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(54) **Title:** A NOVEL APP MUTATION ASSOCIATED WITH AN UNUSUAL ALZHEIMER'S DISEASE PATHOLOGY

(57) **Abstract:** The invention relates to the field of Alzheimer's Disease (AD). In particular, the invention provides a novel mutation (T714I) identified in the amyloid precursor protein (APP), APP714, which leads to a very aggressive form of AD. The mutation involves the 43rd codon of the amyloid β peptide (Aβ) corresponding to the putative gamma 42-secretase cleavage site. The novel mutation alters both Aβ40 and Aβ42 secretion elevating the Aβ42/Aβ40 ratio by 10-fold in vitro. Furthermore, the main amyloid plaque pathology in brains of these patients is of the diffuse 'pre-amyloid' type composed primarily of N-truncated Aβ42. Dense-cored plaques although not absent, were significantly reduced. Also, the usual sites in brain where Aβ40 is predominantly deposited, for instance, in vessels as cerebral amyloid angiopathy (CAA) or senile plaque cores, were composed entirely of Aβ42 form. Together, these indicate that deposition of N-truncated Aβ42 in one of the earliest amyloid deposited in brain, the diffuse plaques, is fully competent of inciting AD either through the well-established 'amyloid cascade' or by a yet unknown mechanism(s).

A novel APP mutation associated with an unusual Alzheimer's disease pathology

Field of the invention

The invention relates to the field of Alzheimer's Disease (AD). In particular, the invention provides a novel mutation (T714I) identified in the amyloid precursor protein (APP), APP714, which leads to a very aggressive form of AD. The mutation involves the 43rd codon of the amyloid ß peptide (Aß) corresponding to the putative γ_{42}-secretase cleavage site. The novel mutation alters both Aß$_{40}$ and Aß$_{42}$ secretion elevating the Aß$_{42}$/Aß$_{40}$ ratio by 10-fold *in vitro*. Furthermore, the main amyloid plaque pathology in brains of these patients is of the diffuse 'pre-amyloid' type composed primarily of N-truncated Aß$_{42}$. Dense-cored plaques although not absent, were significantly reduced. Also, the usual sites in brain where Aß$_{40}$ is predominantly deposited, for instance, in vessels as cerebral amyloid angiopathy (CAA) or senile plaque cores, were composed entirely of Aß$_{42}$ form. Together, these indicate that deposition of N-truncated Aß$_{42}$ in one of the earliest amyloid deposited in brain, the diffuse plaques, is fully competent of inciting AD either through the well-established 'amyloid cascade' or by a yet unknown mechanism(s).

Background of the invention

Alzheimer's disease (AD) is a progressive neurodegenerative disorder defined histologically by the formation in the brain of intracellular neurofibrillary tangles and extracellular amyloid deposits. Particular attention has been focused on the role that the amyloid β-protein (Aβ) plays in the development of AD. Indeed, the predominant protein component of the cortical and cerebrovascular amyloid deposits of AD is the Aß[1-3]. Accumulating evidence suggests that Aß production from amyloid precursor protein (APP)[1-3], its aggregation into fibrils and its deposition are key etiological events in AD[4]. An understanding of these critical steps will be crucial in determining therapeutic targets. There are at least five distinct isoforms of APP: 563, 695, 714, 751, and 770 amino acids, respectively (Wirak et al. (1991) *Science* 253:323). These isoforms of APP are generated by alternative splicing of primary transcripts of the APP gene, which is located on human chromosome 21.

The APP protein is processed by ß secretase (BACE)[5] and as yet unidentified α secretase leading to soluble APP (APPs$_{\alpha}$ and APPs$_{ß}$) and membrane-bound

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C-terminal fragments (α and ß CTFs; for review see reference[6]). While cleavage by ß- and one or many γ-secretase(s) (γ_{40} and γ_{42}) releases 40-42 amino acids Aß peptides (Aß$_{1-40}$ and Aß$_{1-42}$), the major secretory pathway utilizes α secretase that cleaves the Aß sequence between amino acid 16 and 17 of Aß. Further processing of the αCTFs by γ_{40} or γ_{42} secretases releases N-terminally cleaved Aß$_{17-40}$ or Aß$_{17-42}$ peptides (p3). In addition to Aß$_{17-X}$, two other major peptides resulting from activity of ß secretase are noted to be secreted by transfected cells and deposited in brain starting from amino acid 5 (Aß$_{5-X}$) and 11 (Aß$_{11-X}$)[6,7].

As yet, eight missense mutations were identified in APP in families with autosomal dominant early-onset AD[8,9]. All these mutations are clustering in close proximity of the secretase cleavage sites and affect APP metabolism in two distinct ways. The K670N/M671L mutation located near the ß-secretase cleavage site[10], increases the production of both Aß$_{40}$ and Aß$_{42}$[11-13]. In contrast, mutations located near the γ-secretase cleavage sites result in an increased absolute or relative production of Aß$_{42}$ while the total amount of Aß is either unaffected as in APP V717I[12-14] or decreased as in APP V715M[15]. In contrast, mutations in the presenilin genes (PSEN1 and 2) causing autosomal dominant early onset AD[8,9], lead to a preferential increase in Aß$_{42}$. This is of consequence to AD pathology as *in vitro* Aß$_{42}$ was demonstrated to be more fibrillogenic and to aggregate faster than Aß$_{40}$[16].

Immunohistochemistry showed that, although Aß$_{42}$ is deposited first as diffuse plaques in AD and in Down's syndrome (DS) patients[17], Aß$_{40}$ contributes to further growth of plaques resulting in the formation of dense-cored senile plaques[18]. Aβ_{40} is also the predominant constituent of the amyloid deposits in blood vessel walls[19-22]. The amyloid deposited in congophilic dense-cored plaques is certainly fibrillar as it demonstrates Thioflavin-T and Congo red binding. Since most of the neuritic pathology in brain is in

apolipoprotein E (APOE) binding[28,29]. In this respect, an isoform of APOE predisposes carriers of this allele to the common late-onset form of the disease.
At present there is no known effective therapy for Alzheimer's disease. It is important to develop experimental models of AD that can be used to define further the underlying
5 biochemical events involved in AD pathogenesis. Such models can be employed, in one application, to screen for molecules that inhibit, prevent, or reverse the progression of AD.
The present invention provides a novel, mutated nucleic acid encoding human amyloid precursor protein 770 wherein the nucleic acid at codon 714 encodes an Isoleucine (I)
10 instead of Threonine (T). This mutation (APPT714I) is the most 'drastic' mutation in APP described so far. In brain of AD patients with this mutation, the main amyloid plaque pathology is of non-congophilic pre-amyloid nature composed primarily of N-truncated $Aß_{42}$ ($Aß_{X-42}$) in the absence of $Aß_{40}$. These observations implicate a key role for diffuse pre-amyloid $Aß_{X-42}$, rather than or in addition to the fibrillar full-length forms
15 of $Aß_{42}$ and $Aß_{40}$, in neuronal toxicity and cognitive decline in AD patients. Whatever the precise pathogenic mechanisms by which T714I causes AD, it is likely that this unusual APP mutation will help elucidating the mechanism(s) through which AD occurs.

20 **Aims of the invention**

The present invention aims at providing an isolated polynucleotide sequence encoding a codon 714 mutant of human amyoid precursor protein 770, which was identified in patients with a very aggressive form of Alzheimer's disease. In particular, codon 714 of APP770 codes for leucine. The invention further provides a transgenic eukaryotic
25 primary cell, embryonic stem cell line or an immortalized cell line comprising a codon 714 mutant of APP770. The invention also aims at providing a non-human transgenic animal that expresses the codon 714 mutant of APP. Another aim of the invention is to provide methods for screening, in cells and animals, of molecules that can reduce the formation of (N-truncated) beta-amyloid 42 peptide. The invention further aims at using
30 the non-human transgenic animal for the analysis and/or interference of the formation of 'cloudy' diffuse plaques. Finally, the invention aims at providing an assay for the screening of alternative proteases of amyloid beta, gamma-secretase homologues and/or gamma-secretase modulators.

Figure legends

Figure 1: (A) Pedigree of AD156 segregating the APP T714I mutation. Solid symbols indicate affected individuals; †: age at death; arrow denotes the proband where autopsy was performed. **(B)** Sequence analysis for 156.1 and 156.2 showing a heterozygous C to T transition at position 2208 of the cDNA leading to an amino acid substitution of threonine (T) to isoleucine (I) at codon 714 in exon 17 of APP (numbering according to APP770 isoform). **C.** PCR-RFLP analysis of PCR amplified APP exon 17 product followed by *Tsp*RI digestion.

Figure 2: Analysis of soluble APP ($APPs_{\alpha}$ and $APPs_{ß}$). Supernatants were resolved on a NuPAGE gel, and immunoblotted with mAb 6E10 for $APPs_{\alpha}$ (**A**) or 53/4 for $APPs_{ß}$ (**B**). Bands for T714I and WT were quantified using the NIH Image software package (data not shown).

Figure 3: (A) Representative MALDI-TOF mass spectra for APP WT and T714I. Conditioned medium of HEK293T cells transfected with T714I and WT APP were studied by IP/MS using mAb 4G8. Relative peak intensities were normalized with synthetic Aß (12-28) peptide (marked: std) and identities of the observed peaks were inferred as described in Methods. Note that predominant peaks for peptides ending at residue 40 are clearly lost for T714I **(B)** Effect on secreted Aß with different N and C termini were analysed in detail. These were peptides ending at $Aß_{42}$, $Aß_{40}$, $Aß_{39}$, $Aß_{38}$, and $Aß_{37}$ and beginning at E1 ($Aß_{1\text{-}X}$), R5 ($Aß_{5\text{-}X}$), E11 ($Aß_{11\text{-}X}$), and L17 ($Aß_{17\text{-}X}$). Bars are equally scaled to allow inter-panel comparison. Absence of few bars in the WT is due to peaks too low to be measured. *: statistical significance of at least <0.001 versus WT.

Detailed description of the invention

The present invention provides model systems of Alzheimer's disease, wherein the model system comprises a DNA sequence encoding an amyloid precursor protein (APP) isoform or fragment that has an amino acid other than threonine at the amino acid position corresponding to amino acid residue position 714 of APP770.

The APPT714I mutation in an Austrian pedigree is the first APP mutation reported to date that involves amino acid 43 of Aß located directly at the γ_{42} secretase cleavage site. Patients carrying this mutation have a very early onset of AD around 34-35 years, the earliest onset age ever reported for autosomal dominant AD associated with

mutations in APP. The early onset age, as well as rapid progression of the disease, and early death, is comparable to AD caused by mutations in PSEN1[9].

In vitro the T714I mutation drastically affected the γ-secretase cleavage of APP significantly decreasing $Aß_{40}$ and increasing $Aß_{42}$. This effect was not limited to full-length Aß, but also involved N-truncated Aß peptides. Besides a major effect in altering secretion of $Aß_{X\text{-}40}$ and $Aß_{X\text{-}42}$, T714I mutation also lead to significant alternative processing of APP to Aß peptides ending at residues G38 ($Aß_{38}$) and V39 ($Aß_{39}$). The increase by ~2.5-fold of total Aß ($Aß_{X\text{-}X}$) and an unaltered $APPs_{\alpha}$ and $APPs_{ß}$ indicates that processing of CTFs can be a rate-limiting step in APP processing.

A number of terms and expressions are used throughout the detailed description and, to facilitate the understanding thereof, the following definitions are provided:

As used herein, the words "an isolated polynucleotide sequence" may be interpreted to mean the DNA and cDNA sequence as detailed by Yoshikai et al. (1990) *Gene* 87:257, together with the promoter DNA sequence as described by Salbaum et al. (1988) *EMBO J.* 7(9): 2807

As used herein, "isoform", "APP", and "APP isoform" refer to a polypeptide that is encoded by at least one exon of the APP gene (Kitaguchi et al. (1988) *Nature* 331:530; de Sauvage and Octave (1989) *Science* 245:651; Golde et al. (1990) *Neuron* 4:253). An APP isoform may be encoded by an APP allele (or exon thereof) that is associated with a form of AD or that is not associated with an AD disease phenotype.

As used herein, "fragment" refers to a polypeptide of at least about 9 amino acids, typically 50 to 75, or more, wherein the polypeptide contains an amino acid core sequence. A fragment may be a truncated APP isoform, modified APP isoform (as by amino acid substitutions, deletions, or additions outside of the core sequence), or other variant polypeptide sequence, but is not a naturally-occurring APP isoform that is present in a human individual, whether affected by AD or not. If desired, the fragment may be fused at either terminus to additional amino acids, which may number from 1 to 20, typically 50 to 100, but up to 250 to 500 or more.

As used herein, "APP770" refers to the 770 amino acid residue long polypeptides encoded by the human APP gene.

As used herein, "codon 714" refers to the codon (i.e., the trinucleotide sequence) that encodes the 714th amino acid position in APP770, or the amino acid position in an APP isoform or fragment that corresponds to the 714th position in APP770. For example but not limitation, a 670 residue long fragment that is produced by truncating

APP770 by removing the 100 N-terminal amino acids has its 614th amino acid position corresponding to codon 714.

As used herein, "human APP isoform or fragment" refers to an APP isoform or fragment that contains a sequence of at least 9 consecutive amino acids that is
5 identical to a sequence in a human APP770, APP751, or APP695 protein that occurs naturally in a human individual, and wherein an identical sequence is not present in an APP protein that occurs naturally in a non-human species.

A nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For instance, a promoter or enhancer is operably
10 linked to a coding sequence if it affects the transcription of the sequence. With respect to transcription regulatory sequences, operably linked means that the DNA sequences being linked are contiguous and, where necessary to join two protein coding regions, contiguous and in reading frame.

As used herein, the term "mutant" refers to APP alleles having missense mutations
15 that are pathognomonic for a genetic predisposition for developing AD; specifically a mutation at codon 714 (as referenced by the amino acid sequence in APP770) of the APP gene, such that codon 714 encodes one of the nineteen amino acids that are not threonine (i.e., valine, glycine, methionine, alanine, serine, isoleucine, leucine, proline, histidine, cysteine, tyrosine, phenylalanine, glutamic acid, tryptophan, arginine,
20 aspartic acid, asparagine, lysine, and glutamine), but preferably isoleucine. Thus the mutant APP770 polypeptide of the present invention is an APP770 polypeptide that has an amino acid residue at position 714 that is not threonine. Other mutant APP isoforms comprise a non-threonine amino acid at the amino acid residue position that corresponds to codon 714 (i.e., that is encoded by codon 714). Similarly, a mutant
25 APP allele or a variant APP codon 714 allele is an APP allele that encodes an amino acid other than threonine at codon 714 (referenced to the human APP770 deduced translation as described in the "codon 714" definition), preferably isoleucine. Hence, an APP allele that encodes threonine at codon 714 is a "wild-type" APP allele. The sequence of APPT714I is depicted in SEQ ID NO: 1.

30 In a first embodiment, the present invention provides an isolated polynucleotide sequence that encodes an APP isoform or fragment that has an amino acid other than threonine at the amino acid position corresponding to amino acid residue position 714 of APP770, and in a specific embodiment of this invention this amino acid is an isoleucine.

In another embodiment the isolated polynucleotide sequence that encodes said mutant APP isoform or fragment is operably linked with a promotor.

In another embodiment, the present invention provides a human APP isoform or fragment, free from other human proteins, that has an amino acid other than threonine at the amino acid position corresponding to amino acid residue position 714 of APP770. As a not limited example of an expression system, baculovirus expression systems are useful for high level expression of heterologous genes in eukaryotic cells and Knops et al. (1991) *J. Biol. Chem.* 266(11): 7285) describe the expression of APP using said expression system.

In another embodiment, the present invention provides recombinant bacteria and cells, typically eukaryotic cells and preferably mammalian cells and more preferentially of the neural, glial, or astrocytic lineage, that have been transformed or transfected with a heterologous DNA sequence comprising the above described APP714 mutation, or have been derived from a transgenic non-human animal, wherein the cells express an APP isoform or fragment that has an amino acid other than threonine at the amino acid position corresponding to amino acid residue position 714 of APP770. In accordance with standard protocols, cultured human cells, either primary cultures or immortalized cell lines, may be transfected, either transiently or stably, with a mutant APP714 allele so that the cultured human cell expresses said mutant APP714 polypeptide.

In a specific embodiment the cells can also be naturally derived. For this, blood samples or fibroblasts from the affected subject, diagnosed with the APP714 mutation, must be obtained in order to provide the necessary cells which can be permanently transformed into a lymphoblastoid cell line using, for example, Epstein-Barr virus. Once established, such cell lines can be grown continuously in suspension culture and may be used for a variety of *in vitro* experiments to study mutant APP714 expression and processing.

Since the APP714 mutation is dominant, an alternative method for constructing a cell line is to engineer genetically a mutated gene, or a portion thereof spanning codon 714, into an established (either stably or transiently) cell line of choice. Sisodia (1990) *Science* 248:492) has described the insertion of a normal APP gene, by transfection, into mammalian cells. Oltersdorf et al. (1990) *J. Biol. Chem.* 265:4492) describe the insertion of APP into immortalized eukaryotic cell lines.

In another embodiment, the present invention provides a transgenic non-human animal that carries in its somatic and germ cells at least one integrated copy of a human DNA sequence that encodes an APP isoform or fragment that has an amino acid other than threonine at the amino acid position corresponding to amino acid residue position 714 of APP770.

It is expected that the transgenic non-human animal, for example a transgenic mouse, will have a particular value because likewise in the human brain of an AD patient with the APP714 mutation, an exclusive deposition of $A\beta_{42}$ is expected. Overexpression of mutant APP with or without mutant presenilin 1 in mice shows a predominance of $A\beta_{40}$ deposition into dense-cored plaques (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&list_uids=10448051&dopt=Abstract). It also seems that in rodent brain $A\beta_{40}$, but not $A\beta_{42}$, is easily depositable as congophilic amyloid deposits (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&list_uids=9334394&dopt=Abstract) that is important in the light that $A\beta_{42}$, but not $A\beta_{42}$, is proposed to be central to the aetiology of AD (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&list_uids=10196523&dopt=Abstract). In the current invention, both neuronal and non-neuronal recombinant cells for the mutant APP714, increases $A\beta_{42}$ secretion while decreasing $A\beta_{40}$. In brains of the affected patients there is almost no deposition of $A\beta_{40}$ or $A\beta_{39}$ plaques being composed entirely of $A\beta_{42}$. In conclusion, the novel transgenic mouse would mimic more the human disease phenotype of AD because in humans the deposition of $A\beta_{42}$ always supersedes the deposition of $A\beta_{40}$.

In a preferred example it may be possible to excise the mutated APP714 gene for use in the creation of transgenic animals containing the mutated gene. In another example, an entire human APP714 allele may be cloned and isolated, either in parts or as a whole, in a cloning vector (e.g. cosmid or yeast or human artificial chromosome). The human variant APP714 gene, either in parts or in whole, may be transferred to a host non-human animal, such as a mouse or a rat. As a result of the transfer, the resultant transgenic non-human animal will preferably express one or more mutant APP714 polypeptides. Most preferably, a transgenic non-human animal of the invention will express one or more mutant APP714 polypeptides in a neuron-specific manner (Wirak et al. (1991) *EMBO J.* 10:289). This may be accomplished by transferring substantially the entire human APP gene (encoding a codon 714 mutant) including the 4.5 kilobase

sequence that is adjacent to and upstream of the first major APP transcriptional start site.

Alternatively, one may design minigenes encoding variant APP codon 714 polypeptides. Such mini-genes may contain a cDNA sequence encoding a variant APP
5 codon 714 polypeptide, preferably full-length, a combination of APP gene exons, or a combination thereof, linked to a downstream polyadenylation signal sequence and an upstream promoter (and preferably enhancer). Such a mini-gene construct will, when introduced into an appropriate transgenic host (e.g., mouse or rat), express an encoded variant APP codon 714 polypeptide, most preferably a variant APP codon
10 714 polypeptide that contains an isoleucine at codon 714 of APP770 or the corresponding position in an APP isoform or fragment.

Another approach to create transgenic animals is to target a mutation to the desired gene by homologous recombination in an embryonic stem (ES) cell line in vitro followed by microinjection of the modified ES cell line into a host blastocyst and
15 subsequent incubation in a foster mother (see Frohman and Martin (1989) *Cell* 56:145). Alternatively, the technique of microinjection of the mutated gene, or a portion thereof, into a one-cell embryo followed by incubation in a foster mother can be used. Various uses of transgenic animals, particularly transgenic animals that express a wild-type APP isoform or fragment, are disclosed in Wirak et al. (1991) *EMBO J.*, 10(2):289;
20 Schilling et al. (1991) *Gene* 98(2):225; Quon et al. (1991) *Nature* 352:239; Wirak et al. (1991) *Science* 253:323; and Kawabata et al. (1991) *Nature* 354:476. Additional methods for producing transgenic animals are known in the art.

Alternatively, site-directed mutagenesis and/or gene conversion can be used to mutate a murine (or other non-human) APP gene allele, either endogenous or transfected,
25 such that the mutated allele does not encode threonine at the codon position in the mouse APP gene that corresponds to codon 714 (of APP770) of the human APP gene (such position is readily identified by homology matching of the murine APP gene or APP protein to the human APP gene or APP770 protein). Preferably, such a mutated murine allele would encode isoleucine at the corresponding codon position.

30 The procedure for generating transgenic rats is similar to that of mice (Hammer et al., Cell 63; 1099-112 (1990)). Thirty day-old female rats are given a subcutaneous injection of 20 IU of PMSG (0.1 cc) and 48 hours later each female placed with a proven male. At the same time, 40-80 day old females are placed in cages with vasectomized males. These will provide the foster mothers for embryo transfer. The

next morning females are checked for vaginal plugs. Females who have mated with vasectomized males are held aside until the time of transfer. Donor females that have mated are sacrificed (CO_2 asphyxiation) and their oviducts removed, placed in DPBS (Dulbecco's phosphate buffered saline) with 0.5% BSA and the embryos collected. 5 Cumulus cells surrounding the embryos are removed with hyaluronidase (1 mg/ml). The embryos are then washed and placed in EBSS (Earle's balanced salt solution) containing 0.5% BSA in a 37.5 °C incubator until the time of microinjection.

Once the embryos are injected, the live embryos are moved to DPBS for transfer into foster mothers. The foster mothers are anesthetized with ketamine (40 mg/kg, ip) and 10 xylazine (5 mg/kg, ip). A dorsal midline incision is made through the skin and the ovary and oviduct are exposed by an incision through the muscle layer directly over the ovary. The ovarian bursa is torn, the embryos are picked up into the transfer pipet, and the tip of the transfer pipet is inserted into the infundibulum. Approximately 10-12 embryos are transferred into each rat oviduct through the infundibulum. The incision is 15 then closed with sutures, and the foster mothers are housed singly.

In another embodiment of the invention transgenic cell lines transfected with a polynucleotide sequence, or an isoform or fragment thereof, encoding a codon 714 mutant of human amyloid precursor protein 770 can be used in a drug screening assay for the screening of molecules and monitoring the effectiveness of said molecules. 20 Additionally, such model systems provide a tool for defining the underlying biochemistry of APP and β-amyloid metabolism, which thereby provides a basis for rational drug design. The specific effectiveness of a molecule is monitored by measuring the lower formation of the $A\beta_{42}$ peptide or N-truncated $A\beta_{42}$ peptide as compared with the amount of the $A\beta_{42}$ peptide or N-truncated $A\beta_{42}$ peptide without 25 administering said molecule. Suitable molecules may be small molecules, biological polymers, such as polypeptides, polysaccharides, polynucleotides, and the like. Small molecules, e.g. small organic molecules, and other drug candidates can be obtained, for example, from combinatorial and natural product libraries. The test compounds will typically be administered to the culture medium at a concentration in the range from 30 about 1nM to 1mM, usually form about 10 μM to 1mM.

Monitoring of the $A\beta_{42}$ peptide formation and/or truncated forms thereof can be measured using standard biochemical techniques, but preferentially with an ELISA assay. These assays may be performed using conventional techniques developed for these purposes in the course of screening.

To perform drug-screening assays, it is feasible to accommodate automation of the assay. Interaction (e.g., binding of) between the recombinant cells and the target molecules can be accomplished in any vessel suitable for containing the reactants. Examples of such vessels include microtiter plates, test tubes, and microcentrifuge tubes.

Also within the scope of the screening assay are oligonucleotide sequences that include anti-sense RNA and DNA molecules that function to inhibit the translation of specific mRNA targets in the recombinant cell lines. It is very well possible that such a target could for example be a ß-secretase homologue or modulator of said ß - secretase (homologue) or a protease involved in the generation of truncated forms of the $A\beta_{42}$ peptide such as N-truncated $A\beta_{42}$ peptides. For example anti-sense RNA molecules may be generated in a library prepared by any method known in the art for the synthesis of gene libraries. Alternatively said anti-sense sequences can be constructed in an anti-sense cDNA library under expression of an inducible or constitutive promoter.

In another embodiment the non-human transgenic animal, as described above, can be analysed for the formation and the inhibition of 'cloudy' diffuse plaques. The present mutation in APP714 leads to a severe AD pathology with unusual plaque composition, composed mainly of N-truncated $A\beta_{42}$, and morphology. It is found that the $A\beta_{40}$ form is nearly absent from amyloid deposits in the human brain and the formation of typical dense-cored plaques is retarded. The predominant amyloid deposits are diffuse non-congophilic amyloid plaques in association with dystrophic neurites and reactive gliosis. In the present invention novel pathological findings are presented that diffuse, non-congophilic (and thus nonfibrillar) Aβ plaques are pathogenic and cause AD. Furthermore in the affected patients it is shown that dystrophic neurites and a neurofibrillary pathology can develop in a plaque independent way. This demonstrates that nonfibrillar pre-amyloid deposits have the potential to cause neuronal toxicity and that the development of neuritic and amyloid pathology can be uncoupled at some stage of AD disease. In addition the invention presents for the first time evidence, that N-truncated $A\beta_{42}$ plays a necessary and sufficient role to cause neuronal toxicity and cognitive decline in AD patients. In a specific embodiment the non-human transgenic animal as described above can be used for the screening of molecules and monitoring said animal for the effect of said molecules on Aß deposits in its brain. Preferentially

molecules that are identified in the cellular screening assay and have an effect on the formation of N-truncated $A\beta_{42}$ and $A\beta_{42}$-formations are administered to the non-human transgenic animal. Monitoring of the transgenic animals can be done by pathological studies which include, but are not limited to, measuring the amount of plaques in the brain, measuring the amount of abnormally phosphorylated tau protein and/or the increase in the number of glial cells. Another procedure that can be used is the measuring of a reduction in behavioural activity tests. Behavioral tests designed to assess learning and memory deficits are employed. An example of such as test is the Morris Water maze (Morris, *Learn. Motivat.* 12; 239-260 (1981). In this procedure, the animal is placed in a circular pool filled with water, with an escape platform submerged just below the surface of the water. A visible marker is placed on the platform so that the animal can find it by navigating toward a proximal visual cue. Alternatively, a more complex form of the test in which there are no formal cues to mark the platform's location will be given to the animals. In this form, the animal must learn the platform's location relative to distal visual cues.

The procedures applied to test transgenic mice are similar for transgenic rats.

Finally in a last embodiment the polynucleotide sequence that encodes an APP isoform or fragment that has an amino acid other than threonine, but preferably isoleucine, at the amino acid position corresponding to amino acid residue position 714 of APP770, which is a preferred substrate for γ-secretase, can be used for the identification of γ-secretase modulators and/or alternative processing enzymes which lead to N-truncated forms of $A\beta_{42}$ and C- truncated forms. Recently compelling evidence was provided that the activity of γ -secretase is encoded by presenilins, which are a new class of aspartyl proteases (Li et al. (2000) *Nature*, 405, 689). Said identification can be carried out preferentially in a double presenilin knock-out ES cell line in which no detectable γ-secretase activity is present anymore (Herreman A et al. (2000) *Nat. Cell Biology* 2, 461). Therefore the double presenilin mutant ES cell line together with the APP714 mutation of the current invention is an excellent combinatorial tool to isolate and identify γ-secretase homologues, genetic modulators of γ-secretase activity and alternative processing enzymes of the APP protein. By isolation it is meant that standard molecular biology tools such as complementation, screening or selection cloning methods with a genomic or cDNA library are used to transfect the cells and to induce γ-secretase activity. It is obvious that also recombinant virus libraries, such as adenoviral, lentiviral or retroviral libraries can be used. The

aforementioned methodology are only examples and do not rule out other possible approaches that could lead to potential candidates. Restoration of proteolytic activity can be followed by different means, to give only a few examples: ELISA assays or other assays measuring amyloid peptide production, or assays measuring Notch cleavage using luciferase reporter systems or other. To increase the sensitivity of such assays it can be considered to stably transfect the ES cells with cDNA's encoding APP714 mutant or other proteins and reporters useful for such assays.

The following examples more fully illustrate preferred features of the invention, but are not intended to limit the invention in any way. All of the starting materials and reagents disclosed below are known to those skilled in the art, and are available commercially or can be prepared using well-known techniques.

Examples

APP T714I mutation

Family AD156 (figure 1A), an Austrian family consistent with autosomal dominant inheritance of early-onset AD, was referred for DNA diagnosis. The proband, her sister and their mother were diagnosed as probable AD according to NINCDS-ADRDA criteria at age 38, 38 and 44 years respectively. However signs of cognitive impairment and behavior disturbances were apparent several years earlier in all 3 patients suggestive for a mean onset age of ~34 years in the family. Genomic DNA of the proband was examined for mutations in APP, PS1 and PS2. In exon 17 of APP (figure 1B), a heterozygous C to T transition was identified at position 2208 substituting Thr (T) at codon 714 by Ile (I) (T714I, numbering according to APP770 isoform). The mutation abolishes a TspRI restriction site, which was used to confirm the presence of the mutation in the proband (156.1) and her sister (156.2) (figure 1C). The mutation was absent in the father as well as in 50 healthy Austrian individuals. No other mutations were detected.

The Austrian T714I mutation is the first APP mutation reported to date that involves amino acid 43 of Aß located directly at the γ_{42}-secretase cleavage site. The early onset age, as well as rapid progression of the disease, and early death, is comparable to AD caused by mutations in PS1 (http://molgen-www.uia.ac.be/ADMutations).

Drastically altered APP processing *in vitro*

To understand the effect of the T714I mutation on the cleavage specificity of γ_{42}-secretase, we studied APP processing in non-neuronal and neuronal cells. Human embryonic kidney (HEK) 293T cells were transiently transfected with the T714I APP
5 cDNA and secreted $Aß_{1-42}$ and $Aß_{1-40}$ levels were measured in conditioned medium by enzyme linked immunosorbent assay (ELISA)(De Jonghe et al., 1999;De Strooper et al., 1998). Cells over-expressing wild type (WT) and London V717I (Goate et al., 1991) APP cDNA were used as controls. The T714I mutation increased $Aß_{42}$ and at the same time decreased $Aß_{40}$, resulting in a significantly increased $Aß_{1-42}/Aß_{1-40}$ ratio ($p<0.001$)
10 that was 4 times higher than in WT APP. In the same experiment V717I resulted in a 1.8 fold increased $Aß_{1-42}/Aß_{1-40}$ ratio solely due to increased $Aß_{1-42}$, results that are comparable to previous published data (Suzuki et al., 1994). We also measured by ELISA $Aß_{1-42}$ and $Aß_{1-40}$ in plasma of the patient (156.2), her unaffected father (156.3) and 5 unrelated age matched controls. The $Aß_{1-42}/Aß_{1-40}$ ratio was 2.5 fold increased
15 compared to the unaffected father and 1.7 fold compared to the controls. The conditioned medium of the T714I and WT APP transfected HEK293T cells was also analyzed by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (figure 2)(Haass et al., 1992;Sisodia et al., 1990). This method allowed us to assess both full-length and N-truncated Aß. Compared to WT, T714I
20 showed a significant elevation of $Aß_{1-42}$ by 6.4-fold ($p<0.001$), while at the same time $Aß_{1-40}$ decreased significantly by 43% ($p<0.001$). This resulted in nearly equal levels of secreted $Aß_{1-40}$ and $Aß_{1-42}$ and an increased $Aß_{1-42}/Aß_{1-40}$ ratio by 10.8 fold. Similar effects were seen on p3 with equal levels of secreted $Aß_{17-40}$ and $Aß_{17-42}$ increasing $Aß_{17-42}/Aß_{17-40}$ ratio by 10.7 fold over WT. One other pronounced affect of the T714I
25 mutation is the increase of Aß peptides ending at V39 and G38 irrespective of the N-terminal residue. Smaller effects were also seen for full-length Aß and p3 peptides ending at G37. These peptides are not artificially produced by proteolysis of full-length Aß as synthetic $Aß_{1-40}$ and $Aß_{1-42}$ peptides were not degraded when added to the medium of non-transfected cells (Wang et al., 1996). Despite a drastic decrease in
30 $Aß_{40}$, total Aß, irrespective of its N- or C-terminal end ($Aß_{X-X}$) was increased by 2.5-fold for T714I. Compared to other APP mutations located close to the γ_{42}-secretase cleavage site (Ancolio et al., 1999;Cai et al., 1993;Goate et al., 1991;Suzuki et al., 1994), T714I has the highest increase in $Aß_{42}/Aß_{40}$. Interestingly, an *in vitro* decrease in $Aß_{40}$ was also reported recently for the French I715M mutation (Ancolio et al., 1999),

one amino acid downstream of T714I. The mechanisms by which these mutations effect $Aß_{40}$ and $Aß_{42}$ secretion or lead to alternative cleavage to $Aß_{38}$ or $Aß_{39}$, is not yet understood. Distinct γ-secretases (Citron et al., 1996) might have different binding-affinities/cleavage-efficiencies to these mutated CTFs (Klafki et al., 1996) which in this
5 region has an α-helix structure (Lichtenthaler et al., 1997), allowing amino acid residues three or four positions apart, to be spatially close. This might explain why T714I and V715F affect γ_{40}-secretase activity as well. To analyze whether the increase in total Aß was due to an increased processing of CTFs by further action of γ-secretases, or alternatively to an increased trafficking of APP into cellular
10 compartments where α and ß-secretases reside, we quantified $APPs_{\alpha}$ and $APPs_{ß}$. No significant changes were noted (figure 3). Taken together with the increase in total Aß ($Aß_{X-X}$), the unaltered levels of $APPs_{\alpha}$ and $APPs_{ß}$ indicated that processing of CTFs could be a rate-limiting step in APP processing. In a next step we systematically analysed the effect of a series of clinical mutations near the γ-secretase cleavage site
15 of APP in primary neurons. To this end, we expressed human WT APP or APP containing either the Austrian T714I (Kumar-Singh S (2000) Hum. Mol. Genet. 9, 2589), French V715M (Ancolio K (1999) PNAS 96, 4119), German V715A (Van Broeckhoven, unpublished data), Florida I716V (Eckman C (1997) Hum. Mol. Genet. 6, 2087), Indiana V717L (Murrell JR (2000) Arch. Neurol. 57, 885) or London V717I
20 (Goate A (1991) Nature 349, 704) mutation in primary cultures of neurons. We specifically immunoprecipitated $A\beta_{40}$ or $A\beta_{42}$ from the conditioned medium using antibody FCA3340 or antibody FCA3542 respectively (Barelli H (1997) Mol. Med. 3, 695). All APP C-terminal mutations with the exception of V715M, increased $A\beta_{1-42}$ secretion. The increase in $A\beta_{1-42}$ ranged from 1.54 times for T714I to 2.71 times for
25 V717L. This was accompanied by an increase in the N-truncated Aβ-isoforms ending at residue 42 ($A\beta_{x-42}$). These isoforms run in the gels as doublet bands, and are presumably generated by alternative β-secretase activity at amino acid residue 11 or by α-secretase activity at amino acid residue 17 of the Aβ sequence. $A\beta_{1-40}$, on the other hand, is decreased with most mutations. The T714I, V715M and V715A
30 mutations reduce $A\beta_{1-40}$ secretion most drastically to 20%, 30% and 55% of the wild type levels, respectively. The V717I and V717L mutations affect $A\beta_{40}$ secretion to a lesser extent and the I716V mutation has apparently no effect on $A\beta_{40}$ secretion. The secretion of N-truncated $A\beta_{40}$ isoforms ($A\beta_{x-40}$) was decreased to an extent comparable to $A\beta_{1-40}$. Hence, when the $A\beta_{1-42}/A\beta_{1-40}$ ratio is compared for each mutation to WT

APP (arbitrarily set equal to 1), this ratio is increased in all APP mutations. This increase ranges from 1.89 times for V717I to 8.20 times for T714I. Moreover, the $A\beta_{1-42}/A\beta_{1-40}$ ratio correlates inversely (r= -0,86) with the mean onset age for the different mutations.

5

Unusual plaque morphology and composition

Neuropathological examination of the proband (156.1) showed extensive neuronal loss accompanied by diffuse gliosis, amyloid plaques and neurofibrillary tangles confirming the diagnosis of AD. We performed an in-depth analysis by histo- and 10 immunohistochemistry on serial sections from 3 select brain areas, viz. hippocampus including entorhinal cortex (ERC), and temporal and frontal cortices. Immunostaining with an antibody recognizing both full-length Aß and p3 (mAb 4G8), remarkably stained a huge plaque load predominantly as 'cloud-like' diffuse plaques that sometimes enclosed a central lacuna. In the molecular layer of the dentate gyrus, the 15 amyloid plaques had the same non-neuritic 'cotton wool' plaques as described for PS1 Δ9 patients (Crook et al., 1998). We determined the fibrillar nature of the amyloid plaques by Congo red and thioflavin staining. Dense-cored plaques were congophilic and fluorescent with thioflavin, while the majority of the diffuse plaques were non-congophilic. In the few diffuse plaques faintly positive for thioflavin, the fibrillar amyloid 20 occupied only a small proportion of total amyloid recognized immunohistochemically. In the layer III of ERC, the amyloid plaques were congophilic while in its superficial and deep layers, the diffuse plaques were again nonfibrillar. A strong neuritic pathology was evident in CA1 and subicular fields of hippocampus and ERC. Neurites accumulated hyperphosphorylated tau (AT8), ubiquitin, and APP, irrespective of 25 plaque pathology. Using endothelial cell markers (CD31 and CD34) with amyloid staining, senile plaque cores but not the diffuse plaques were closely associated with blood vessels. This feature was most remarkable in the end plate region where all fibrillar amyloid deposits were associated with blood vessels. Next we examined whether *in vivo* the mutation had similar effects on APP processing. Reactivity to a C-30 terminal $Aß_{40}$ specific monoclonal antibody (mAb), JRF/cAb40/10, was completely absent in both blood vessel walls and amyloid plaques in the isocortex, while a faint immunoreactivity was occasionally present in the endplate region of the hippocampus (2-3 amyloid deposits per 10 fields of 0.7 mm^2 each). By contrast, a C-terminal $Aß_{42}$ specific mAb, JRF/cAb42/12, imparted a strong reactivity to all amyloid deposits

including diffuse plaques, blood vessel walls and 'infrequent' dense-cored plaques. Sections were stained with 4G8 and compared to immunoreactivity for antibodies specific for $Aß_{40}$ and $Aß_{42}$ by image analysis. In hippocampus $Aß_{40}$ constituted only 1-7% of amyloid deposits while in neocortex was completely absent. Multi-spectral analysis by confocal laser scanning microscope (CLSM) also demonstrated the absence of $Aß_{40}$ and a full histochemical overlap for 4G8 and $Aß_{42}$ specific antibody. These observations were confirmed using other $Aß_{40}$ specific (FCA3340; Clements et al., 1993, and R209; Mehta et al., 2000) and $Aß_{42}$ specific antibodies (21F12, Johnson-Wood et al., 1997; FCA3542, Clements et al., 1993; R226, Mehta et al., 2000). We also checked for presence of Aß ending at Val39 (Aß39) and Thr43 (Aß43), however, neither of these peptides were significantly deposited in APP714 brain. We studied the N-truncation of Aß in these amyloid deposits and confirmed whether the diffuse plaques were capable of inciting any glial reaction. Reactivity for a panel of antibodies against N-terminal Aß (6E10, 6F/3D, and JRF/AßN/11) when compared to 4G8 reactivity, indicated that diffuse plaques constituted N-truncated Aß while full-length Aß was confined to blood vessel walls, dense amyloid cores and amyloid plaques in ERC layer III and remarkably negative for deep cortical layers. Abundant glial and inflammatory pathology was noted in association with diffuse as well as compact plaques using astroglial (GFAP), microglial (CD68, HLA-DR), and complement (C1q) markers. Microglia were present in the vicinity of compact plaques, although an intense glial activation was noted in many regions without plaque pathology as in white matter.

Generation of transgenic mice

We are currently generating transgenic mice. The sequence of a construct where the mutant APP714 is under control of the platelet derived growth factor (PDGF) promoter is depicted in SEQ ID NO: 2 (construct with nucleotide numbering in SEQ ID NO: 2, 114-1576 PDGF promoter; 1676-1905 Gorman's synthetic intron; 1648-1932 sequences flanking Gorman's intron derived from pIRES-EYFP; 1938-4022 APP coding sequence (1938-ATG, 4023-TAG); 4026-4678 APP 3'UTR; 4691-4899 SV40 pA addition; 3853 C to T transition (APP714 mutation)) A linearized construct such as SEQ ID NO:2, is purified and microinjected into 1.5 days old pre-nuclear embryos isolated from superovulated females of four kinds of F1 hybrid strains: C57/Bl/6 X CBA, C57/Bl/6 X DBA, C57/Bl/6 X D2, C57/Bl/6 X C3H.

Materials and Methods

AD diagnosis

Patients in family AD156 were diagnosed with AD based on neurological examination, neuropsychological testing, neuro-imaging and neuropathology (Kleinert et al., in
5 preparation). The mother was diagnosed at age 44 years. She had progressive memory problems and was disoriented in time. EEG showed moderate but generalized unspecific changes while CT showed brain atrophy. The proband as well as her sister had a neurological examination at age 38 years. They both suffered from severe depression. Mini metal state examination (MMSE) confirmed the presence of
10 dementia. SPECT showed clear hypoactivity while CT confirmed the presence of brain atrophy. In both sisters the dementia was rapidly progressing as measured repeatedly by MMSE. For example at age 39 years the proband scored 18/30 and her sister 11/30, at age 40 years the scores had already dropped to respectively 10/30 and 3/30. The actual age of onset of the symptoms was several years earlier according to the
15 neurologists who treated the patients. Onset age in the mother was estimated 5-7 years and in the daughters 4-5 years earlier. Therefore mean onset age in family AD156 was estimated at ~34 years. The APOE genotype of the proband was E3E3, that of the sister E2E3. The APOE genotype of the mother E2E3 was inferred from that of the father and siblings. The proband died at age 41 years and had brain autopsy,
20 the sister is still alive at age 42 years. Macroscopic examination of the brain showed gross atrophy weighing ~1000 g. Sections derived from the fore-, mid- and hindbrain were stained with haematoxylin-eosin (HE), Nissl, Congo red, and modified Bielshowsky. A definite diagnosis of presenile AD was made.

25 Genetic analysis

Exons 16 and 17 of APP were PCR amplified from genomic DNA of patient 156.1 using published primer sets and PCR conditions (Bakker et al., 1991) and PCR fragments were sequenced using the 'Ready Reaction Rhodamine Dye Terminator Cycle Sequencing' kit (Applied Biosystems, Foster City, USA). The products were
30 analyzed on an ABI310 capillary DNA sequencer (Applied Biosystems). The APP T714I mutation was analyzed by *Tsp*RI digestion of PCR amplified APP exon 17. Wild-type (WT) fragments of 354 bp are cut into 2 fragments of 232 and 122 bp respectively, while the T714I mutant fragments are not cut (figure 1). All coding exons of PS1 and PS2 were PCR amplified from genomic DNA using published primer sets

(Cruts et al., 1998) and screened for mutations by single strand conformational polymorphism (SSCP) analysis as described (Cruts et al., 1998). APOE genotype was determined as described (van Duijn et al., 1994; Wenhan et al., 1991).

In vitro mutagenesis

5 Site-directed mutagenesis was performed on WT APP_{695} cDNA cloned in pCDNA3 (Hendriks et al., 1995) using the 'QuikChange site-directed mutagenesis' system (Stratagene, La Jolla, CA, USA). Primers app714s (5'-CGGTGTTGTCATAGCGA**T**-AGTGATCGTCATCACC-3') and app714as (5'-GGTGATGACGATCACT**A**TCGCTAT-GACAACACCG-3') were used to insert the APP T714I mutation into the construct. The

10 sequence of the constructs was confirmed by direct PCR sequencing of the insertion fragment using the '*Taq* dye terminator sequenase II sequencing' kit (Applied Biosystems, Foster City, USA). The products were analyzed on an ABI373 automated DNA sequencer (Applied Biosystems). Mutant APP V717I were constructed as described previously (Hendriks et al., 1995).

15

cDNA transfection

Human embryonic kidney (HEK-293T) cells were transiently transfected with pCDNA3 vector containing the T714I, V717I or WT APP_{695} cDNA constructs using Fugene (Roche Diagnostics) according to the manufacturer's procedures. The presence of the

20 constructs in the cells was confirmed by Western Blotting. To normalize for APP expression, cells were lyzed in 300 l RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS + complete protease inhibitors). A dilution series of a 5μl aliquot was separated on a 4-12% NuPAGE polyacrylamide gel. Proteins were blotted on a PVDF membrane and immunodetection was performed with

25 antibody 10B4 (Senetek) using the Western Star Chemiluminescence system (Tropix). The full-length APP immunoreactive band was quantified using the NIH Image software package.

Aß ELISA

30 HEK293T cells were transfected in triplicate with WT or T714I APP cDNA in a 6 well plate. One day after transfection, 1 ml OPTIMEM medium without additives was added to the HEK-293T cells and conditioned for 24 hrs. Medium was collected and pooled from 6 transfections. A 1 ml aliquot was used for Aß ELISA. $Aß_{42}$ concentrations were measured in the conditioned media by ELISA, using a prototype version of the

INNOTEST ß-amyloid$_{1-42}$ HS ELISA detecting Aß$_{42}$ peptide (De Strooper et al., 1998). Aß$_{40}$ was measured by ELISA using rabbit antiserum R209 (Mehta et al., 2000) as capturing antibody and biotinylated 3D6 (Johnson-Wood et al., 1997) as detector antibody as described (De Jonghe et al., 1999;De Strooper et al., 1998). Each
5 experiment was performed in triplicate and the results were averaged. A two-tailed unpaired t-test was used to compare the mean level of Aß produced by the WT and mutant transfectants.

Mass spectrometric Aß analysis

10 In a second aliquot of supernatant, collected as described above, proteinase inhibitors (2 mM EDTA-Na, 10µM leupeptin, 1µM pepstatin A, 1mM PMSF, 0.1 mM TLCK, 0.2 mM TPCK) were added. Aß peptides were analyzed by immunoprecipitation/mass spectrometric Aß assay (IP/MS) as described previously (Wang et al., 1996). The Aß peptides were immunoprecipitated from 1.0 ml of conditioned media using mAb 4G8
15 (Senetek, Maryland Heights, MO) and protein G Plus/Protein A-agarose beads (Oncogene Science, Inc., Cambridge, MA) and analyzed using a MALDI-TOF mass spectrometer (Voyager-DE STR BioSpectrometry Workstation, PE/PerSeptive Biosystem). Each mass spectrum was averaged from 256 measurements and calibrated by using bovine insulin as internal mass calibrant. For comparing the
20 peptide levels in the conditioned media, synthetic Aß (12-28) peptide (10 nM) was used as internal standard and the relative peak intensity was used. Both ELISA and MALDI-TOF mass spectrometric analysis were performed by experimenters 'blinded' to sample identity.

25 Quantification of APPs$_{\alpha}$ and APPs$_{ß}$

5 µl conditioned medium was separated on a 4-12% NuPage gel (Novex). Proteins were transferred to a PVDF membrane, the membrane was blocked in PBS + 0.2% I-block + 0.1% tween-20, incubated over night at 4°C with primary antibodies 6E10 diluted 1:2000 (for APPs$_{\alpha}$) or 53/4 diluted 1/500 (for APPs$_{ß}$), incubated with alkaline
30 phosphatase labeled secondary antibody (1/4000 diluted), and detected with either Western Star chemiluminescent reagent (Tropix) or ECL (Amersham).

Immunohistochemistry

MAb JRF/cAb40/10 and JRF/cAb42/12 specific for the C-terminus of $Aß_{40}$ and $Aß_{42}$, respectively, were raised by immunizing mice with synthetic peptides corresponding to Aß residues 36-40 (VGGVV) or residues 33-42 (GLMVGGVVIA) (Mercken *et al.*, unpublished data). Specificity of the $Aß_{40}$ and $Aß_{42}$ mAbs was validated by ELISA and Western blotting showing no cross reactivity. Similarly mAb JRF/AßN/11 specific for N-terminus of Aß was raised against Aß residues 1-7 (DAEFRHD) and recognizes full-length Aß. In addition for immunohistochemistry, following antibodies were used: mAb 6E10 (Senetek; raised against Aß 1-17, recognizes Aß 5-13, Rong Wang, personal communication), mAb 4G8 (Senetek; Aß residues 17-24), 6F3D (Dako; raised against Aß residues 7-17), mAb 21F12 (for $Aß_{42}$, Innogenetics, Belgium), rabbit antisera FCA3542 and FCA3340 (Barelli et al., 1997), rabbit $Aß_{40}$ antisera R209 (Mehta et al., 2000) and $Aß_{42}$ R226 (Mehta et al., 2000), rabbit anti-$Aß_{1-40}$ (Sigma, St. Louis, MO, USA), rabbit anti-$Aß_{39}$ and anti-$Aß_{43}$ (T. Saido, Laboratory for Proteolytic Neuroscience, RIKEN Brain Science Institute, Wako-shi 351-0198 Japan), mAb 22C11 (N-terminus APP; Roche), mAb AT8 (abnormally phosphorylated tau; Innogenetics), and anti-glial fibrillary acidic protein (GFAP; Dako, Glostrup, Denmark), CD68 (macrophage; Dako), rabbit anti-ubiquitin (Dako), C1q complement (Dako), HLA-DR (HLA-DP,DQ,DR; Dako), CD31(Dako), and CD34 (QBEnd; Dako). Antigen retrieval for Aß immunohistochemistry was performed on sections treated with 98% formic acid for 10 min, and for other antibodies as recommended by the supplier. Staining for single antigen was performed using streptavidin-biotin-horse radish peroxidase (ABC/HRP) or peroxidase-anti-peroxidase (PAP), utilizing 3'3'diaminobenzidine (DAB) as a chromogen as described elsewhere (Kumar-Singh et al., 1997). Immunohistochemistry involving detection of more than one antigen was done using species-specific or IgG subtype-specific secondary antibodies conjugated directly with biotin, HRP, alkaline phosphotase or Galactosidase (Southern Biotechnology, Birmingham, USA). This was followed by color development using one of the following chromogens (Roche): DAB, 3-amino-9-ethylcarbazole (AEC), Fast-red, 5-bromo-4-chloro-3indolul phosphate/nitroblue tetrazolium solution (BCIP/NBT) or 5-Bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (X-gal). For $Aß_{40}$ immunohisto-chemistry, a sensitive tyramide amplification system (NEN, MA, USA) was utilized.

Densitometric analysis

Densitometric analysis was performed for staining 5 µm thick serial sections stained with 21F12, JRF/cAb42/12, and FCA3542 (for $Aß_{42}$), JRF/cAb40/10, R209 (Mehta et al., 2000), and FCA3340 (for $Aß_{40}$), and 4G8, was performed using the Vidas image analysis system (Kontron) and the obtained results were compared with staining of similar brain regions of patients with sporadic AD cases and PS1 (I143T) related familial AD. Pixels representing the immunocytochemical stain were counted to calculate the size of each plaque. Also, the relative area occupied by Aß staining in five fields from hippocampus or ERC with most intense staining and envisaging an area of 0.7 mm^2, was determined as described elsewhere (Kumar-Singh et al., 1997).

Fluorescent microscopy

For multiple labeling on a CLSM, 10 µm sections were incubated overnight with mAb 21F12 and a JRF/cAb42/12, washed and labeled with an anti-mouse TRITC conjugated and an anti-rabbit FITC antibody (Molecular probes, Oregon, USA). Images were acquired with a Zeiss CLM-410 using either 488-nm line of argon single laser or 632-nm helium-neon double laser for excitation. One µm thick consecutive optical slices were captured for both fluorochromes separately, and the relative $Aß_{42}/Aß_{40}$ content and ratio in amyloid plaques were determined.

Preparation of recombinant SFV virus

The Austrian T714I, German V715A, French V715M, Florida I716V and Indiana V717L mutations were introduced in human WT APP cDNA (695 isoform) by site-directed mutagenesis using appropriate oligonucleotides and the Quick-change mutagenesis system (Stratagene, La Jolla, CA). The mutant APP cDNA was subsequently cloned into the SmaI-site of pSFV-1 (Gibco BRL, Bethesda, MD). Recombinant Semliki Forest Viruses were produced as described (Annaert WG et al. (1999) J. Cell Biol. 147, 277).

Primary neuronal cultures

Primary cortical neurons were isolated from E14 embryonic mice as described (Annaert WG et al. (1999) J. Cell Biol. 147, 277). Briefly, after dissection of the brain and dissociation of the cells by trypsinisation, cells were plated on poly-lysine coated dishes and incubated in neurobasal medium + B27 supplement. Proliferation of non-neuronal cells was prevented by adding 5µM cytosine arabinoside.

Infection of primary neurons

Recombinant SFV was diluted 1:10 in culture medium and added to 3 to 5 day-old primary cortical neurons. After adsorption for one hour, the viral vector containing 5 solution was replaced with normal culture medium and transduction continued for 2 hours. Medium was then replaced by methionine-free medium containing 100 µCi ^{35}S-methionine (ICN, Irvine, CA). After 4 hours of metabolic labelling, culture supernatants were collected, cells were washed in PBS and finally lysed in DIP buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0,1% SDS), 10 containing protease inhibitors (5mM EDTA, trasylol, 1µg/ml pepstatin).

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Claims

1. An isolated polynucleotide sequence, or an isoform or fragment thereof, encoding a codon 714 mutant of human amyloid precursor protein 770.

2. An isolated polynucleotide sequence according to claim 1, wherein the amino acid at the position encoded by codon 714 is an isoleucine.

3. An isolated polynucleotide according to any of claims 1 to 2, wherein the polynucleotide sequence is a cDNA.

4. A polynucleotide according to claims 1-3, wherein said polynucleotide sequence is operably linked with a promoter.

5. A polynucleotide according to claims 1-4, wherein the polynucleotide incorporates at least one substitution other than at codon 714.

6. A protein encoded by a polynucleotide according to claims 1-5.

7. A transgenic cell comprising a polynucleotide according to claims 1-5.

8. A transgenic cell according to claim 7, which is a eukaryotic primary cell, embryonic stem cell line or an immortalized cell line.

9. A transgenic cell according to claim 7, which is a bacterium.

10. A transgenic cell according to claims 7 or 8, wherein said polynucleotide according to claims 1-5 is integrated into the cell's genome.

11. A non-human transgenic animal of which somatic and germ cells comprise a polynucleotide according to claims 1-5.

12. A method of screening a molecule which is able to reduce the formation of beta-amyloid 42 peptide comprising:

- administering said molecule to a cell line according to claims 7-10;
- determining the amount of beta-amyloid 42 peptide formed; and
- comparing said amount with the amount of beta-amyloid 42 formed without administering said molecule.

13. A method of screening a molecule curing Alzheimer's Disease, comprising:

- administering said molecule to a transgenic animal according to claim 11 and
- monitoring said animal for the effects of said molecule on beta-amyloid deposits in its brain and/or neuronal cell death and/or abnormally-phosphorylated tau protein and/or an increase in the number of glial cells and/or reduced behavioural activity tests.

14. A molecule obtained by a screening method according to claims 12 and 13.

15. Use of the non-human transgenic animal according to claim 11 to analyse the formation and inhibition of 'cloudy' diffuse plaques.

16. Use of the polynucleotide sequence according to claims 1-5 to develop an assay for the screening of alternative proteases of Amyloid β and/or γ-secretase-
5 homologues and/or γ-secretase-modulators comprising:

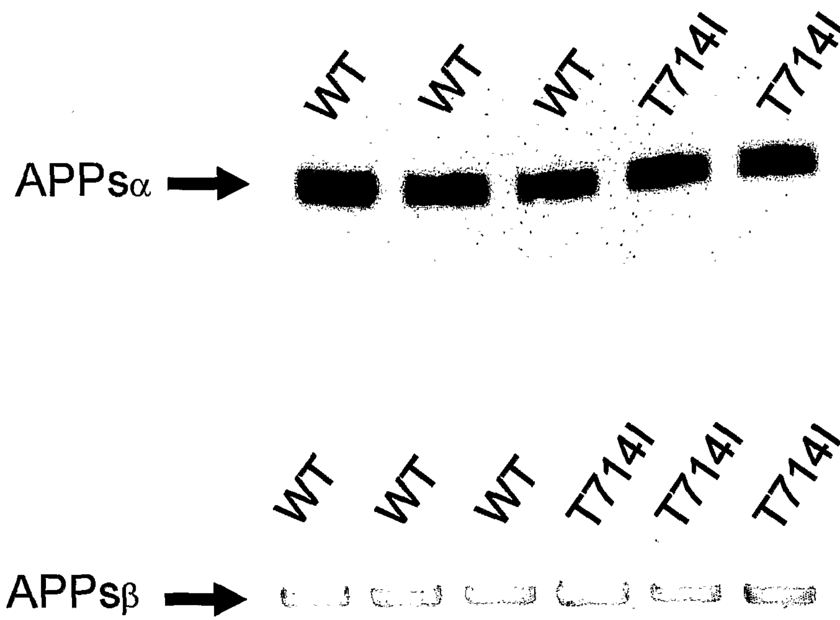
- transfecting said sequence into a presenilin 1 and presenilin 2 negative cell line;
- super-transfecting said cell line with a genetic library, and
- monitoring the production of beta amyloid production.

10

Figure 1

1/4

Fig. 2



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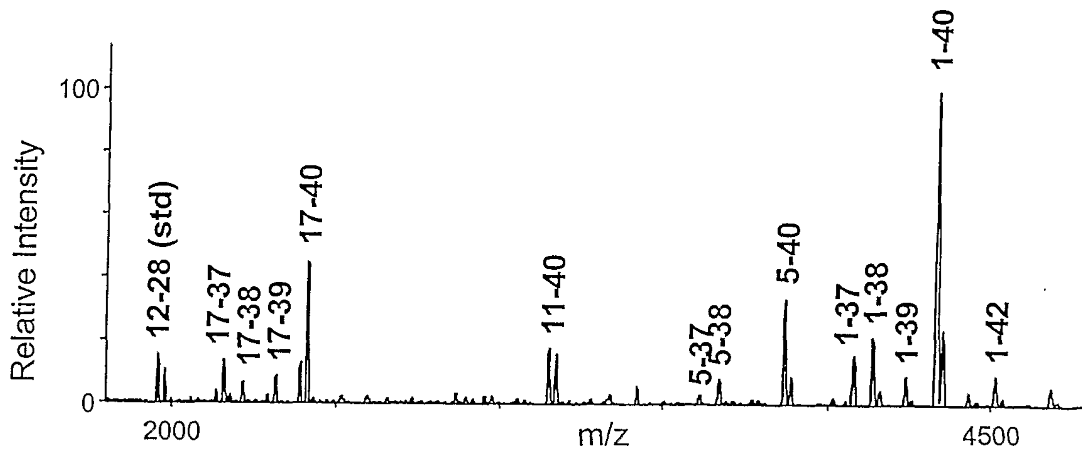

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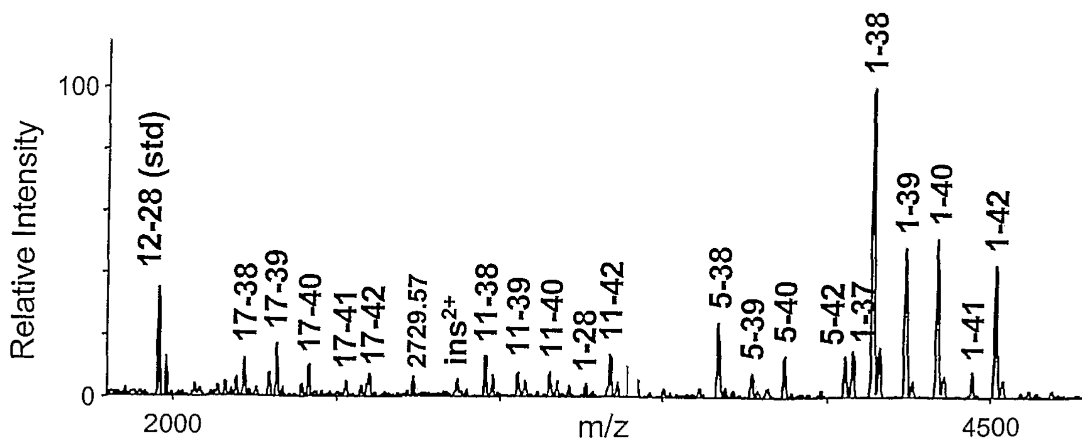

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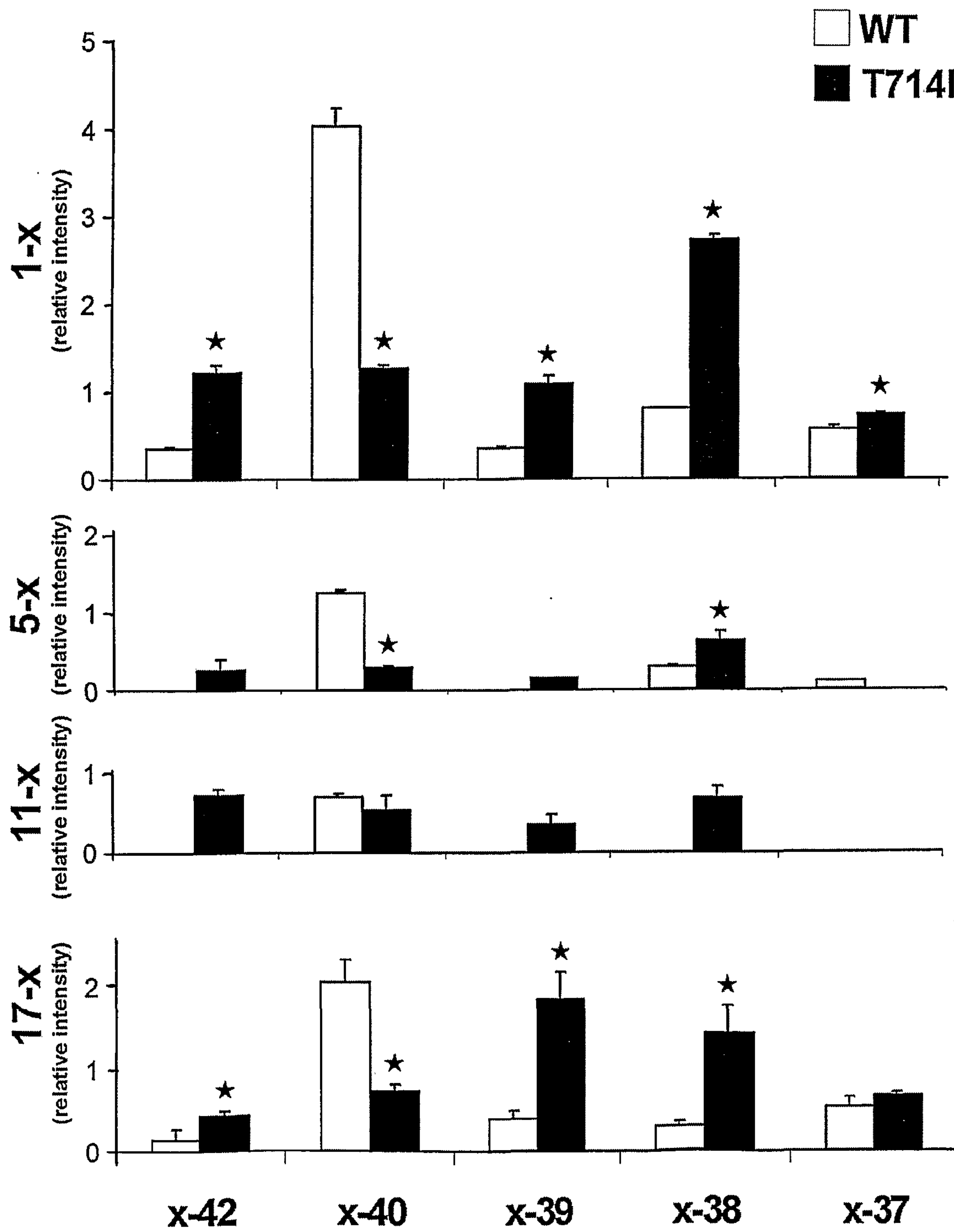
Figure 3 B



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INTERNATIONAL SEARCH REPORT

Inter[illegible] [illegible]l Application No
PC[illegible]/[illegible] 01/07830

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/12 C12N5/10 C12N1/21 C07K14/47 A01K67/027
G01N33/50

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K A01K

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

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	KUMAR-SINGH S. ET AL.: "Nonfibrillar diffuse amyloid deposition due to a gamma42-secretase site mutation points to an essential role for N-truncated Abeta42 in Alzheimer's disease." HUM. MOL. GENET., vol. 9, no. 18, 1 November 2000 (2000-11-01), pages 2589-2598, XP002154668 the whole document ---	1-13,15, 16
A	ALZHEIMER RESEARCH FORUM, "Mutations in APP known to be pathogenic". Updated February 7,2000. http://www.alzforum.org/members/resources/app_mutations/pathogenic.html XP002154669 the whole document ---	1-13,15, 16

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

[X] Patent family members are listed in annex.

° Special categories of cited documents :

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 October 2001	29/10/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Galli, I

Form PCT/ISA/210 (second sheet) (July 1992)

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	WO 92 13069 A (IMPERIAL COLLEGE) 6 August 1992 (1992-08-06) abstract claims 1-39 figure 3 -----	1-13,15, 16

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FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 14

Claim 14 relates to a molecule for curing Alzheimer's disease, but it provides no true technical characterization. Moreover, no such compound is described or defined in the application. In consequence, said claim is ambiguous and vague, and the subject-matter lacks sufficient disclosure and support (Art. 5 and 6 PCT). No search can be carried out for such purely speculative claim, the wording of which is, in fact, a mere recitation of the results to be achieved.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Intern Application No
PCT/EP 01/07830

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
WO 9213069	A	06-08-1992	AU	652997 B2	15-09-1994
			AU	1169492 A	27-08-1992
			CA	2101774 A1	22-07-1992
			DE	971033 T1	03-05-2001
			EP	0568575 A1	10-11-1993
			EP	0971033 A2	12-01-2000
			WO	9213069 A1	06-08-1992
			JP	6504441 T	26-05-1994
			US	5877015 A	02-03-1999