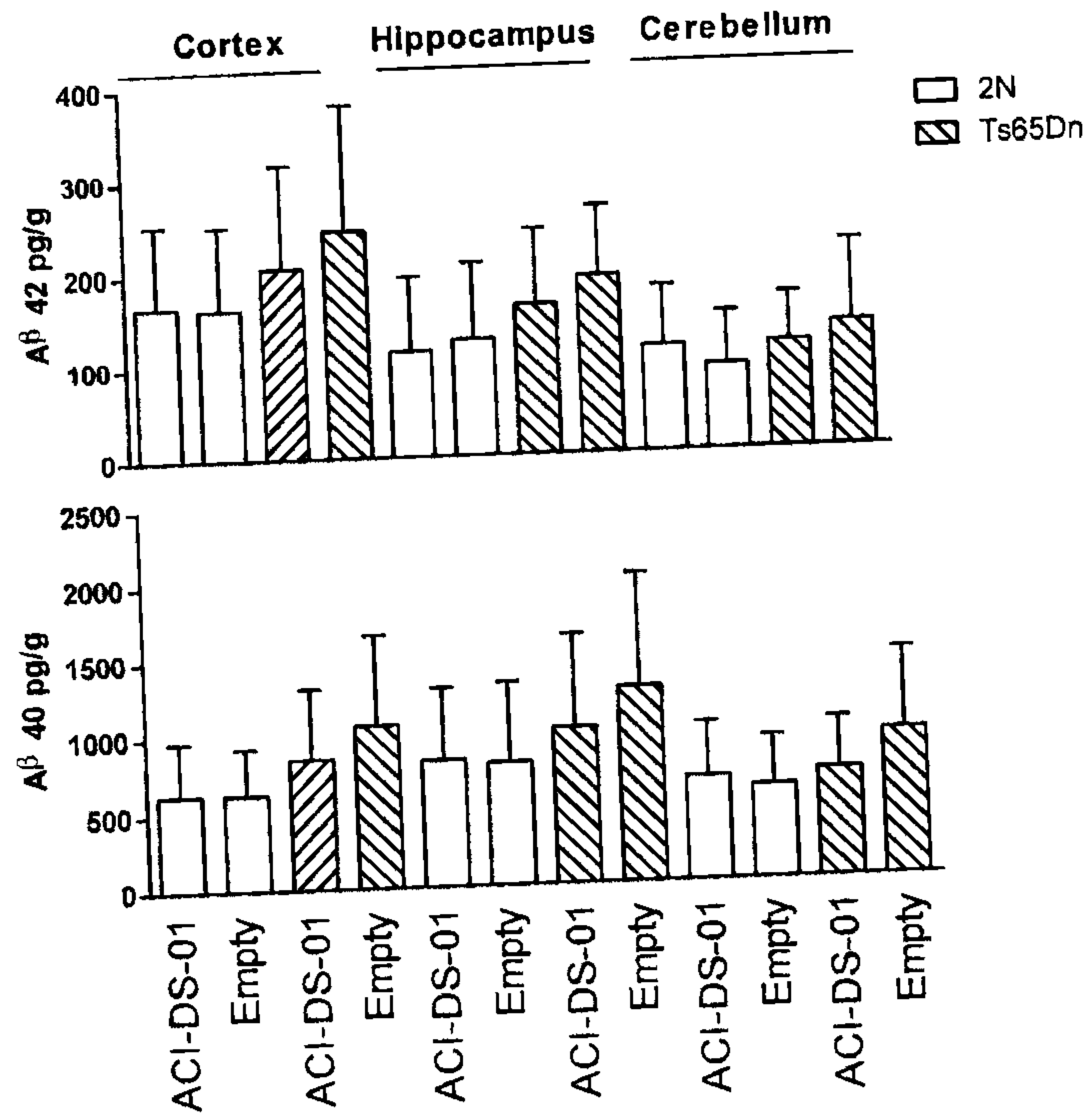
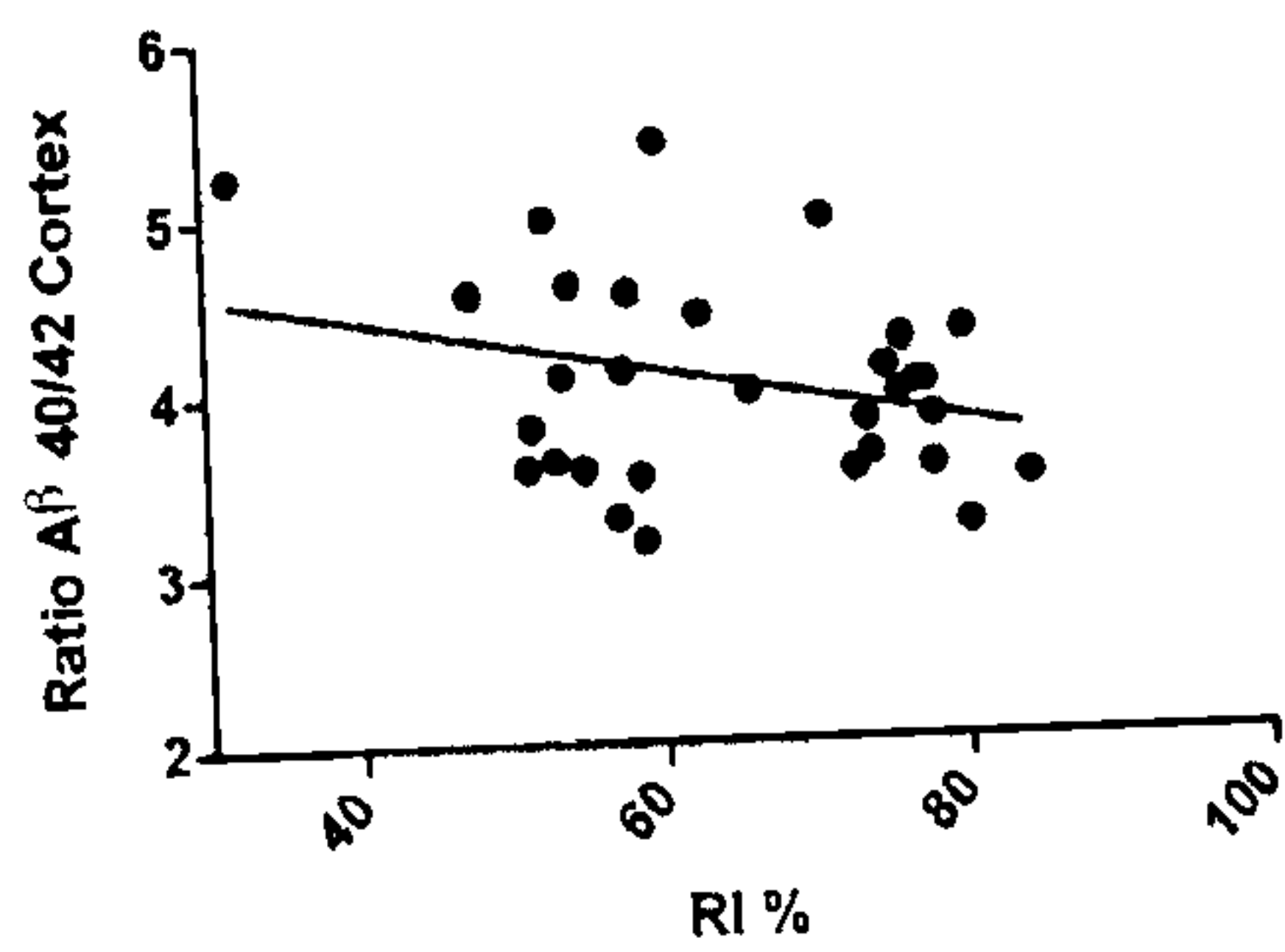
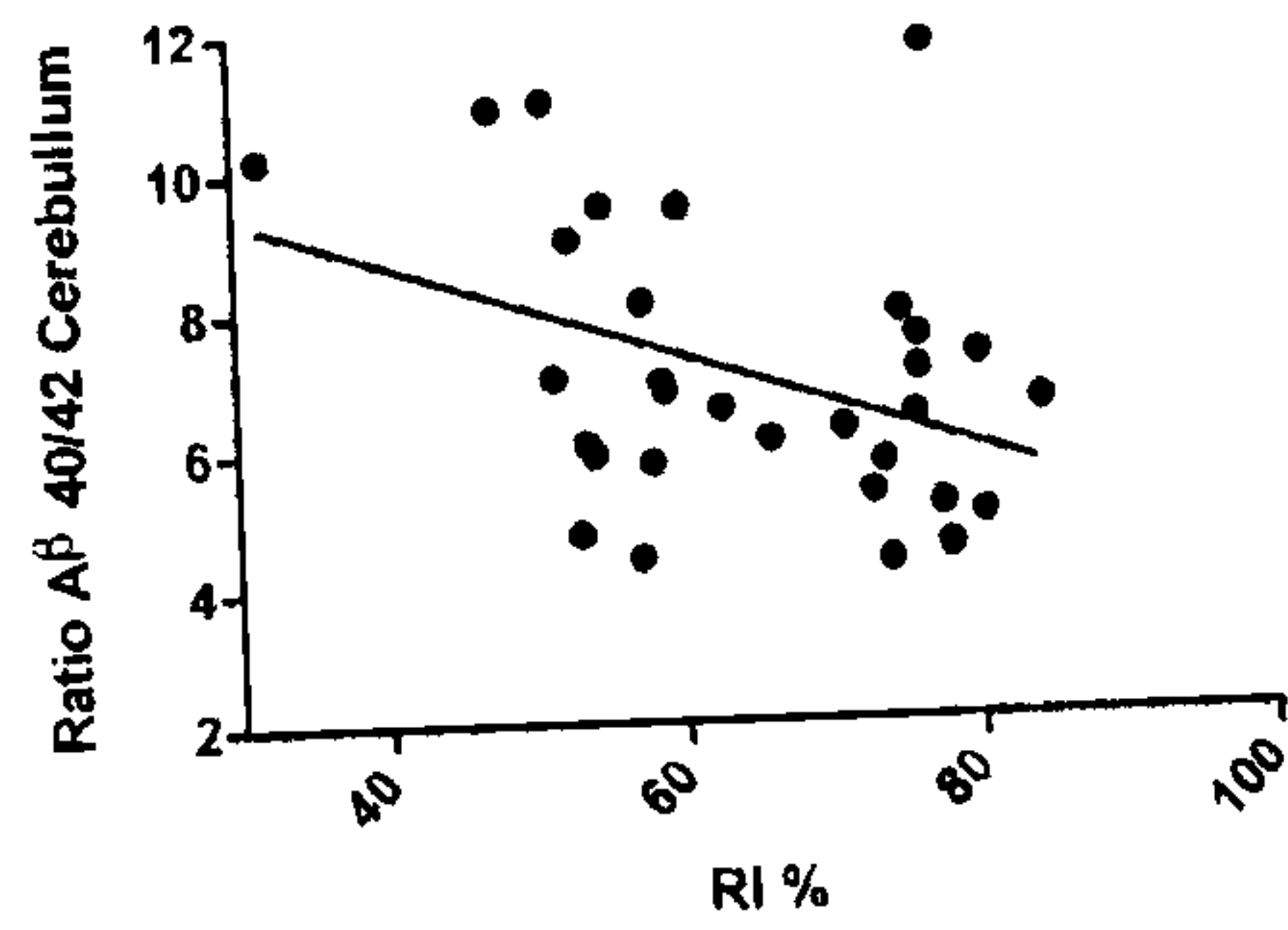
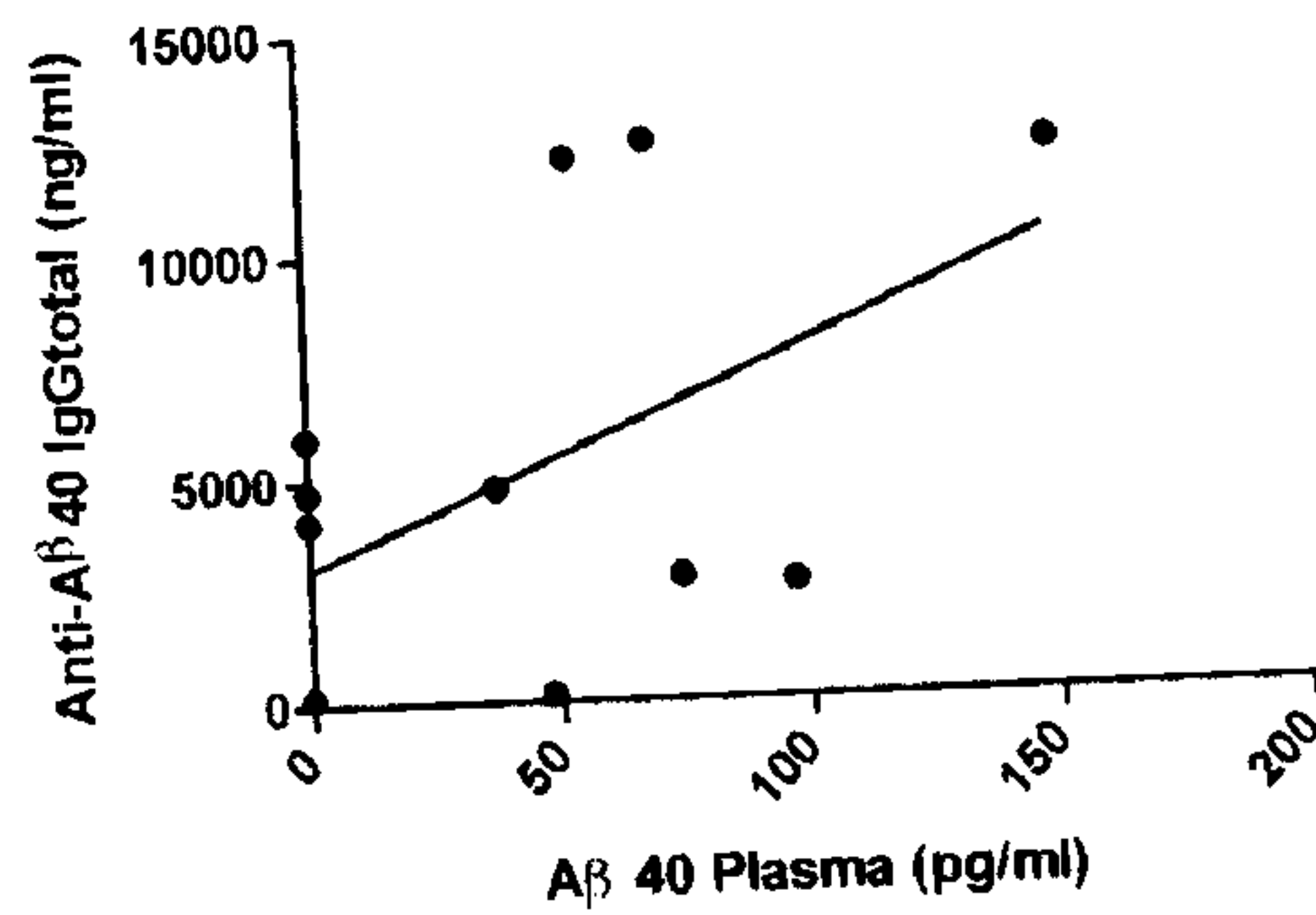
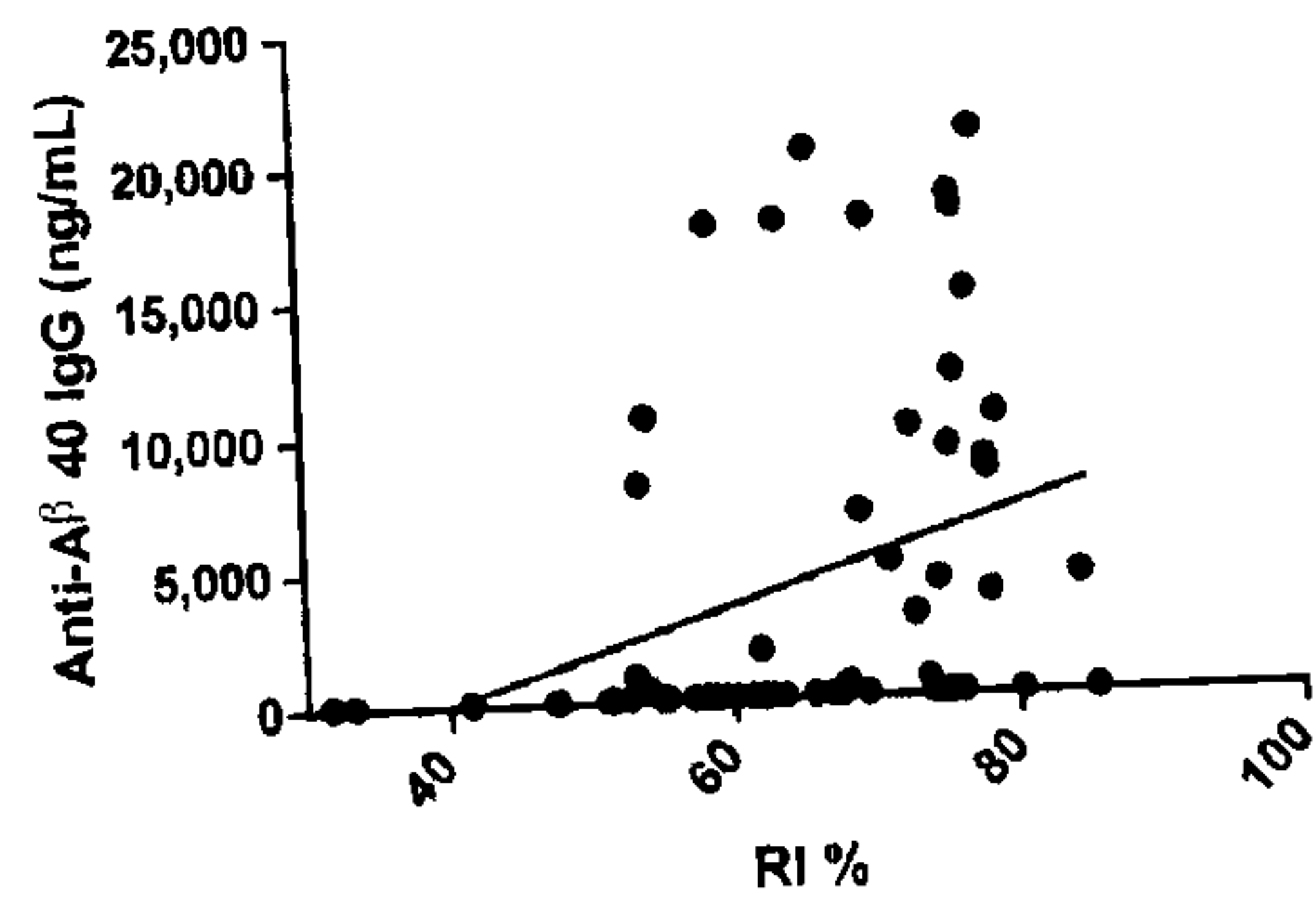
A**B****C****D****E**

Figure 5

