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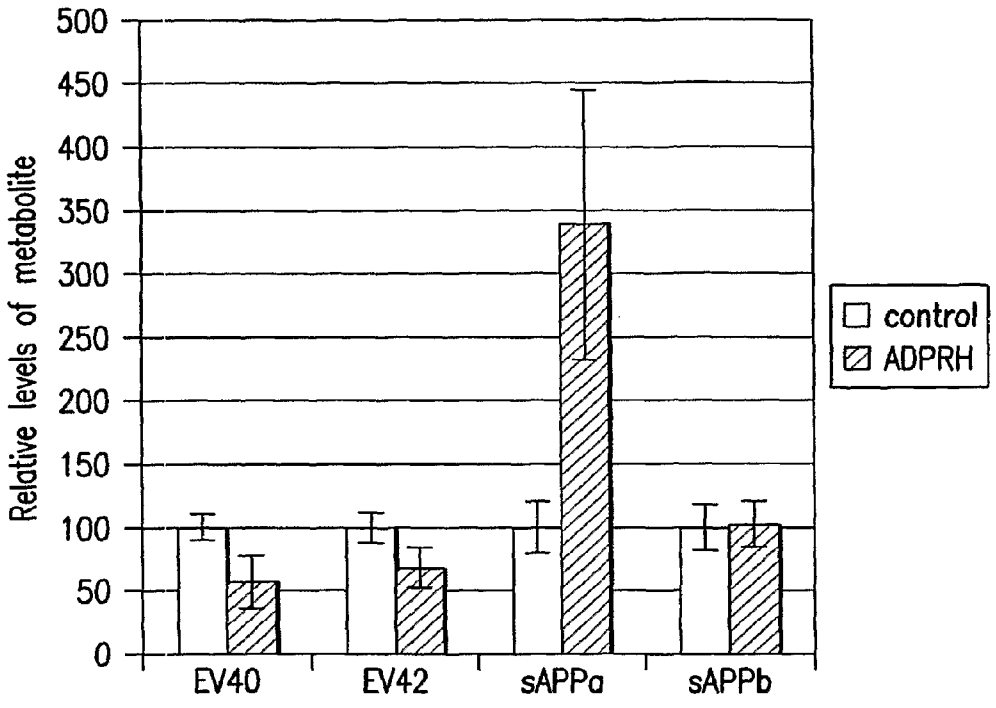
(54) **Title:** METHOD FOR IDENTIFYING MODULATORS OF ADPRH USEFUL FOR TREATING ALZHEIMER'S DISEASE



(57) **Abstract:** Methods for identifying modulators of ADPRH are described. The methods are particularly useful for identifying analytes that antagonize ADPRH 's effect on processing of amyloid precursor protein to AjS peptide and thus useful for identifying analytes that can be used for treating Alzheimer disease.

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TITLE OF THE INVENTION

METHOD FOR IDENTIFYING MODULATORS OF ADPRH USEFUL FOR TREATING ALZHEIMER'S DISEASE

BACKGROUND OF THE INVENTION

(1) Field of the Invention

The present invention relates to methods for identifying modulators of ADPRH. The methods are particularly useful for identifying analytes that antagonize ADPRH's effect on processing of amyloid precursor protein to Aβ peptide and thus useful for identifying analytes that can be used for treating Alzheimer disease.

(2) Description of Related Art

Alzheimer's disease is a common, chronic neurodegenerative disease characterized by a progressive loss of memory and sometimes severe behavioral abnormalities, as well as an impairment of other cognitive functions that often leads to dementia and death. It ranks as the fourth leading cause of death in industrialized societies after heart disease, cancer, and stroke. The incidence of Alzheimer's disease is high, with an estimated 2.5 to 4 million patients affected in the United States and perhaps 17 to 25 million worldwide. Moreover, the number of sufferers is expected to grow as the population ages.

A characteristic feature of Alzheimer's disease is the presence of large numbers of insoluble deposits, known as amyloid plaques, in the brains of those affected. Autopsies have shown that amyloid plaques are found in the brains of virtually all Alzheimer's patients and that the degree of amyloid plaque deposition correlates with the degree of dementia (Cummings and Cotman, Lancet 326: 1524-1587 (1995)). While some opinion holds that amyloid plaques are a late stage by-product of the disease process, the consensus view is that amyloid plaques and/or soluble aggregates of amyloid peptides are more likely to be intimately, and perhaps causally, involved in Alzheimer's disease.

A variety of experimental evidence supports this view. For example, amyloid β (Aβ) peptide, a primary component of amyloid plaques, is toxic to neurons in culture and transgenic mice that overproduce Aβ peptide in their brains show significant deposition of Aβ into amyloid plaques as well as significant neuronal toxicity (Yankner, Science 250: 279-282 (1990); Mattson *et al.*, J. Neurosci. 12: 379-389 (1992); Games *et al.*, Nature 373: 523-527 (1995); LaFerla *et al.*, Nature Genetics 9: 21-29 (1995)). Mutations in the APP gene, leading to elevated Aβ production, have been linked to heritable forms of Alzheimer's disease (Goate *et al.*, Nature 349:704-706 (1991); Chartier-Harlan *et al.*, Nature 353:844-846 (1991); Murrel *et al.*, Science 254: 97-99 (1991); Mullan *et al.*, Nature Genetics 1: 345-347 (1992)). Presenilin-1 (PS1) and presenilin-2 (PS2) related familial early-onset Alzheimer's disease (FAD) shows disproportionately increased production of Aβ1-42, the 42 amino acid isoform of Aβ, as opposed to Aβ1-40, the 40 amino acid isoform (Scheuner *et al*, Nature Medicine 2: 864-870 (1996)). The longer isoform

of Aβ is more prone to aggregation than the shorter isoform (Jarrett *et al*, Biochemistry 32:4693-4697 (1993). Injection of the insoluble, fibrillar form of Aβ into monkey brains results in the development of pathology (neuronal destruction, tau phosphorylation, microglial proliferation) that closely mimics Alzheimer's disease in humans (Geula *et al.*, Nature Medicine 4:827-831 (1998). See, Selkoe, J. Neuropathol. Exp. Neurol. 53: 438-447 (1994) for a review of the evidence that amyloid plaques have a central role in Alzheimer's disease.

Aβ peptide, a 39-43 amino acid peptide derived by proteolytic cleavage of the amyloid precursor protein (APP), is the major component of amyloid plaques (Glenner and Wong, Biochem. Biophys. Res. Comm. 120: 885-890 (1984)). APP is actually a family of polypeptides produced by alternative splicing from a single gene. Major forms of APP are known as APP695, APP751, and APP770, with the subscripts referring to the number of amino acids in each splice variant (Ponte *et al.*, Nature 331: 525-527 (1988); Tanzi *et al.*, Nature 331: 528-530 (1988); Kitaguchi *et al.*, Nature 331: 530-532(1988)). APP is a ubiquitous membrane-spanning (type 1) glycoprotein that undergoes proteolytic cleavage by at least two pathways (Selkoe, Trends Cell Biol. 8: 447-453 (1998)). In one pathway, cleavage by an enzyme known as α-secretase occurs while APP is still in the trans-Golgi secretory compartment (Kuentzel *et al.*, Biochem. J. 295:367-378 (1993)). This cleavage by α- secretase occurs within the Aβ peptide portion of APP, thus precluding the formation of Aβ peptide. In an alternative proteolytic pathway, cleavage of the Met596-Asp597 bond (numbered according to the 695 amino acid protein) by an enzyme known as β-secretase occurs. This cleavage by β-secretase generates the N-terminus of Aβ peptide. The C-terminus is formed by cleavage by a second enzyme known as γ-secretase. The C-terminus is actually a heterogeneous collection of cleavage sites rather than a single site since γ-secretase activity occurs over a short stretch of APP amino acids rather than at a single peptide bond. Peptides of 40 or 42 amino acids in length (Aβ1-40 and Aβ1-42, respectively) predominate among the C-termini generated by γ-secretase. Aβ1-42 peptide is more prone to aggregation than Aβ1-40 peptide, the major secreted species (Jarrett *et al.*, Biochemistry 32: 4693-4697 91993); Kuo *et al.*, J. Biol. Chem. 271: 4077-4081 (1996)), and its production is closely associated with the development of Alzheimer's disease (Sinha and Lieberburg, Proc. Natl. Acad. Sci. USA 96: 11049-11053 (1999)). The bond cleaved by γ-secretase appears to be situated within the transmembrane domain of APP. For a review that discusses APP and its processing, see Selkoe, Trends Cell. Biol. 8: 447-453 (1998).

While abundant evidence suggests that extracellular accumulation and deposition of Aβ peptide is a central event in the etiology of Alzheimer's disease, recent studies have also proposed that increased intracellular accumulation of Aβ peptide or amyloid containing C-terminal fragments may play a role in the pathophysiology of Alzheimer's disease. For example, over-expression of APP harboring mutations which cause familial Alzheimer's disease results in the increased intracellular accumulation of C99, the carboxy-terminal 99 amino acids of APP containing Aβ peptide, in neuronal cultures and Aβ42 in HEK 293 cells in neuronal cultures and Aβ42 peptide in HEK 293 cells. Moreover, evidence suggests that intra- and extracellular Aβ peptide are formed in distinct cellular pools in hippocampal neurons and

that a common feature associated with two types of familial Alzheimer's disease mutations in APP ("Swedish" and "London") is an increased intracellular accumulation of Aβ42 peptide. Thus, based on these studies and earlier reports implicating extracellular Aβ peptide accumulation in Alzheimer's disease pathology, it appears that altered APP catabolism may be involved in disease progression.

Much interest has focused on the possibility of inhibiting the development of amyloid plaques as a means of preventing or ameliorating the symptoms of Alzheimer's disease. To that end, a promising strategy is to inhibit the activity of β- and γ- secretase, the two enzymes that together are responsible for producing Aβ. This strategy is attractive because, if the formation of amyloid plaques is a result of the deposition of Aβ is a cause of Alzheimer's disease, inhibiting the activity of one or both of the two secretases would intervene in the disease process at an early stage, before late- stage events such as inflammation or apoptosis occur. Such early stage intervention is expected to be particularly beneficial (see, for example, Citron, Molecular Medicine Today 6:392-397 (2000)).

To that end, various assays have been developed that are directed to the identification of substances that may interfere with the production of Aβ peptide or its deposition into amyloid plaques. U.S. Patent No. 5,441,870 is directed to methods of monitoring the processing of APP by detecting the production of amino terminal fragments of APP. U.S. Patent No. 5,605,811 is directed to methods of identifying inhibitors of the production of amino terminal fragments of APP. U.S. Patent No. 5,593,846 is directed to methods of detecting soluble Aβ by the use of binding substances such as antibodies. US Published Patent Application No. US20030200555 describes using amyloid precursor proteins with modified β-secretase cleavage sites to monitor beta-secretase activity. Esler *et al.*, Nature Biotechnology 15: 258-263 (1997) described an assay that monitored the deposition of Aβ peptide from solution onto a synthetic analogue of an amyloid plaque. The assay was suitable for identifying substances that could inhibit the deposition of Aβ peptide. However, this assay is not suitable for identifying substances, such as inhibitors of β- or γ-secretase, that would prevent the formation of Aβ peptide.

Various groups have cloned and sequenced cDNA encoding a protein believed to be β-secretase (Vassar *et al.*, Science 286: 735-741 (1999); Hussain *et al.*, Mol. Cell. Neurosci. 14: 419- 427 (1999); Yan *et al.*, Nature 402: 533-537 (1999); Sinha *et al.*, Nature 402: 537-540 (1999); Lin *et al.*, Proc. Natl. Acad. Sci. USA 97: 1456-1460 (2000)). U.S. Pat. Nos. 6,828,117 and 6,737,510 disclose a β-secretase, which the inventors call aspartyl protease 2 (Asp2), variant Asp-2(a) and variant Asp-2(b), respectively, and U.S Pat. No. 6,545,127 discloses a catalytically active enzyme known as memapsin. Hong *et al.*, Science 290: 150-153 (2000) determined the crystal structure of the protease domain of human β-secretase complexed with an eight- residue peptide-like inhibitor at 1.9 angstrom resolution. Compared to other human aspartic proteases, the active site of human β-secretase is more open and less hydrophobic, contributing to the broad substrate specificity of human β-secretase (Lin *et al.*, Proc. Natl. Acad. Sci. USA 97: 1456-1460 (2000)).

Ghosh *et al.*, J. Am. Chem. Soc. 122: 3522-3523 (2000) disclosed two inhibitors of β-secretase, OM99-1 and OM99-2, that are modified peptides based on the β-secretase cleavage site of the

Swedish mutation of APP (SEVNL/DAEFR, with "/" indicating the site of cleavage). OM99-1 has the structure VNL*AAEF (with "L*A" indicating the uncleavable hydroxyethylene transition-state isostere of the LA peptide bond) and exhibits a Ki towards recombinant β-secretase produced in *E. coli* of 6.84×10^{-8} M±2.72×10^{-9} M. OM99-2 has the structure EVNL*AAEF (with "L*A" indicating the uncleavable hydroxyethylene transition-state isostere of the LA peptide bond) and exhibits a Ki towards recombinant β-secretase produced in E. coli of 9.58×10^{-9} M±2.86×10^{-10} M. OM99-1 and OM99-2, as well as related substances, are described in International Patent Publication WO0100665.

Currently, most drug discovery programs for Alzheimer's disease have targeted either aceytlcholinesterase or the secretase proteins directly responsible for APP processing. While acetylcholinesterase inhibitors are marketed drugs for Alzheimer's disease, they have limited efficacy and do not have disease modifying properties. Secretase inhibitors, on the other hand, have been plagued either by mechanism-based toxicity (γ-secretase inhibitors) or by extreme difficulties in identifying small molecule inhibitors with appropriate pharmacokinetic properties to allow them to become drugs (BACE inhibitors). Identifying novel factors involved in APP processing would expand the range of targets for Alzheimer's disease treatments and therapy.

BRIEF SUMMARY OF THE INVENTION

The present invention provides methods for identifying modulators of ADPRH. The methods are particularly useful for identifying analytes that antagonize ADPRH's effect on processing of amyloid precursor protein to Aβ peptide and thus useful for identifying analytes that can be used for treating Alzheimer disease.

Therefore, in one embodiment, the present invention provides a method for screening for analytes that antagonize processing of amyloid precursor protein (APP) to Aβ peptide, comprising providing recombinant cells, which ectopically expresses ADPRH and the APP; incubating the cells in a culture medium under conditions for expression of the ADPRH and APP and which contains an analyte; removing the culture medium from the recombinant cells; and determining the amount of at least one processing product of APP selected from the group consisting of sAPPβ and Aβ peptide in the medium wherein a decrease in the amount of the processing product in the medium compared to the amount of the processing product in medium from recombinant cells incubated in medium without the analyte indicates that the analyte is an antagonist of the processing of the APP to Aβ peptide.

In further aspects of the method, the recombinant cells each comprises a first nucleic acid that encodes ADPRH operably linked to a first heterologous promoter and a second nucleic acid that encodes an APP operably linked to a second heterologous promoter. In preferred aspects of the present invention, the APP is APP_{NFEV}. In preferred aspects, the method includes a control which comprises providing recombinant cells that ectopically express the APP but not ADPRH.

The present invention further provides a method for screening for analytes that antagonize processing of amyloid precursor protein (APP) to amyloid β (Aβ) peptide, comprising

providing recombinant cells, which ectopically express ADPRH and a recombinant APP comprising APP fused to a transcription factor that when removed from APP during processing of the APP produces an active transcription factor, and a reporter gene operably linked to a promoter inducible by the transcription factor; incubating the cells in a culture medium under conditions for expression of the ADPRH and recombinant APP and which contains an analyte; and determining expression of the reporter gene wherein a decrease in expression of the reporter gene compared to expression of the reporter gene in recombinant cells in a culture medium without the analyte indicates that the analyte is an antagonist of the processing of the APP to Aβ peptide.

In further aspects of the method, the recombinant cells each comprises a first nucleic acid that encodes ADPRH operably linked to a first heterologous promoter, a second nucleic acid that encodes the recombinant APP operably linked to a second heterologous promoter, and a third nucleic acid that encodes a reporter gene operably linked to promoter responsive to the transcription factor comprising the recombinant APP.

In light of the analytes that can be identified using the above methods, the present invention further provides a method for treating Alzheimer's disease in an individual which comprises providing to the individual an effective amount of an antagonist of ADPRH activity.

Further still, the present invention provides a method for identifying an individual who has Alzheimer's disease or is at risk of developing Alzheimer's disease comprising obtaining a sample from the individual and measuring the amount of ADPRH in the sample.

Further still, the present invention provides for the use of an antagonist of ADPRH for the manufacture of a medicament for the treatment of Alzheimer's disease.

Further still, the present invention provides for the use of an antibody specific for ADPRH for the manufacture of a medicament for the treatment of Alzheimer's disease.

Further still, the present invention provides a vaccine for preventing and/or treating Alzheimer's disease in a subject, comprising an antibody raised against an antigenic amount of ADPRH wherein the antibody antagonizes the processing of APP to Aβ peptide.

The term "analyte" refers to a compound, chemical, agent, composition, antibody, peptide, aptamer, nucleic acid, or the like, which can modulate the activity of ADPRH.

The term "ADPRH" refers to ADP-ribosylarginine hydrolase (Official Gene Symbol ADPRH, NP_001116), which is a gene from a human or another mammal having an open reading frame coding for a protein of 357 amino acids in length (SEQ ID NO:2). The term further includes mutants, variants, alleles, and polymorphs of ADPRH. Where appropriate, the term further includes fusion proteins comprising all or a portion of the amino acid sequence of ADPRH fused to the amino acid sequence of a heterologous peptide or polypeptide, for example, hybrid immuoglobulins comprising the amino acid sequence of ADPRH or ADPRH fused at its C-terminus to the N-terminus of an immunoglobulin constant region amino acid sequence (*see*, for example, U.S. Patent No. 5,428,130 and related patents).

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a nucleic sequence encoding the human ADPRH.

Figure 2 is the amino acid sequence of the human ADPRH.

Figure 3 is a graph showing the Relative expression of the metabolites expressed as a percent of the mean control non-silencing siRNA value of 100. ADPRH $p<0.05$ for EV40, EV42, and sAPPα and $p \approx 0.5$ for sAPPβ.

Figure 4 shows the tissue distribution of ADPRH mRNA in various human tissues.

DETAILED DESCRIPTION OF THE INVENTION

One potential way to inhibit the processing of APP to Aβ is to identify enzymes that modify APP, BACE1, or other factors involved in APP metabolism. Many studies show that the activity and localization of proteins are regulated by post-translational modification. The multitude of modifications than can regulate molecular activity include, but are not limited to, adding proteins such as ubiquitin (Welchman, R.L., *et al.*, Nat. Rev. Mo.l Cell Biol. 6(8): 599-609 (2005)) and small molecules such as glutathione (O'Brian, C.A. and Chu, F., Free Radical Res. 39(5): 471-480 (2005)). Recently, ADP ribosylation has been reported to play a similar regulatory role (DiGirolamo, M., FEBS J. 272(18): 4565-4575 (2005)). Methods to target the protein modifying pathways affecting APP processing offer a new and unprecedented approach to Alzheimer's disease.

The protein referred to herein as ADPRH is a protein that the Applicants have discovered to have a role in processing of amyloid precursor protein (APP) to amyloid β (Aβ) peptide. ADP-ribosylation is a post translational modification of proteins, in which the ADP-ribose moiety of β-NAD is transferred to specific amino acid residues in a target protein (Takada, T., *et al.*, J. Biol. Chem. 268 (24): 17837-17843 (1993)). ADP-ribosylarginine hydrolase has been cloned and purified (U.S. Pat. No. 5,716,816). ADP-ribosylarginine hydrolases release ADP-ribose from the ADP-ribosylated proteins (Takada, *et al.*, supra). It is believed that the ADP-ribosyltransferases and hydrolases act together to regenerate an unmodified protein, i.e. active form by releasing ADP-ribose from the enzyme. Id. The precise mechanism by which ADPRH acts to increase Aβ secretion is not known. However, as noted above, post-translational modification of other proteins with ADP has been shown to be a key regulatory step in several physiological processes (DiGirolam, M., FEBS J. 272(18): 4565-4575 (2005)).

A defining characteristic of Alzheimer's disease (AD) is the deposition of aggregated plaques containing Aβ peptide in the brains of affected individuals. The applicant's discovery that ADPRH has a role processing APP to Aβ peptide suggests that ADPRH has a role in the progression of Alzheimer's disease in an individual. Therefore, in light of the applicants' discovery, identifying molecules which target the activity or expression of ADPRH would be expected to lead to treatments or therapies for Alzheimer's disease. Expression or activity of ADPRH may also be useful as a diagnostic

marker for identifying individuals who have Alzheimer's disease or are at risk of developing Alzheimer's disease.

The deposition of aggregated plaques containing amyloid β (Aβ) peptide in the brains of individuals affected with Alzheimer's disease is believed to involve the sequential cleavage of APP by two secretase-mediated cleavages to produce Aβ peptide. The first cleavage event is catalyzed by the type I transmembrane aspartyl protease BACE1. BACE1 cleavage of APP at the BACE cleavage site (between amino acids 596 and 597) generates a 596 amino acid soluble N-terminal sAPPβ fragment and a 99 amino acid C-terminal fragment (βCTF) designated C99. Further cleavage of C99 by γ-secretase (a multicomponent membrane complex consisting of at least presenilin, nicastrin, aph1, and pen2) releases the 40 or 42 amino acid Aβ peptide. An alternative, non-amyloidogenic pathway of APP cleavage is catalyzed by γ-secretase, which cleaves APP to produce a 613 amino acid soluble sAPPα N-terminal fragment and an 83 amino acid βCTF fragment designated C83. While ongoing drug discovery efforts have focused on identifying antagonists of BACE1 and γ-secretase mediated cleavage of APP, the complicated nature of Alzheimer's disease suggests that efficacious treatments and therapies for Alzheimer's disease might comprise other targets for modulating APP processing. ADPRH of the present invention is another target for which modulators (in particular, antagonists) of are expected to provide efficacious treatments or therapies for Alzheimer's disease, either alone or in combination with one or more other modulators of APP processing, for example, antagonists selected from the group consisting of BACE1 and γ-secretase.

ADPRH was identified by screening a siRNA library for siRNA that inhibited APP processing. As described in Example 1, a library of about 15,200 siRNA pools, each targeting a single gene, was transfected individually into recombinant cells ectopically expressing a recombinant APP (APP_{NFEV}). APP_{NFEV} has been described in U.S. Pub. Pat. Appl. No. 2003/0200555, comprises isoform 1-695 and has a HA, Myc, and FLAG sequences at the amino acid position 289, an optimized β-cleavage site comprising amino acids NFEV, and a K612V mutation. Metabolites of APP_{NFEV} produced during APP BACE1/γ-secretase or α-secretase processing are sAPPβ with NF at the C-terminus, EV40, and EV42 or sAPPα. EV40 and EV42 are unique Aβ40-like and Aβ42-like peptides that contain the glutamic acid and valine substitutions of APP_{NFEV} and sAPPβ and sAPPα each contain the HA, FLAG, and myc sequences. sAPPβ, sAPPα, EV40, and EV42 were detected by an immunodetection method that used antibodies that were specific for the various APP_{NFEV} metabolites. Expression levels were determined relative to a non-silencing siRNA control.

Following two rounds of screening, which consisted of a primary screen done with the entire library of siRNAs and secondary screening of about 1600 siRNAs performed in triplicate repeats, a siRNA designed to target ADPRH RNA was found to consistently alter processing of APP to sAPPβ, EV40, and EV42. The ADPRH protein was found in the cerebrospinal fluid of rodents (Miyaoka, T., *et al.*, Brain Res. 746 (1-2):1-9 (1997)) and was expressed in the brains of bovines (Moss, J., *et al.*, J. Biol.

Chem., 267(15): 10481-10488 (1992)). Thus, ADPRH is expressed in CNS tissues of mammals, including presumably humans.

The nucleic acid sequence encoding the human ADPRH (SEQ ID NO:1) is shown in Figure 1 and the amino acid sequence for the human ADPRH (SEQ ID NO:2) is shown in Figure 2.

The mRNA encoding ADPRH was found to be expressed in regions of the brain subject to Alzheimer's disease pathology (Example 2).

In light of the applicants' discovery, ADPRH or modified mutants or variants thereof is useful for identifying analytes which antagonize processing of APP to produce Aβ peptide. These analytes can be used to treat patients afflicted with Alzheimer's disease. ADPRH can be used alone or in combination with acetylcholinesterase inhibitors, NMDA receptor partial agonists, secretase inhibitors, amyloid-reactive antibodies, growth hormone secretagogues, and other treatments for Alzheimer's disease.

The present invention provides methods for identifying ADPRH modulators that modulate expression of ADPRH by contacting ADPRH with a substance that inhibits or stimulates ADPRH expression and determining whether expression of ADPRH polypeptide or nucleic acid molecules encoding an ADPRH are modified. The present invention also provides methods for identifying modulators that antagonize ADPRH's effect on processing APP to Aβ peptide or formation of Aβ-amyloid plaques in tissues where ADPRH is localized or co-expressed. For example, ADPRH protein can be expressed in cell lines that also express APP and the effect of the modulator on Aβ production is monitored using standard biochemical assays with Aβ-specific antibodies or by mass spectrophotometric techniques. Inhibitors for ADPRH are identified by screening for a reduction in the release of Aβ peptide which is dependent on the presence of ADPRH protein for effect. Both small molecules and larger biomolecules that antagonize ADPRH-mediated processing of APP to Aβ peptide can be identified using such an assay. A method for identifying antagonists of ADPRH's effect on the processing APP to Aβ peptide includes the following method which is amenable to high throughput screening. In addition, the methods disclosed in U.S. Pub. Pat. Appl. No. 2003/0200555 can be adapted to use in assays for identifying antagonists of ADPRH activity.

A mammalian ADPRH cDNA, encompassing the first through the last predicted codon contiguously, is amplified from brain total RNA with sequence-specific primers by reverse-transcription polymerase chain reaction (RT-PCR). Alternatively the ADPRH cDNA can be purchased from commercial vendors such as Invitrogen (Carlsbad, CA) and Origene (Rockville, MD). The cDNA sequence is cloned into pcDNA3.zeo or other appropriate mammalian expression vector. Fidelity of the sequence and the ability of the plasmid to encode full-length ADPRH is validated by DNA sequencing of the ADPRH plasmid (pcDNA_ADPRH).

Commercially available mammalian expression vectors which are suitable for recombinant ADPRH expression include, but are not limited to, pcDNA3.neo (Invitrogen, Carlsbad, CA), pcDNA3.1 (Invitrogen, Carlsbad, CA), pcDNA3.1/Myc-His (Invitrogen), pCI-neo (Promega, Madison,

WI), pLITMUS28, pLITMUS29, pLITMUS38 and pLITMUS39 (New England Bioloabs, Beverly, MA), pcDNAI, pcDNAIamp (Invitrogen), pcDNA3 (Invitrogen), pMC1neo (Stratagene, La Jolla, CA), pXT1 (Stratagene), pSG5 (Stratagene), EBO-pSV2-neo (ATCC 37593) pBPV-1(8-2) (ATCC 37110), pdBPV-MMTneo (342-12) (ATCC 37224), pRSVgpt (ATCC 37199), pRSVneo (ATCC 37198), pSV2-dhfr (ATCC 37146), pUCTag (ATCC 37460), lZD35 (ATCC 37565), pMC1neo (Stratagene), pcDNA3.1, pCR3.1 (Invitrogen, San Diego, Calif.), EBO-pSV2-neo (ATCC 37593), pCI.neo (Promega), pTRE (Clontech, Palo Alto, Calif.), pV1Jneo, pIRESneo (Clontech, Palo Alto, Calif.), pCEP4 (Invitrogen,), pSC11, and pSV2-dhfr (ATCC 37146). The choice of vector will depend upon the cell type in which it is desired to express the ADPRH, as well as on the level of expression desired, cotransfection with expression vectors encoding APP_{NFEV}, and the like.

Cells transfected with plasmid vector comprising APP_{NFEV}, for example the HEK293T/APP_{NFEV} cells used to detect ADPRH activity in the siRNA screening experiment described in Example 1, are used as described in Example 1 with the following modifications. Cells are either cotransfected with a plasmid expression vector comprising APP_{NFEV} operably linked to a heterologous promoter and a plasmid expression vector comprising the ADPRH operably linked to a heterologous promoter or the HEK293T/APP_{NFEV} cells described in Example 1 and U.S. Pub. Pat. Appl. No. 2003/0200555 are transfected with a plasmid expression vector comprising the ADPRH operably linked to a heterologous promoter. The promoter comprising the plasmid expression vector can be a constitutive promoter or an inducible promoter. Preferably, the assay includes a negative control comprising the expression vector without the ADPRH.

After the cells have been transfected, the transfected or cotransfected cells are incubated with an analyte being tested for ability to antagonize ADPRH's effect on processing of APP to Aβ peptide. The analyte is assessed for an effect on the ADPRH transfected or cotransfected cells that is minimal or absent in the negative control cells. In general, the analyte is added to the cell medium the day after the transfection and the cells incubated for one to 24 hours with the analyte. In particular embodiments, the analyte is serially diluted and each dilution provided to a culture of the transfected or cotransfected cells. After the cells have been incubated with the analyte, the medium is removed from the cells and assayed for secreted sAPPα, sAPPβ, EV40, and EV42 as described in Examples 1 and 5. Briefly, the antibodies specific for each of the metabolites is used to detect the metabolites in the medium. Preferably, the cells are assessed for viability.

Analytes that alter the secretion of one or more of EV40, EV42, sAPPα, or sAPPβ in the presence of ADPRH protein are considered to be modulators of ADPRH and potentially useful as therapeutic agents for ADPRH-related diseases. Direct inhibition or modulation of ADPRH can be confirmed using binding assays using the full-length ADPRH, extracellular or intracellular domain thereof or a ADPRH fusion proteins comprising the intracellular or extracellular domains coupled to a C-terminal FLAG, or other, epitopes. A cell-free binding assay using full-length ADPRH, extracellular or intracellular domain thereof or an ADPRH fusion proteins or membranes containing the ADPRH

integrated therein and labeled-analyte can be performed and the amount of labeled analyte bound to the ADPRH determined.

The present invention further provides a method for measuring the ability of an analyte to modulate the level of ADPRH mRNA or protein in a cell. In this method, a cell that expresses ADPRH is contacted with a candidate compound and the amount of ADPRH mRNA or protein in the cell is determined. This determination of ADPRH levels may be made using any of the above-described immunoassays or techniques disclosed herein. The cell can be any ADPRH expressing cell such as cell transfected with an expression vector comprising ADPRH operably linked to its native promoter or a cell taken from a brain tissue biopsy from a patient.

The present invention further provides a method of determining whether an individual has a ADPRH-associated disorder or a predisposition for a ADPRH-associated disorder. The method includes providing a tissue or serum sample from an individual and measuring the amount of ADPRH in the tissue sample. The amount of ADPRH in the sample is then compared to the amount of ADPRH in a control sample. An alteration in the amount of ADPRH in the sample relative to the amount of ADPRH in the control sample indicates the subject has a ADPRH-associated disorder. A control sample is preferably taken from a matched individual, that is, an individual of similar age, sex, or other general condition but who is not suspected of having a ADPRH related disorder. In another aspect, the control sample may be taken from the subject at a time when the subject is not suspected of having a condition or disorder associated with abnormal expression of ADPRH.

Other methods for identifying inhibitors of ADPRH can include blocking the interaction between ADPRH and the enzymes involved in APP processing or trafficking using standard methodologies for analyzing protein-protein interaction such as fluorescence energy transfer or scintillation proximity assay. Surface Plasmon Resonance can be used to identify molecules that physically interact with purified or recombinant ADPRH.

In accordance with yet another embodiment of the present invention, there are provided antibodies having specific affinity for the ADPRH or epitope thereof. The term "antibodies" is intended to be a generic term which includes polyclonal antibodies, monoclonal antibodies, Fab fragments, single V_H chain antibodies such as those derived from a library of camel or llama antibodies or camelized antibodies (Nuttall *et al.*, Curr. Pharm. Biotechnol. 1: 253-263 (2000); Muyldermans, J. Biotechnol. 74: 277-302 (2001)), and recombinant antibodies. The term "recombinant antibodies" is intended to be a generic term which includes single polypeptide chains comprising the polypeptide sequence of a whole heavy chain antibody or only the amino terminal variable domain of the single heavy chain antibody (V_H chain polypeptides) and single polypeptide chains comprising the variable light chain domain (V_L) linked to the variable heavy chain domain (V_H) to provide a single recombinant polypeptide comprising the Fv region of the antibody molecule (scFv polypeptides) (*see* Schmiedl *et al.*, J. Immunol. Meth. 242: 101-114 (2000); Schultz *et al.*, Cancer Res. 60: 6663-6669 (2000); Dübel *et al.*, J. Immunol. Meth. 178: 201-209 (1995); and in U.S. Patent No. 6,207,804 B1 to Huston *et al.*). Construction of recombinant single

V_H chain or scFv polypeptides which are specific against an analyte can be obtained using currently available molecular techniques such as phage display (de Haard *et al.*, J. Biol. Chem. 274: 18218-18230 (1999); Saviranta *et al.*, Bioconjugate 9: 725-735 (1999); de Greeff *et al.*, Infect. Immun. 68: 3949-3955 (2000)) or polypeptide synthesis. In further embodiments, the recombinant antibodies include modifications such as polypeptides having particular amino acid residues or ligands or labels such as horseradish peroxidase, alkaline phosphatase, fluors, and the like. Further still embodiments include fusion polypeptides which comprise the above polypeptides fused to a second polypeptide such as a polypeptide comprising protein A or G.

The antibodies specific for ADPRH can be produced by methods known in the art. For example, polyclonal and monoclonal antibodies can be produced by methods well known in the art, as described, for example, in Harlow and Lane, Antibodies: A Laboratory Manual. Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY (1988). The ADPRH or fragments thereof can be used as immunogens for generating such antibodies. Alternatively, synthetic peptides can be prepared (using commercially available synthesizers) and used as immunogens. Amino acid sequences can be analyzed by methods well known in the art to determine whether they encode hydrophobic or hydrophilic domains of the corresponding polypeptide. Altered antibodies such as chimeric, humanized, CDR-grafted, or bifunctional antibodies can also be produced by methods well known in the art. Such antibodies can also be produced by hybridoma, chemical synthesis or recombinant methods described, for example, in Sambrook *et al.*, *supra.*, and Harlow and Lane, *supra*. Both anti-peptide and anti-fusion protein antibodies can be used (*see*, for example, Bahouth *et al.*, Trends Pharmacol. Sci. 12: 338 (1991); Ausubel *et al.*, Current Protocols in Molecular Biology, (John Wiley and Sons, N.Y. (1989)).

Antibodies so produced can be used for the immunoaffinity or affinity chromatography purification of ADPRH or ADPRH/ligand or analyte complexes. The above referenced anti-ADPRH antibodies can also be used to modulate the activity of ADPRH in living animals, in humans, or in biological tissues isolated thereof. Accordingly, contemplated herein are compositions comprising a carrier and an amount of an antibody having specificity for ADPRH effective to block naturally occurring ADPRH from binding its ligand or for effecting the processing of APP to Aβ peptide.

Therefore, in another aspect, the present invention further provides pharmaceutical compositions that antagonize ADPRH's effect on processing of APP to Aβ peptide. Such compositions include a ADPRH nucleic acid, ADPRH peptide, fusion protein comprising ADPRH or fragment thereof coupled to a heterologous peptide or protein or fragment thereof, an antibody specific for ADPRH, nucleic acid or protein aptamers, siRNA inhibitory to ADPRH mRNA, analyte that is a ADPRH antagonist, or combinations thereof, and a pharmaceutically acceptable carrier or diluent.

In a further still aspect, the present invention further provides a kit for in vitro diagnosis of disease by detection of ADPRH in a biological sample from a patient. A kit for detecting ADPRH preferably includes a primary antibody capable of binding to ADPRH; and a secondary antibody conjugated to a signal-producing label, the secondary antibody being capable of binding an epitope

different from, i.e., spaced from, that to which the primary antibody binds. Such antibodies can be prepared by methods well-known in the art. This kit is most suitable for carrying out a two-antibody sandwich immunoassay, e.g., two-antibody sandwich ELISA.

Using derivatives of ADPRH protein or cDNA, dominant negative forms of ADPRH that could interfere with ADPRH-mediated APP processing to Aβ release can be identified. These derivatives could be used in gene therapy strategies or as protein-based therapies top block ADPRH activity in afflicted patients. ADPRH can be used to identify endogenous brain proteins that bind to ADPRH using biochemical purification, genetic interaction, or other techniques common to those skilled in the art. These proteins or their derivatives can subsequently be used to inhibit ADPRH activity and thus be used to treat Alzheimer's disease. Additionally, polymorphisms in the ADPRH RNA or in the genomic DNA in and around ADPRH could be used to diagnose patients at risk for Alzheimer's disease or to identify likely responders in clinical trials.

The following examples are intended to promote a further understanding of the present invention.

EXAMPLE 1

ADPRH was identified in a screen of an siRNA library for modulators of APP processing.

A cell plate was prepared by plating HEK293T/APP_{NFEV} cells to the wells of a 384-well Corning PDL-coated assay plate at a density of about 2,000 cells per well in 40 µL DMEM containing 10% fetal bovine serum (FBS) and antibiotics. The cell plate was incubated overnight at 37°C in 5% CO_2. HEK293T/APP_{NFEV} cells are a subclone of HEK293T cells stably transformed with the APP_{NFEV} plasmid described in U.S. Pub. Pat. Appl. No. 2003/0200555. In brief, APP_{NFEV} encodes human amyloid precursor protein (APP), isoform 1-695, modified at amino acid position 289 by an in-frame insertion of HA, Myc, and FLAG epitope amino acid sequences and at amino acid positions 595, 596, 597, and 598 by substitution of the amino acid sequence NFEV for the endogenous amino acid sequence KMDA sequence comprising the BACE1 cleavage site. Thus, the BACE cleavage site is a modified BACE1 cleavage site and BACE1 cleaves between amino acids F and E of NFEV. Maintenance of the plasmid within the subclone is achieved by culturing the cells in the presence of the antibiotic puromycin.

The next day, the cells in each of the wells of the cell plate were transfected with a siRNA library as follows. Oligofectamine™ (Invitrogen, Inc., Carlsbad, CA) was mixed with Opti-MEM® (Invitrogen, Inc., Carlsbad, CA) at a ratio of 1 to 40 and 20 µL of the mixture was added to each well of a different 384-well plate. To each well of the plate, 980 nL of a particular 10 µM siRNA species was added and the plate incubated for ten minutes at room temperature. Afterwards, five µL of each the siRNA/Oligofectamine™ /Opti-MEM® mixtures was added to a corresponding well in the cell plate containing the HEK293/APP_{NFEV} cells. The cell plate was incubated for 24 hours at 37°C in 5% CO_2. Controls were provided which contained non-silencing siRNA or a siRNA that inhibited BACE1.

On the next day, for each of the wells of the cell plate, the siRNA and Oligofectamine™/Opti-MEM® mixture was removed and replaced with 70 μL DMEM containing 10% FBS and Merck compound A (see, WO2003/093252, Preparation of spirocyclic [1,2,5]thiadiazole derivatives as γ-secretase inhibitors for treatment of Alzheimer's disease, Collins *et al.*), a γ-secretase inhibitor given at a final concentration equal to its IC_{50} in cell-based enzyme assays. The cell plate was incubated for 24 hours at 37°C in 5% CO_2.

On the next day, for each of the wells of the cell plate, 64 μL of the medium (conditioned medium) was removed and transferred to four 384-well REMP plates in 22, 22, 10, and 10 μL aliquots for subsequent use in detecting sAPPα, EV42, EV40, sAPPβ using AlphaScreen™ (PerkinElmer, Wellesley, MA) detection technology. Viability of the cells was determined by adding 40 μL 10% AlamarBlue (Serotec, Inc., Raleigh, NC) in DMEM containing 10% FBS to each of the wells of the cell plate with the conditioned medium removed. The cell plate was then incubated at 37°C for two hours. The Acquest™ (Molecular Devices Corporation, Sunnyvale, CA) plate reader was used to assay fluorescence intensity (ex. 545 nm, em. 590 nm) as a means to confirm viability of the cells.

Assays for detecting and measuring sAPPβ, EV42, EV40, and sAPPα were detected using antibodies as follows. In general, detection-specific volumes (8 or 0.5 μL) were transferred to a Greiner 384-well white small-volume detection plate. In the case of the smaller volume, 7.5 μL of assay medium was added for a final volume of eight μL per well. One μL of Antibody/Donor bead mixture (see below) was dispensed into the solution, and one μL antibody/Acceptor bead mixture was added. Plates were incubated in the dark for 24 hours at 4°C. Then the plates were read using AlphaQuest™ (PerkinElmer, Wellesley, MA) instrumentation. In all protocols, the plating medium was DMEM (Invitrogen, Carlsbad, CA; Cat. No. 21063-029); 10% FBS, the AlphaScreen™ buffer was 50 mM HEPES, 150 mM NaCl, 0.1% BSA, 0.1% Tween-20, pH 7.5, and the AlphaScreen™ Protein A kit was used.

Anti-NF antibodies and anti-EV antibodies were prepared as taught in U.S. Pub. Pat. Appl. No. 2003/0200555. β-secretase cleaves between amino acids F and E of the NFEV cleavage site of APP_{NFEV} to produce a sAPPβ peptide with NF at the C-terminus and an EV40 or EV42 peptide with amino acids EV at the N-terminus. Anti-NF antibodies bind the C-terminal neoepitope NF at the C-terminus of the sAPPβ peptide produced by β-secretase cleavage of the NFEV sequence of APP_{NFEV}. Anti-EV antibodies bind the N-terminal neoepitope EV at the N-terminus of EV40 and EV42 produced by β-secretase cleavage of the NFEV sequence of APP_{NFEV}. Anti-Bio-G2-10 and anti-Bio-G2-11 antibodies are available from the Genetics Company, Zurich, Switzerland. Anti-Bio-G2-11 antibodies bind the neoepitope generated by the γ-secretase cleavage of Aβ or EV peptides at the 42 amino acid position. Anti-Bio-G2-10 antibodies bind the neoepitope generated by the γ-secretase cleavage of Aβ or EV peptides at the 40 amino acid position. Anti-6E10 antibodies are commercially available from Signet Laboratories, Inc., Dedham, MA. Anti-6E10 antibodies bind the epitope within amino acids 1 to 17 of the N-terminal region of the Aβ and the EV40 and EV42 peptides and also binds sAPPα because the same

epitope resides in amino acids 597 to 614 of sAPPα. Bio-M2 anti-FLAG antibodies are available from Sigma-Aldrich, St. Louis, MO.

Detecting sAPPβ. An AlphaScreen™ assay for detecting sAPPβ–NF produced from cleavage of APP_{NFEV} at the β-secretase cleavage site was performed as follows. Conditioned medium for each well was diluted 32-fold into a final volume of eight µL. As shown in Table 1, biotinylated-M2 anti-FLAG antibody, which binds the FLAG epitope of the APP_{NFEV}, was captured on streptavidin-coated donor beads by incubating a mixture of the antibody and the streptavidin coated beads for one hour at room temperature in AlphaScreen buffer. The amount of antibody was adjusted such that the final concentration of antibody in the detection reaction was 3 nM. Anti-NF antibody was similarly captured separately on protein-A acceptor beads in AlphaScreen™ buffer and used at a final concentration of 1 nM (Table 1). The donor and acceptor beads were each used at final concentrations of 20 µg/mL.

Table 1

Donor/Antibody Bead Mixture			Acceptor/Antibody Bead Mixture		
	Vol. (µL)	Final Conc. in 50 µL assay		Vol. (µL)	Final Conc. in 50 µL assay
Anti-Bio-Flag (16 µM)	1	3 nM	NF-IgG (1.1 µM)	5	1 nM
SA Coated Donor Beads (5 mg/mL)	23	20 µg/mL	Protein A Acceptor Beads (5 mg/mL)	23	20 µg/mL
Alpha Buffer	1131		Alpha Buffer	1127	
Final Vol.	1155		Final Vol.	1155	

Detecting EV42: Conditioned medium for each well was used neat (volume eight µL). As shown in Table 2, anti-Bio-G2-11 antibody was captured on streptavidin-coated donor beads by incubating a mixture of the antibody and the streptavidin coated beads for one hour at room temperature in AlphaScreen™ buffer. The amount of antibody was adjusted such that the final concentration of antibody in the detection reaction was 20 nM. Anti-EV antibody was similarly captured separately on protein-A acceptor beads in AlphaScreen™ buffer and used at a final concentration of 5 nM (Table 2). The donor and acceptor beads were used at final concentrations of 20 µg/mL.

Table 2

Donor/Antibody Bead Mixture			Acceptor/Antibody Bead Mixture		
	Vol. (µL)	Final Conc. in 50 µL assay		Vol. (µL)	Final Conc. in 50 µL assay
Anti-Bio-G2-11 (8.27 µM)	14	20 nM	EV-IgG (1.27 µM)	23	5 nM
SA Coated Donor Beads (5 mg/mL)	23	20 µg/mL	Protein A Acceptor Beads (5 mg/mL)	23	20 µg/mL
Alpha Buffer	1118		Alpha Buffer	1109	
Final Vol.	1155		Final Vol.	1155	

Detecting EV40: Conditioned medium for each well was diluted four-fold into a final volume eight µL. As shown in Table 3, anti-Bio-G2-10 antibody was captured on streptavidin-coated donor beads by incubating a mixture of the antibody and the streptavidin coated beads for one hour at room temperature in AlphaScreen™ buffer. The amount of antibody was adjusted such that the final concentration of antibody in the detection reaction was 20 nM. Anti-EV antibody was similarly captured separately on protein-A acceptor beads in AlphaScreen™ buffer and used at a final concentration of 5 nM. The donor and acceptor beads were used at final concentrations of 20 µg/mL.

Table 3

Donor/Antibody Bead Mixture			Acceptor/Antibody Bead Mixture		
	Vol. (µL)	Final Conc. in 50 µL assay		Vol. (µL)	Final Conc. in 50 µL assay
Anti-Bio-G2-10 (6.07 µM)	5	5 nM	EV-IgG (1.27 µM)	23	5 nM
SA Coated Donor Beads (5 mg/mL)	23	20 µg/mL	Protein A Acceptor Beads (5 mg/mL)	23	20 µg/mL
Alpha Buffer	1127		Alpha Buffer	1109	
Final Vol.	1155		Final Vol.	1155	

Detecting sAPPα: Conditioned medium for each well was diluted four-fold into a final volume eight µL. As shown in Table 4, Bio-M2 anti-FLAG antibody was captured on streptavidin-coated donor beads by incubating a mixture of the antibody and the streptavidin coated beads for one hour at room temperature in AlphaScreen™ buffer. Anti-6E10 antibody acceptor beads were obtained from the manufacturer (PerkinElmer, Inc., which makes the beads and conjugates antibody 6E10 to them). Antibody 6E10 (made by Signet Laboratories, Inc., a Covance Company, Dedham, MA) were used at 30 µg/ml final concentration. The donor beads were used at final concentrations of 20 µg/mL.

Table 4

Donor/Antibody Bead Mixture			Acceptor/Antibody Bead Mixture		
	Vol. (µL)	Final Conc. in 50 µL assay		Vol. (µL)	Final Conc. in 50 µL assay
Anti-Bio-Flag (16 µM)	1	5 nM	6E10-IgG (5 mg/mL)	34.65	30 µg/mL
SA Coated Donor Beads (5 mg/mL)	23	20 µg/mL			
Alpha Buffer	1131		Alpha Buffer	1120.35	
Final Vol.	1155		Final Vol.	1155	

About 15,200 single replicate pools of siRNAs were tested for modulation of sAPPβ, sAPPα, EV40 and EV42 by the AlphaScreen™ immunodetection method as described above. Based on the profile from this primary screen, 1,622 siRNA were chosen for an additional round of screening in triplicate. An siRNA was defined as "secretase-like" if a significant decrease in sAPPβ, EV40 and EV42 was detected, as well as either no change or an increase in sAPPα.

An siRNA was identified which inhibited an mRNA having a nucleotide sequence encoding a protein which had 100% identity to the nucleotide sequence encoding ADPRH. Compared to control non-silencing siRNAs (set to 100%), ADPRH siRNA pool significantly decreased EV40 (56.5%), EV42 (68.4%), while increasing sAPPα (339.1%).

The results are shown schematically in Figure 3 and show that ADPRH has a role in APP processing, in particular, the cleavage of APP at the β-secretase cleavage site, an event necessary in the processing of APP to Aβ peptide. Aβ peptide is a defining characteristic of Alzheimer's disease. Because of its role APP processing, ADPRH appears to have a role in the establishment or progression of Alzheimer's disease.

EXAMPLE 2

Because ADPRH appeared to have a role in APP processing to Aβ peptide and thus, a role in progression of Alzheimer's disease, expression of ADPRH was examined in a variety of tissues to determine whether ADPRH was expressed in the brain.

A proprietary database, the TGI Body Atlas, was used to show that the results of a microarray analysis of the expression of a majority of characterized genes, including ADPRH, in the human genome in a panel of different tissues. In this database, total RNA from multiple human tissue sources was subjected to microarray analysis to determine the expression levels of individual genes in various organs and tissues. Differences in hybridization intensities across tissues reflects the abundance of an RNA. ADPRH mRNA was found to be expressed in several organs, including the brain and within cortical structures which are subjected to amyloid Aβ deposition and Alzheimer pathology. The results are summarized in Figure 4. Arrows indicate brain expression levels.

The results strengthen the conclusion of the Example 1 that ADPRH has a role in APP processing and thus, a role in the establishment or progression of Alzheimer's disease.

EXAMPLE 3

The results of Examples 1 and 2 have shown that the ADPRH has a role in the establishment or progression of Alzheimer's disease. The results suggest that analytes that antagonize ADPRH activity will be useful for the treatment or therapy of Alzheimer's disease. Therefore, there is a need for assays for identifying analytes that antagonize ADPRH activity, for example, inhibit binding of ADPRH to its natural ligand or to BACE1. The following is an assay that can be used to identify analytes that antagonize ADPRH activity.

HEK293T/APP_{NFEV} cells are transfected with a plasmid encoding the human ADPRH or a homolog of the human ADPRH, for example, the primate, rodent, or other mammalian ADPRH, using a standard transfection protocols to produce HEK293T/APP_{NFEV}/ADPRH cells. For example, HEK293T/APP_{NFEV} are plated into a 96-well plate at about 8000 cells per well in 80 μL DMEM containing 10%FBS and antibiotics and the cell plate incubated at 37°C at 5% CO_2 overnight.

On the next day, a mixture of 600 µL Oligofectamine™ and 3000µL Opti-MEM® is made and incubated at room temperature for five minutes. Next, 23 µL Opti-MEM® is added to each well of a 96-well mixing plate. 50 ng pcDNA_ADPRH and empty control vector (in 1 µL volume) are added into adjacent wells of the mixing plate in an alternating fashion. The mixing plate is incubated at room temperature for five minutes. Next, 6 µL of the Oligofectamine™ mixture is added to each of the wells of the mixing plate and the mixing plate incubated at room temperature for five minutes. After five minutes, 20 µL of the plasmid/Oligofectamine™ mixture is added to the corresponding well in the plate of HEK293/APP_{NFEV} cells plated in the cell plate and the plates incubated overnight at 37°C in 5% CO_2.

The next day, the medium is removed from each well and replaced with 100 µL DMEM containing 10% FBS. Analytes being assayed for the ability to antagonize ADPRH-mediated activation of Aβ secretion are added to each well individually. The analytes are assessed for an effect on the APP processing to Aβ peptide in ADPRH transfected cells that is either minimal or absent in cells transfected with the vector-alone as follows. The cells are incubated at 37°C at 5% CO_2 overnight.

The next day, conditioned media is collected the amount of sAPPβ, EV42, EV40, and sAPPα in the conditioned media is determined as described in Example 1. Analytes that effect a decrease in the amounts of sAPPβ , EV42, and EV40 and either an increase or no change in the amount of sAPPα are antagonists of ADPRH. Viability of the cells is determined as in Example 1.

EXAMPLE 4

Analytes that alter secretion of EV40, EV42, sAPPa, or sAPPb only, or more, in the presence of ADPRH are considered to be modulators of ADPRH and potential therapeutic agents for treating ADPRH-related diseases. The following is an assay that can be used to confirm direct inhibition or modulation of ADPRH.

To confirm direct inhibition or modulation of ADPRH, ADPRH intracellular or extracellular domains are subcloned into expression plasmid vectors such that a fusion protein with C-terminal FLAG epitopes are encoded. These fusion proteins are purified by affinity chromatography, according to manufacturer's instructions, using an ANTI-FLAG M2 agarose resin. ADPRH fusion proteins are eluted from the ANTI-FLAG column by the addition of FLAG peptide (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys) (Sigma Aldrich, St. Louis, MO) re-suspended in TBS (50 mM Tris HCl pH 7.4, 150 mM NaCl) to a final concentration of 100 µg/ml. Fractions from the column are collected and concentrations of the fusion proteins determined by A280.

A PD-10 column (Amersham, Boston, MA) is used to buffer exchange all eluted fractions containing the ADPRH-fusion proteins and simultaneously remove excess FLAG peptide. The FLAG-ADPRH fusion proteins are then conjugated to the S series CM5 chip surface (Biacore™ International AB, Uppsala, Sweden) using amine coupling as directed by the manufacturer. A pH scouting protocol is followed to determine the optimal pH conditions for immobilization. Immobilization

is conducted at an empirically determined temperature in PBS, pH 7.4, or another similar buffer following a standard Biacore™ immobilization protocol. The reference spot on the CM5 chip (a non-immobilized surface) serves as background. A third spot on the CM5 chip is conjugated with bovine serum albumin in a similar fashion to serve as a specificity control. Interaction of the putative ADPRH modulating analyte identified in the assay of Example 3 at various concentrations and ADPRH are analyzed using the compound characterization wizard on the Biacore™ S51. Binding experiments are completed at 30°C using 50 mM Tris pH 7, 200 uM MnCl2 or MgCl2 (+ 5% DMSO) or a similar buffer as the running buffer. Prior to each characterization, the instrument is equilibrated three times with assay buffer. Default instructions for characterization are a contact time of 60 seconds, sample injection of 180 seconds and a baseline stabilization of 30 seconds. All solutions are added at a rate of 30 µL/min. Using the BioEvaluation software (Biacore™ International AB, Uppsala, Sweden), each set of sensorgrams derived from the ligand flowing through the ADPRH-conjugated sensor chip is evaluated and, if binding is observed, an affinity constant determined.

EXAMPLE 5

This example describes a method for making polyclonal antibodies specific for the ADPRH or particular peptide fragments or epitope thereof.

The ADPRH is produced as described in Example 1 or a peptide fragment comprising a particular amino acid sequence of ADPRH is synthesized and coupled to a carrier such as BSA or KLH. Antibodies are generated in New Zealand white rabbits over a 10-week period. The ADPRH or peptide fragment or epitope is emulsified by mixing with an equal volume of Freund's complete adjuvant and injected into three subcutaneous dorsal sites for a total of about 0.1 mg ADPRH per immunization. A booster containing about 0.1 mg ADPRH or peptide fragment emulsified in an equal volume of Freund's incomplete adjuvant is administered subcutaneously two weeks later. Animals are bled from the articular artery. The blood is allowed to clot and the serum collected by centrifugation. The serum is stored at -20°C.

For purification, the ADPRH is immobilized on an activated support. Antisera is passed through the sera column and then washed. Specific antibodies are eluted via a pH gradient, collected, and stored in a borate buffer (0.125M total borate) at ~0.25 mg/mL. The anti-ADPRH antibody titers are determined using ELISA methodology with free cS1P5 receptor bound in solid phase (1 pg/well). Detection is obtained using biotinylated anti-rabbit IgG, HRP-SA conjugate, and ABTS.

EXAMPLE 6

This example describes a method for making monoclonal antibodies specific for ADPRH.

BALB/c mice are immunized with an initial injection of about 1 µg of purified ADPRH per mouse mixed 1:1 with Freund's complete adjuvant. After two weeks, a booster injection of about 1

μg of the antigen is injected into each mouse intravenously without adjuvant. Three days after the booster injection serum from each of the mice is checked for antibodies specific for ADPRH.

The spleens are removed from mice positive for antibodies specific for the ADPRH and washed three times with serum-free DMEM and placed in a sterile Petri dish containing about 20 mL of DMEM containing 20% fetal bovine serum, 1 mM pyruvate, 100 units penicillin, and 100 units streptomycin. The cells are released by perfusion with a 23 gauge needle. Afterwards, the cells are pelleted by low-speed centrifugation and the cell pellet is resuspended in 5 mL 0.17 M ammonium chloride and placed on ice for several minutes. Then 5 mL of 20% bovine fetal serum is added and the cells pelleted by low-speed centrifugation. The cells are then resuspended in 10 mL DMEM and mixed with mid-log phase myeloma cells in serum-free DMEM to give a ratio of 3:1. The cell mixture is pelleted by low-speed centrifugation, the supernatant fraction removed, and the pellet allowed to stand for 5 minutes. Next, over a period of 1 minute, 1 mL of 50% polyethylene glycol (PEG) in 0.01 M HEPES, pH 8.1, at 37°C is added. After 1 minute incubation at 37°C, 1 mL of DMEM is added for a period of another 1 minute, then a third addition of DMEM is added for a further period of 1 minute. Finally, 10 mL of DMEM is added over a period of 2 minutes. Afterwards, the cells are pelleted by low-speed centrifugation and the pellet resuspended in DMEM containing 20% fetal bovine serum, 0.016 mM thymidine, 0.1 hypoxanthine, 0.5 μM aminopterin, and 10% hybridoma cloning factor (HAT medium). The cells are then plated into 96-well plates.

After 3, 5, and 7 days, half the medium in the plates is removed and replaced with fresh HAT medium. After 11 days, the hybridoma cell supernatant is screened by an ELISA assay. In this assay, 96-well plates are coated with the ADPRH. One hundred μL of supernatant from each well is added to a corresponding well on a screening plate and incubated for 1 hour at room temperature. After incubation, each well is washed three times with water and 100 μL of a horseradish peroxide conjugate of goat anti-mouse IgG (H+L), A, M (1:1,500 dilution) is added to each well and incubated for 1 hour at room temperature. Afterwards, the wells are washed three times with water and the substrate OPD/hydrogen peroxide is added and the reaction is allowed to proceed for about 15 minutes at room temperature. Then 100 μL of 1 M HCl is added to stop the reaction and the absorbance of the wells is measured at 490 nm. Cultures that have an absorbance greater than the control wells are removed to two cm^2 culture dishes, with the addition of normal mouse spleen cells in HAT medium. After a further three days, the cultures are re-screened as above and those that are positive are cloned by limiting dilution. The cells in each two cm^2 culture dish are counted and the cell concentration adjusted to 1×10^5 cells per mL. The cells are diluted in complete medium and normal mouse spleen cells are added. The cells are plated in 96-well plates for each dilution. After 10 days, the cells are screened for growth. The growth positive wells are screened for antibody production; those testing positive are expanded to 2 cm^2 cultures and provided with normal mouse spleen cells. This cloning procedure is repeated until stable antibody producing hybridomas are obtained. The stable hybridomas are progressively expanded to larger culture dishes to provide stocks of the cells.

Production of ascites fluid is performed by injecting intraperitoneally 0.5 mL of pristane into female mice to prime the mice for ascites production. After 10 to 60 days, 4.5 x 10^6 cells are injected intraperitoneally into each mouse and ascites fluid is harvested between 7 and 14 days later.

While the present invention is described herein with reference to illustrated embodiments, it should be understood that the invention is not limited hereto. Those having ordinary skill in the art and access to the teachings herein will recognize additional modifications and embodiments within the scope thereof. Therefore, the present invention is limited only by the claims attached herein.

WHAT IS CLAIMED:

1. A method for screening for analytes that antagonize processing of amyloid precursor protein (APP) to Aβ peptide, comprising:

(a) providing recombinant cells, which ectopically expresses ADPRH and the APP;

(b) incubating the cells in a culture medium under conditions for expression of the ADPRH and APP and which contains an analyte;

(c) removing the culture medium from the recombinant cells; and

(d) determining the amount of at least one processing product of APP selected from the group consisting of sAPPβ and Aβ peptide in the medium wherein a decrease in the amount of the processing product in the medium compared to the amount of the processing product in medium from recombinant cells incubated in medium without the analyte indicates that the analyte is an antagonist of the processing of the APP to Aβ peptide.

2. The method of Claim 1 wherein the recombinant cells each comprises a first nucleic acid that encodes ADPRH operably linked to a first heterologous promoter and a second nucleic acid that encodes an APP operably linked to a second heterologous promoter.

3. The method of Claim 2 wherein the APP is APP_{NFEV}.

4. The method of Claim 1 wherein a control is provided which comprises providing recombinant cells which ectopically express the APP but not the ADPRH.

5. A method for screening for analytes that antagonize processing of amyloid precursor protein (APP) to amyloid β (Aβ) peptide, comprising:

(a) providing recombinant cells, which ectopically express ADPRH and a recombinant APP comprising APP fused to a transcription factor that when removed from the APP during processing of the APP produces an active transcription factor, and a reporter gene operably linked to a promoter inducible by the transcription factor;

(b) incubating the cells in a culture medium under conditions for expression of the ADPRH and recombinant APP and which contains an analyte; and

(c) determining expression of the reporter gene wherein a decrease in expression of the reporter gene compared to expression of the reporter gene in recombinant cells in a culture medium without the analyte indicates that the analyte is an antagonist of the processing of the APP to Aβ peptide.

6. A method for treating Alzheimer's disease in an individual comprising providing to the individual an effective amount of an antagonist of ADPRH activity.

7. A method for identifying an individual who has Alzheimer's disease or is at risk of developing Alzheimer's disease comprising obtaining a sample from the individual and measuring the amount of ADPRH in the sample.

8. The use of an antagonist of ADPRH for the manufacture of a medicament for the treatment of Alzheimer's disease.

9. The use of an antibody specific for ADPRH for the manufacture of a medicament for the treatment of Alzheimer's disease.

10. A vaccine for preventing and/or treating Alzheimer's disease in a subject, comprising an antibody raised against an antigenic amount of ADPRH wherein the antibody antagonizes the processing of APP to $A\beta$ peptide.

(SEQ ID NO:1)

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FIG.1

TCCCTTCCTTCTCCTTTTGAGAAAACCACAGAACTATGACTCTCATGATTTTTTCCTTTGTGAAATCCCTGGTTT
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FIG.1 (cont.)

(SEQ ID NO:2)

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FIG.2

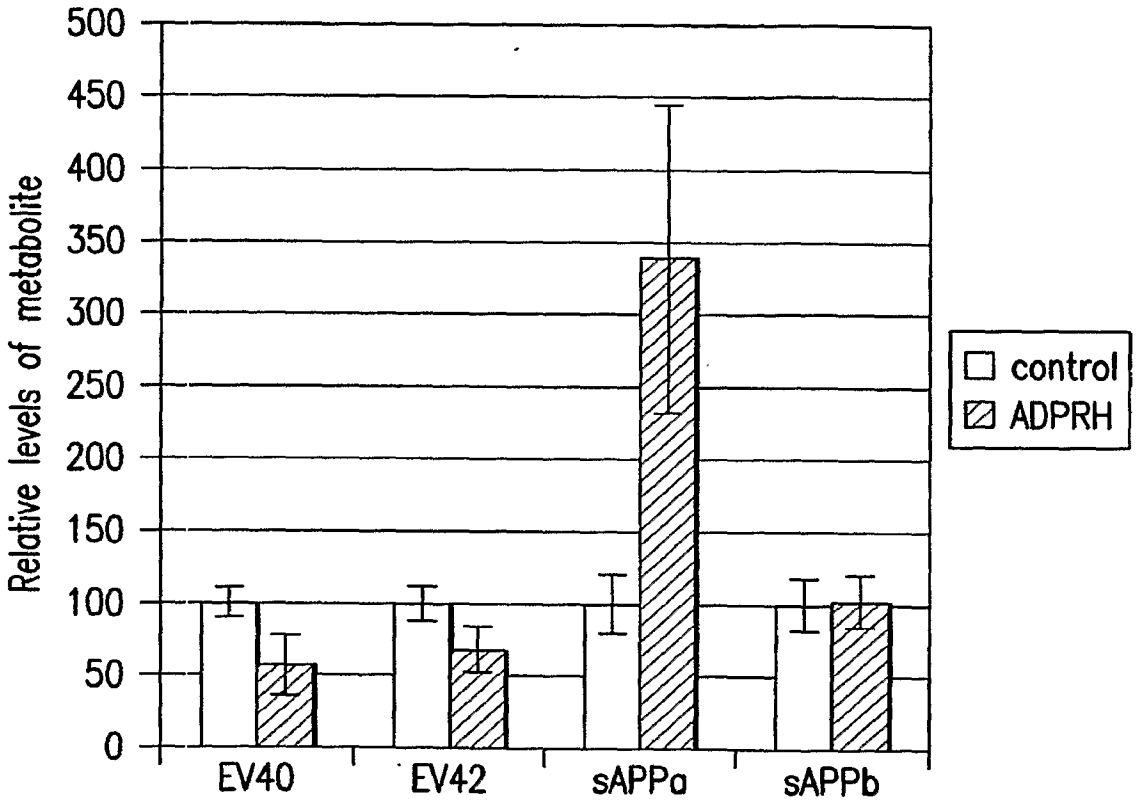
500
450
400
350
300
250
200
150
100
50
0
Relative levels of metabolite
EV40
EV42
sAPPα
sAPPb
control
ADPRH

FIG.3

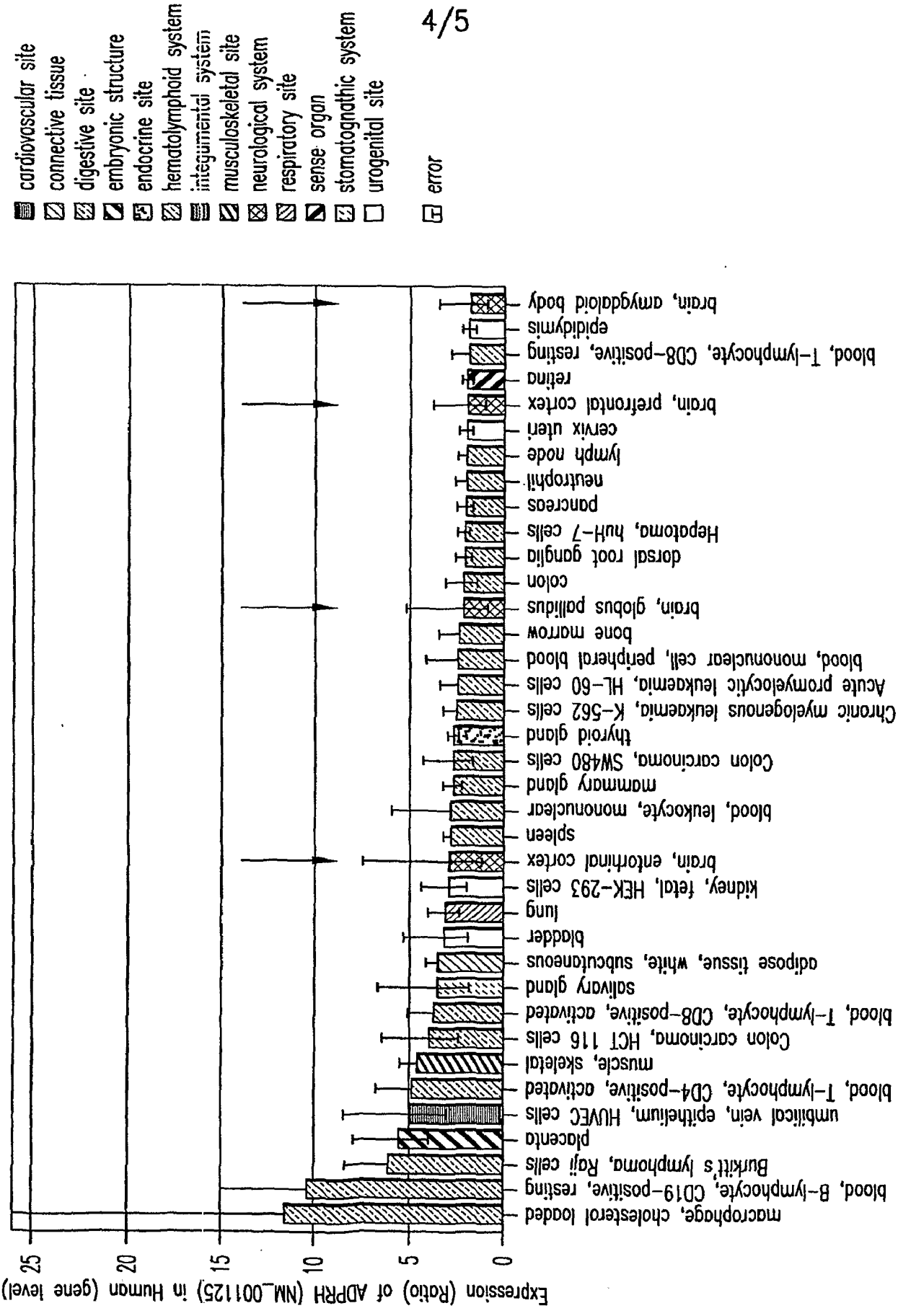
Expression (Ratio) of ADPRH (NM_001125) in Human (gene level)
0
5
10
15
20
25
macrophage, cholesterol loaded
blood, B-lymphocyte, CD19-positive, resting
Burkitt's lymphoma, Raji cells
placenta
umbilical vein, epithelium, HUVEC cells
blood, T-lymphocyte, CD4-positive, activated
muscle, skeletal
Colon carcinoma, HCT 116 cells
blood, T-lymphocyte, CD8-positive, activated
salivary gland
adipose tissue, white, subcutaneous
bladder
lung
kidney, fetal, HEK-293 cells
brain, entorhinal cortex
spleen
blood, leukocyte, mononuclear
mammary gland
Colon carcinoma, SW480 cells
thyroid gland
Chronic myelogenous leukaemia, K-562 cells
Acute promyelocytic leukaemia, HL-60 cells
blood, mononuclear cell, peripheral blood
bone marrow
brain, globus pallidus
colon
dorsal root ganglia
Hepatoma, huH-7 cells
pancreas
neutrophil
lymph node
cervix uteri
brain, prefrontal cortex
retina
blood, T-lymphocyte, CD8-positive, resting
epididymis
brain, amygdaloid body
cardiovascular site
connective tissue
digestive site
embryonic structure
endocrine site
hematolymphoid system
integumental system
musculoskeletal site
neurological system
respiratory site
sense organ
stomatognathic system
urogenital site
error

FIG.4A

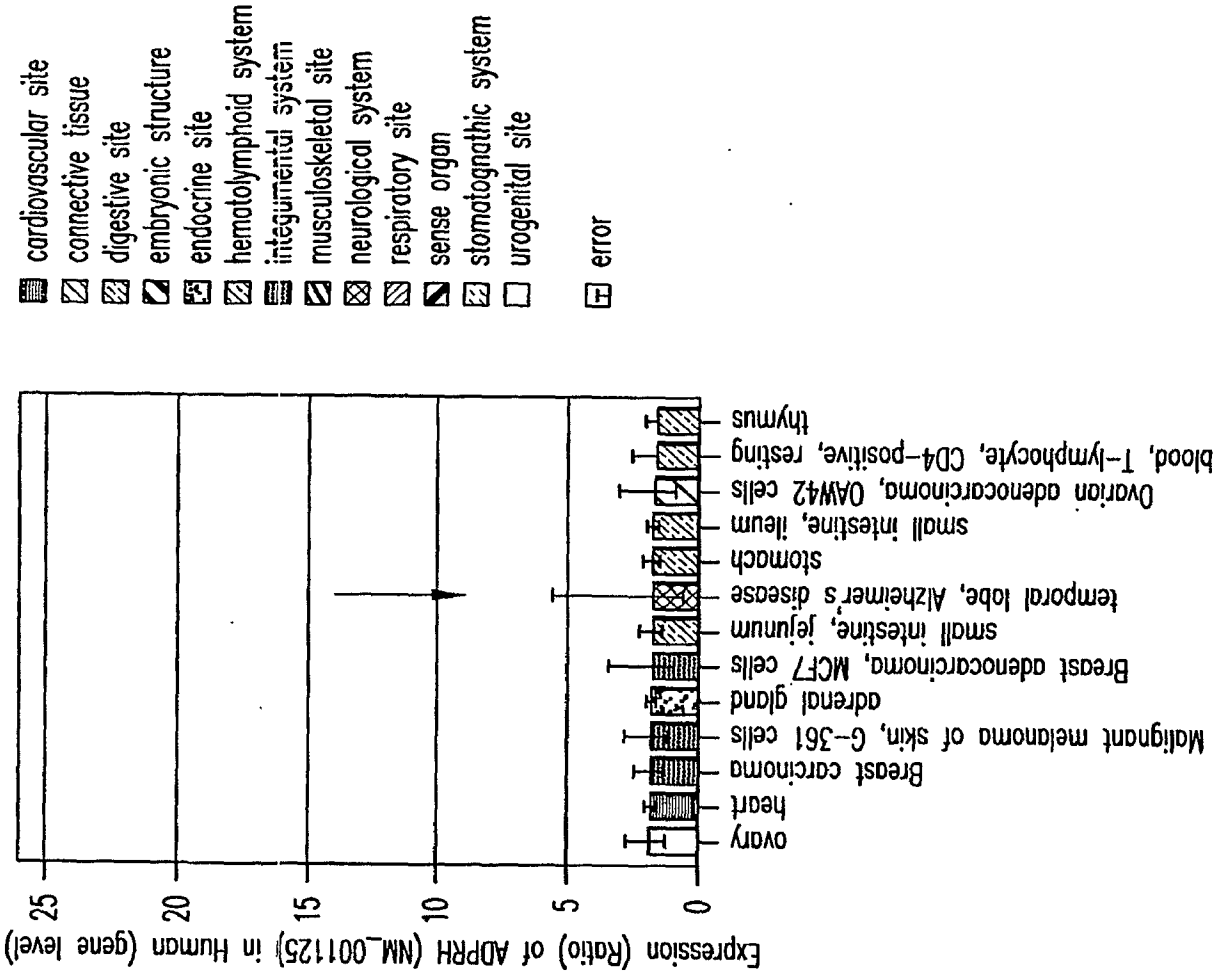
cardiovascular site
connective tissue
digestive site
embryonic structure
endocrine site
hematolymphoid system
integumental system
musculoskeletal site
neurological system
respiratory site
sense organ
stomatognathic system
urogenital site
error
Expression (Ratio) of ADPRH (NM_001125) in Human (gene level)
0
5
10
15
20
25
ovary
heart
Breast carcinoma
Malignant melanoma of skin, G-361 cells
adrenal gland
Breast adenocarcinoma, MCF7 cells
small intestine, jejunum
temporal lobe, Alzheimer's disease
stomach
small intestine, ileum
Ovarian adenocarcinoma, OAW42 cells
blood, T-lymphocyte, CD4-positive, resting
thymus

FIG.4B

SEQUENCE LISTING

<110> Merck & Co., Inc.
Majercak, John M.
Ray, William J.
Stone, David J.

<120> METHOD FOR IDENTIFYING MODULATORS OF ADPRH USEFUL FOR TREATING ALZHEIMER'S DISEASE

<130> 21979 PCT

<150> US60/748,465
<151> 2005-12-08

<160> 2

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35 40 45
Gly Arg Trp Arg Val Ser Asp Asp Thr Val Met His Leu Ala Thr Ala
50 55 60
Glu Ala Leu Val Glu Ala Gly Lys Ala Pro Lys Leu Thr Gln Leu Tyr
65 70 75 80
Tyr Leu Leu Ala Lys His Tyr Gln Asp Cys Met Glu Asp Met Asp Gly
85 90 95
Arg Ala Pro Gly Gly Ala Ser Val His Asn Ala Met Gln Leu Lys Pro
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Gly Lys Pro Asn Gly Trp Arg Ile Pro Phe Asn Ser His Glu Gly Gly
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Cys Gly Ala Ala Met Arg Ala Met Cys Ile Gly Leu Arg Phe Pro His
130 135 140
His Ser Gln Leu Asp Thr Leu Ile Gln Val Ser Ile Glu Ser Gly Arg
145 150 155 160
Met Thr His His His Pro Thr Gly Tyr Leu Gly Ala Leu Ala Ser Ala
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Leu Phe Thr Ala Tyr Ala Val Asn Ser Arg Pro Pro Leu Gln Trp Gly
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Lys Gly Leu Met Glu Leu Leu Pro Glu Ala Lys Lys Tyr Ile Val Gln
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            260                 265                 270
Asp Ala Pro Met Ile Ala Tyr Asp Ala Val Leu Ala Ala Gly Asp Ser
        275                 280                 285
Trp Lys Glu Leu Ala His Arg Ala Phe Phe His Gly Gly Asp Ser Asp
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Thr Val Ile Ser Leu
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