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Lifespan profiles of Alzheimer's disease–associated genes and their products in monkeys and mice

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Abstract

Alzheimer's disease (AD) is characterized by plaques of amyloid-beta ($A\beta$) peptide, cleaved from amyloid- β precursor protein (A β PP). Our hypothesis is that lifespan profiles of AD-associated mRNA and protein levels in monkeys would differ from mice, and that differential lifespan expression profiles would be useful to understand human AD pathogenesis. We compared profiles of A β PP mRNA, A β PP protein, and $A\beta$ levels in rodents and primates. We also tracked a transcriptional regulator of the A β PP gene, specificity protein 1 (SP1), and the β amyloid precursor cleaving enzyme (BACE1). In mice, A β PP and Sp1 mRNA and their protein products were elevated late in life; $A\beta$ levels declined in old age. In monkeys, Sp1, A β PP, and BACE1 mRNA declined in old age, while protein products and $A\beta$ levels rose. Proteolytic processing in both species did not match production of $A\beta$. In primates, A β PP and Sp1 mRNA levels coordinate, but an inverse relationship exists with corresponding protein products, as well as $A\beta$ levels. Comparison of human DNA and mRNA sequences to monkey and mouse counterparts revealed structural features that may explain differences in transcriptional and translational processing. These findings are important for selecting appropriate models for AD and other age-related diseases.

Keywords

A β PP; amyloidogenesis; BACE1; development; primates; rodents; SP1; transcription

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Introduction

Alzheimer's disease (AD) is a progressive, irreversible neurodegenerative disorder resulting in memory loss, dementia, and death. It is characterized by senile plaques composed of aggregated amyloid-beta ($A\beta$), neurofibrillary tangles, and synaptic loss in the brain [1-6]. Autosomally inherited AD can be caused by mutations in the amyloid- β precursor protein (A β PP) and presenilin 1 (PSEN1) genes. However, this accounts for no more than 10% of cases [6]. In over 90% of cases, patients have "sporadic" AD not characterized by autosomal inheritance. The APOE ϵ 4 allele [7, 8] the only confirmed genetic risk factor for sporadic AD, accounts for a minority of the disorder [9], and the etiology of most sporadic AD cases remains unknown.

The accumulation of amyloidogenic $A\beta$ peptides from cleavage of A β PP has been studied extensively. Human A β PP and/or PS-1 transgenic rodent animal models [10-14] are widely used to study molecular and biological processes underlying AD pathogenesis. While these animal models demonstrate AD-like pathologies and behavioral changes compared to wild-type animals, they carry mutant forms of AD-related genes, driven by promoters other than those of the respective genes. Thus, A β PP is over-expressed and processed to speed up AD pathogenesis in a manner that may not be clinically relevant. Transgenic human A β PP in these rodents is cleaved by intrinsic β -secretase (BACE1) to produce $A\beta$. The preferred cleavage site by rodent BACE1 in human A β PP is different from that by which human BACE1 cleaves A β PP.

Although rodents and primates share more than 95% amino acid sequence homology in their respective A β PP proteins [15], the amyloid products from the two differ. Murid rodents do not aggregate $A\beta$ peptides and, therefore, are non plaque-forming, but primates are human-like in their ability for $A\beta$ aggregation to form senile plaques. The $A\beta$ peptide sequence from primates and murid rodents differ in three amino acids at residues 5 (R \rightarrow G), 10 (Y \rightarrow F) and 13 (H \rightarrow R). However, the primary sequence difference may not explain plaque-forming or non plaque-forming nature of the peptides, since *in vitro* experiments [16, 17] indicate that either peptide could aggregate. A difference has been noted in the 5'-untranslated region of the mRNA of non-plaque-forming vs. plaque-forming mammalian species, specifically, the absence of a "CAGA" box in non-plaque-forming species mRNA [18]. However, differences in processing of A β PP or $A\beta$ metabolism *in vivo* may, likewise, determine amyloidogenesis. This is especially true if physiologic manifestations of aging are considered, and this paper seeks to address this issue.

The 5'-flanking regulatory region of the A β PP gene is rich in GC box elements and contains consensus sites that are recognized by several transcription factors, including specificity protein 1 (SP1) [19, 20]. SP1 binds to both the human and rat A β PP promoters and accelerates the production of A β PP mRNA [21-23], which can be further spliced to generate several cell-specific species. SP1 belongs to a family of zinc finger protein (ZFP) transcription factors that includes other members such as Sp2, Sp3, and Sp4 [24, 25]. Sp1 is a transcription factor involved in the pathology of AD [26, 27]. Furthermore, SP1 regulates the expression of BACE1 [28], the main form of β -secretase that cleaves A β PP to generate $A\beta$ [29]. Likewise, silencing Sp1 gene dramatically reduces A β PP promoter activity [26]. In

addition, SP1 regulates the expression of the microtubule associated protein tau (MAPT) gene [30]. The buildup of hyper-phosphorylated tau protein results in the formation of the pathogenic tangles found in AD and some other neurodegenerative disorders like Pick's disease [27].

AD incidence increases exponentially after 65 years of age, making age the greatest risk factor for AD [31]. It is well known that the most common animal model species for AD (mouse) does not develop any similar conditions, requiring the use of transgenics or drug-induced defects. Monkeys, on the other hand, have shown the spontaneous presence of β -amyloid plaque [32], and many genes known to be involved in human neurological disorders have been shown to be present and regulated in cynomolgus monkeys [33]. Likewise, administration of a modified version of the Cambridge Automated Neuropsychological Test Battery has determined that aged monkeys reveal patterns of age-related cognitive impairment that mirror in quality and severity those of aged humans [34].

We hypothesize that profiles of gene expression and protein levels of the AD-critical SP1, A β PP, and BACE1 genes, in mice would differ over the lifespan in a non-trivial fashion from those of a species closer to human, specifically cynomolgus monkey. These differences would be qualitative and appear at transcriptional, translational, and protein processing levels of gene expression. Therefore, we examined the pathways of A β PP production, processing, and accumulation of its A β cleavage product as a function of age. We compared differences in AD-related gene expression between rodents and primates. Our results suggest fundamental differences between rodents and primates in expression and processing of important AD-related genes and proteins. These differences occur at transcriptional, translational, and post-translation levels, potentially affecting applicability of animal model studies at several stages of understanding AD.

Materials and Methods

Animals and tissues

The C57Bl/6 mice were bred in house at University of Rhode Island. Male pups from different dams were pulled and selected randomly at the time of sacrifice. Brains were dissected at different ages, specifically three mice at each post-natal day (PND) 5, 20, 365, and 630, and brain frontal cortex tissues were stored at -80°C until use. The Cynomolgus monkey brain cortex tissues were obtained from National Institutes of Health (NIH), and were control monkeys from two separate studies [35]. They were kept at the NIH facility until termination at 3–6, 12, and 23 years of age, three animals at each of three timepoints. All animal procedures were conducted under the supervision of a licensed veterinarian according to a National Institute on Environmental Health Sciences–NIH-approved animal protocol. The tissues were kept frozen until use in our study.

Total RNA isolation, synthesis of cDNA, and Real Time PCR

RNA from the brain tissue was isolated according to the TRIzol method (Invitrogen, Carlsbad, CA). The RNA was reverse transcribed to obtain cDNAs, catalyzed by SuperScript III (Invitrogen) reverse transcriptase (RT). The RNA/primer mixture containing

500 ng of total RNA, 1 µl of 10mM dNTP mixture, and 1 µl Oligo(dT) were incubated at 65°C for 5 min. A reaction mixture containing 2 µl of 10× RT buffer [200 mM Tris-HCl (pH 8.4), 500 mM KCl], 4 µl of 25 mM MgCl₂, 2 µl of 0.1 M DTT and 1 µl of RNaseOUT (Invitrogen) recombinant RNase inhibitor (40 U/µl) was added. One microliter of SuperScript III RT (200 U/µl) was then added and incubated at 50°C for 50 min. The reaction was terminated at 85°C for 5 min. One microliter of RNase H was added and the reaction was incubated for 20 min at 37°C. The resulting cDNA was stored at -20°C and used in the real time PCR step. The primer pairs used for mouse AβPP, SP1, and β-actin are described in Table 1. Each real time PCR reaction mix contained 1 µl of cDNA, 1 µl of primer mix (final concentration 200 nM), 10.5 µl of nuclease free water and 12.5 µl SYBR® GREEN PCR Master Mix (Applied Biosystems, CA). Each sample was done in triplicate. Real Time PCR was conducted for all above genes with respective primer pairs in a 7500 Real-Time PCR System following standard protocol. The initial step was 50 °C for 2 min followed by 95 °C for 10 min, then 40 cycles of 95 °C for 15 sec and 60 °C for 1min. Real time PCR products were checked with agarose gel to confirm no non-specific products formed. Results were analyzed with 7500 system software with relative quantification method using β-actin or GAPDH as endogenous controls.

Western Blot

Brain tissues were homogenized in RIPA buffer containing protease inhibitor cocktail (Promega), incubated on ice for one hour then centrifuged at 3500 × g for 20 min [26]. The supernatant was collected and used for western blot. Protein concentration was determined by BCA kit (Pierce Biotechnology, Inc. Rockford, IL). A 10 µg of total protein from each sample was applied for western blot. Protein samples were denatured and resolved in 8% SDS-PAGE then transferred to PVDF membrane. The membrane was blocked with 5% non-fat milk in Tris buffered saline (TBS, pH 7.4) for one hour, then 22C11 AβPP N-terminal specific antibody (1:1000) was incubated with 5% milk in TBS overnight at 4°C. For SP1, the membrane was incubated with SP1 (H-225) polyclonal antibody (1:200). The membrane was washed with TBST (TBS + 0.5% Tween-20) 4 times before adding horseradish peroxidase conjugated goat anti-rabbit IgG (1:1000, Pierce). Each membrane was incubated at room temperature for 60 min followed by sequential wash with TBST 4 times and TBS 4 times then developed by Amersham ECL Plus Western Blotting Detection System and exposed with Typhoon 9410 MultiMode Scanner. Signals were normalized to GAPDH or Actin signal of same lanes.

Beta amyloid (Aβ) 1–40 assay

The levels of Aβ were measured with human Aβ 1–40 assay kits (Immuno-Biological Laboratories, Gunma Japan). These kits were designed as solid phase sandwich ELISA with highly specific antibody. The assay conditions were followed according to the method described previously, [36] with slight modifications. Brain tissue was homogenized in Tris-Saline (TS) [50 mM Tris-HCl buffer, pH 7.4; 150 mM NaCl; 1 µg/mL TLCK (N-Alpha-p-tosyl-L-Lysine chloromethyl ketone); 1 µg/mL antipain; 0.5 mM DIFP (Diisopropyl fluorophosphates); 1 mM PMSF; 1 mM NaF; 0.25% sodium deoxycholate; 0.5% SDS; 0.1% Protease Inhibitor cocktail], incubated on ice for one hour, then centrifuged at 3500 g for 20 min at 4°C. The resultant supernatant was collected and diluted by EIA buffer (supplied with

the kit) to 1 µg/µl (100 µg of protein in 100 µl EIA buffer) and assay standards were added to a 96 well plate (pre-coated with anti-human Aβ (35–40) (1A10) Mouse IgG monoclonal antibody) and incubated overnight at 4°C. The wells were washed 7 times with EIA buffer. Then 100 µl of labeled antibody was added to each well containing sample or standard and incubated at 4°C for 1 hour. The wells were washed 9 times with EIA buffer followed by the addition of 100 µl of TMB buffer and incubated in the dark for 30 min at room temperature. The reaction was stopped by adding 100 µl of 1N H₂SO₄ and the colorimetric absorption was taken at 450 nm. The levels of Aβ in the test samples were calculated relative to the standard curve generated on each plate.

BACE1 activity assay

β-secretase activity was conducted with BACE1 activity kit (R&D Systems, Inc. Minneapolis, MN) following manufacturer's protocol with small modifications. The enzymatic reaction for secretase activity was carried out in the black uncoated microplate provided, with controls provided in the kit. Total protein from brain tissue homogenate was diluted to 1 µg/µl with 1X Cell Extraction Buffer. Then 50 µl of protein sample was added to each well. Each sample was run in duplicates for greater accuracy. Next, to each well, 50 µl of 2X Reaction Buffer was added to each well plus 5 µl of substrate at room temperature to each well. Negative controls run were a) no protein sample and b) no substrate. The plate was covered by provided film, tapped gently to mix, and incubated at 37° C for 2 hours in the dark. The plate was read on a fluorescent microplate reader using light filters that allow for wavelength excitation between 335–355 nm and emitted light between 495–510 nm was recorded.

Statistical analysis

Results from mRNA expression, protein or peptide level, and enzymatic activity assays were subject to t-test against “adult” data points, “adult” defined as PND 365 for mice and 12 years age for monkeys. Average values with $p < 0.05$ vs. their respective “adult” data points were deemed significant.

DNA sequence homology analysis of mouse and monkey Sp1 and AβPP genes vs. human Sp1 and AβPP genes

The NCBI human genome database (build 36.2) was consulted for the reference sequences of the human Sp1 and AβPP genomic sequences. A portion of reference sequence NC_000012 from 52056246 to 52096497 was selected for Sp1 BLAST, and the AβPP reference sequences NG_007376.1 was used for BLAST. Cynomolgus monkey (*Macaca fascicularis*) genomic sequences were not available; therefore, rhesus monkey (*Macaca mulatta*) sequences were used. BLAST comparison of the available cynomolgus sequence vs. rhesus sequences showed 99% or greater homology (data not shown) between the two. Each sequence was used to probe the rhesus monkey (build 1.1) and mouse (build 37.1) NCBI reference genome databases. In addition, sequences of 4kb upstream of the +1 transcription start site (TSS) of the Sp1 genes of mouse, rhesus monkey, and human were extracted from their respective NCBI genomic databases, and the monkey and mouse Sp1 sequences were each compared with the human Sp1 sequence via ClustalX [37].

Transcription factor binding site analysis of promoters of Sp1 for human, mouse, and monkey

Sequences 4kb upstream of the +1 TSS of the Sp1 genes of mouse, rhesus monkey, and human were used to search for putative transcription factor binding sites via the MatInspector utility of the Genomatix analysis package [38]. Results for each species were compared, and predicted factor binding sites unique to mouse or shared by monkey and human but absent in mouse were recorded.

Motif analysis of 5'– and 3'–untranslated regions (UTRs) of A β PP for human, mouse, and monkey

The RegRNA database/analysis utility [39] was employed to search the sequences of the human (GenBank Accession #NM_000484), cynomolgus monkey (M58727), and mouse (NM_007471) A β PP mRNA 5'– and 3'–UTRs. For consistency with our previous publications, the +1 transcription start site (TSS) producing a 147bp 5'–UTR [19] was selected for the human A β PP sequence rather than a further–upstream TSS that produces a 194bp 5'–UTR. UTR sequences were searched for conventional regulatory motifs and miRNA homology sites.

Results

Our purpose in comparing AD–associated gene expression between monkeys and mice is to establish comparative non–pathological “baselines” for disease–critical genes and their protein products. We hypothesized that non–trivial, qualitative differences exist between primate and rodent expression in fundamental AD–associated genes at transcriptional, post–transcriptional, and protein processing levels. Profiling of AD–associated gene expression and protein levels in the lifespan of monkeys and mice produces control profiles of animal models, which develop an understanding of “baseline” normal aging effects on transcriptional, translational, and post–translational mechanisms. These baselines then form a basis for evaluating overall model performance in comparison to human aging and pathological processes. The data reported below are the results of measuring A β PP, BACE1 and Sp1 gene expression as well as their protein levels in the brain frontal cortices of wild–type mice and primates. In addition, comparative results of pertinent DNA and mRNA sequences and sub–sequences of the A β PP, BACE1, and Sp1 genes are presented.

A β PP, BACE1, and Sp1 mRNA expression in mice at different ages

The mRNA expression of A β PP and Sp1 was examined by real time PCR at 6 different times within the mouse life span and normalized to β –actin PCR signal, PND 5 through 630. In mice, normalized A β PP mRNA signal increased dramatically from PND 5 to PND 20, remained steady throughout adulthood, and increased again upon aging (Fig. 1A). normalized Sp1 mRNA expression showed the same trend as that of the A β PP mRNA (Fig. 1B); however, normalized BACE1 mRNA did not show any trend with aging (data not shown).

A β PP, SP1, and A β 1–40 levels in mice at different ages

SP1 and A β PP protein levels were determined by Western blot analysis and normalized to β -actin, and A β levels were measured using an ELISA assay (Fig. 2A, 2B, and 2C). Normalized A β PP levels slightly increased during development, remained steady during adulthood, and sharply rose in old age (Fig. 2A). Normalized SP1 levels on the other hand did not change on PND 20 but showed a similar increase in old age (Fig. 2B). A β levels on the other hand dropped on PND 20, rose to adult levels by PND 365, but exhibited a sharp decline in old age at PND 630 (Fig. 2C). The profile of the SP1 and A β PP proteins was similar to the pattern of A β PP and Sp1 mRNA expression in old age (Fig. 1 vs. 2).

A β PP, BACE1, and Sp1 mRNA expression in monkeys at different ages

In the three monkey age groups, both GAPDH-normalized A β PP and normalized BACE1 mRNA were independently elevated in the adult group (12-year old), but decreased in the aged 23-year old group (Fig. 3A and 3B). As was the case in mice, normalized Sp1 mRNA expression showed the same trend as normalized mRNA of A β PP (Fig. 1 vs. 2). Unlike in mice, BACE1 mRNA decreased in monkey.

A β PP, SP1 protein expression and A β 1–40 levels in monkeys at different ages

The total β -actin-normalized A β PP protein level in monkeys increased linearly as they aged (Fig. 4A). The plaque-forming primates accumulated A β PP protein in their brain as they aged despite their inverse relationship to the A β PP mRNA (Fig. 3 vs. 4). Normalized SP1 levels on the other hand, remained steady during adulthood but sharply rose in old age (Fig. 4B). In monkeys, there was an age-dependent increase in A β 1–40 levels (Fig. 4C), which was consistent with normalized A β PP protein levels.

BACE1 activity in mice and monkeys at different ages

BACE1 is the proteolytic enzyme that generates the N-terminus of A β peptide. Our study showed that BACE1 activity remained relatively steady in young and aging mice (Fig. 5A). In monkeys, BACE1 activity appears to decrease in old age (Fig. 5B), although this decrease is not significant at $p < 0.05$. In both mice and monkeys, BACE1 activity does not appear to follow the same pattern as A β PP or A β levels.

Genomic sequence homologies of monkey and mouse Sp1 and A β PP sequences with human sequence

Structural similarities and differences in genomic sequences are often reflected in differences in transcription and hnRNA processing. We examined the respective Sp1 genes of monkey (40kb) and mouse (39kb) vs. human (40kb) genomic sequences via BLAST. When the mouse genomic sequence database was BLAST probed with the human genomic sequence for Sp1 (Fig. 6A), coverage of homologous regions attained 19% of total human genomic sequence with an identity of 91% over the longest homologous sub-sequence. Coverage of homologous regions between human (300kb) A β PP and mouse (290kb) A β PP sequences (Fig. 6B) was 3%, with an identity of 85% over the longest homologous sub-sequence. Comparison of monkey and human Sp1 genomic sequences (Fig. 6C) revealed 63% coverage with a 95% homology for the longest homologous sub-sequence. Coverage

of homologous regions between human and monkey (300kb) A β PP sequences (Fig. 6D) was 91% with an identity of 91% over the longest homologous sequence. These differences between mouse and monkey in structural homology to the human AD-related genes Sp1 and A β PP are not unexpected and indicate that fundamental gene regulatory variation should, likewise, be expected between rodent model systems and human AD.

Sp1 promoter sequence homologies vs. human

Promoter homology comparison in a 50bp window (up to 4kb upstream of the +1 TSS) of Sp1 (Fig. 7A) produced two distinct running profiles when comparing mouse vs. human and monkey vs. human. Homology between mouse and human sequences was mostly between 25% and 50% from -4000 to -1600 on the human sequence, counting the TSS as +1, when it rose to around 75% from -1400 to -100 (human sequence numbering). From -100 through the 5'-UTR (+1 to +101), homology was between 90% and 100%. When comparing monkey to human Sp1 5'-flanking sequences (Fig. 7B), there was no region of homology between -4000 and -3709 on the human sequence. From -3709 to -2450, homology was 85%–100%. A sharp drop in homology occurred at -2450 to -1950, where homology dropped to 25%, rose to 75% and then dropped back to 25%. From -1950 to the end of the 5'-UTR (+101), homology of between 85% and 100% resumed.

Unique transcription factor binding sites predicted in Sp1 promoter of mouse vs. primate (monkey and human)

We used the human, monkey, and mouse Sp1 promoter sequences, 4kb upstream of the +1TSS, to probe the transcription factor database included in MatInspector (v. 7.7). Hundreds of sites were shared between both rodents and primates (data not shown). However, when the output was screened to include only those sites that were unique by transcription factor and transcription factor family, 18 different transcription factor families, each represented by a single factor, remained (Table 2). For mouse, the families were represented by transcription factors activator protein 2 (AP2), autoimmune regulatory element binding factor (AIRE), a barbiturate-inducible element (BARBIE), downstream immunoglobulin control element, critical for B cell activity and specificity (DICE), GTF2I-like repeat 4 of GTF3 (GTF3R4), and Nuclear DEAF-1-related protein (NUDR).

Transcription factor families unique to the primate (human and monkey) sequences were represented by the specific factors of amino acid response element (AARE), T-box transcription factor 5 (TBX5), kaiso (a zinc-finger transcription factor), the factor for Friend leukemia integration site 1 (FLI), hypoxia inducible factor 1 (HIF1), hypermethylated in cancer 1 (HIC1), transcription factor 11 (TCF11), myocyte expression factor 3 (MEF3), Neuron-restrictive silencer factor (NSRF), Pancreas transcription factor 1 (PTF1), the Notch-associated factor RBPJ κ (RBPJ κ), and THAP domain containing, apoptosis associated protein 1 (THAP1).

Regulatory factor and miRNA binding sites in the 5'-UTR of human, monkey, and mouse A β PP

To assess potential pathways whereby differences in posttranscriptional regulation of primate vs. murid rodent A β PP may operate, the 5'- and 3'-UTRs of the human,

cynomolgus monkey, and mouse A β PP gene were analyzed via the RegRNA utility. In addition, some microRNA (miRNA) sites determined were redundant homologies, since both the human and mouse miRNA databases were used to search these sequences. In the 5'-UTRs (Table 3), we noticed that all three species had terminal oligopyrimidine tracts (TOP), although the mouse sequence had only one while the human and monkey sequences had three. Furthermore, three different human miRNA homology sites (hsa-miR-346, 502, 504) were found in both monkey and human sequences but not in the mouse (along with three sites for the homologous mouse miRNAs). One mouse miRNA homology site (mmu-miR-328) was found in the mouse sequence (homologous to a single human miRNA) but neither in monkey nor human.

Regulatory sites in 3'-UTR in different species

Examining the 3'-UTR sequences of human, cynomolgus monkey, and mouse also revealed potentially important differences in regulatory motifs (Table 4). All three sequences had the A β PP mRNA stability control element (A β PP SCE). However, the mouse sequence alone possessed (two copies of) the cytoplasmic polyadenylation element (CPE). While all sequences had at least two copies of the gamma interferon activated inhibitor of ceruloplasmin mRNA translation (GAIT) motif, the human and monkey sequences had five more or four more such sites, respectively, than did the mouse. Similarly, human and monkey sequences had more selenocysteine insertion sequence (SECIS) motifs than did the mouse. The major difference noticed in regard to miRNA homology sites was that human and monkey sequences shared five (hsa-miR-17-5p, 20b, 106b, 483, 485-5p) human sites (with five homologous mouse miRNAs) found in mouse, while the mouse sequence had one unique mouse miRNA (mmu-miR-99b) site (with a homologous human miRNA).

Discussion

We undertook our study specifically to test a critical unspoken assumption in the field of AD research, that conventional rodent animal models can be presumed to be sufficiently similar to humans that results from the animal models can be “linearly applied” to the human condition. We, therefore, compared levels of proteins shown to be critical for AD etiology between murine rodents and primates, specifically to see how much actual similarity existed for the expression of these proteins between rodent models and primates (which would include humans).

Animal disease models are not necessarily “non-reactive vessels”—the substrate species has its own normal expression patterns and pathways for etiologically interesting genes. These patterns and pathways may or may not significantly resemble those of the ultimate target species, which for AD would be humans. It is not known whether or not “normal” expression patterns do or do not interact with AD models imposed on mice. An indispensable step in learning whether or not the mouse functions more as a “vessel” or as an “environment” for AD models is to understand lifetime non-diseased expression patterns of AD-associated genes. This understanding is enlarged in the context of translation of animal results to human subjects by comparing these patterns between mice and primates that are, presumably, more closely related to humans.

The single most important risk factor in the etiology of Alzheimer's disease is age. The majority of research within the field “short–circuits” this risk factor by utilizing transfected cell cultures and short–lived natural or transgenic models. These “time–compressed” models of AD have not been evaluated by comparison to actual aging, particularly not for comparison of non–pathological lifespan expression of genes and proteins associated with AD, such as A β PP, BACE1, and A β between plaque forming (primate) and non–plaque forming (rodent) species.

In this study, constitutive A β PP and Sp1 mRNA expression mirror each other across the rodent lifespan and thus either gene could be biomarker for the other. These findings are consistent with previous siRNA studies, which showed a dramatic drop in A β PP promoter activity, if SP1 levels are depleted [26] and with other surveys of A β PP and Sp1 mRNA [40–42]. Immunohistochemical studies have shown that A β PP and SP1 co–localize to neurons and have a similar distribution in the brain [43]. These lifetime signatures establish SP1 as a partner transcription factor that controls A β PP expression across the lifespan.

BACE1 mouse mRNA levels remained unchanged (data not shown). This may indicate that, in mice, SP1 does not drive BACE1 mRNA expression in the same manner as does A β PP. Other studies have shown that, even when A β PP is transgenically up–regulated, BACE1 mRNA levels do not alter significantly at different ages [44]. BACE1 over–expression with elevated mRNA level was only seen in genomic BACE1 transgenic mice [45].

In primates, the mRNA expression of A β PP, BACE1, and Sp1 showed the same trend among all age groups. Higher levels of Sp1 mRNA expression were accompanied by greater A β PP and BACE1 mRNA expression, and vice–versa. This would not be inconsistent with SP1 coordinately regulating both A β PP and BACE1 gene expression in primates. However, there was no elevation of mRNA expression for the genes in old age, instead, mRNA levels for those genes decreased in the oldest age group of mice.

Several studies have reported age related brain A β PP mRNA changes in cynomolgus monkeys, normal humans, and AD patients. A primate study [46] showed unchanged mRNA levels of A β PP751 and A β PP695 in cortical regions in the aged group in comparison to the young group, but expression in thalamus was significantly increased with a decreased A β PP695/A β PP751 ratio in aged monkeys. Another report found no differential expression of alternatively spliced A β PP mRNA transcripts in AD cases or aged monkeys [47]. A study reported that the mRNA ratio A β PP770+751/A β PP695 increased with age in AD patients [48], while another study found expression of major A β PP mRNA in neocortex declined as a function of age in both AD and control cases [49]. On the other hand, there was a report of no alteration of total A β PP mRNA as well as A β PP with Kunitz protease inhibitor (KPI) in a wide range of age group of control human subjects [50]. These contradictory results are probably caused by the methods for studying mRNA used by different studies. For example, solution hybridization–RNase protection assay [48, 50] is more sensitive and quantitative than the Northern blot analysis utilized by another group [49]. Moreover, several factors, such as gender, postmortem interval, and brain pH would affect the quantification of mRNA when postmortem brain tissues are utilized [51].

In mice, A β PP and Sp1 gene expression and their protein products were elevated late in life; however A β levels declined in old age. In monkeys, Sp1, BACE1, and A β PP mRNA expression declined in old age, while their protein products, as well as A β levels, rose. In rodents, A β levels in old age do not appear to be maintained by the transcriptional or translational status of the A β PP gene but instead by enhanced degradation and clearance. In primates, there is an inverse relationship between A β PP and Sp1 mRNA levels and their corresponding protein products, as well as A β levels. While the specific mechanism of this disparity is not apparent, it suggests that enhanced translational efficiency or reduced protein degradation may account for a rise in A β PP consequently elevating A β levels in primates.

We found that BACE1 activity remains practically unchanged across the entire adult lifespan of mice and has a non-significant decrease in monkeys. Thus, BACE1 activity does not appear to mirror A β levels nor is it influenced by A β PP levels. Neither does it appear to match the decline in BACE1 mRNA levels in monkeys. We must note that currently available β -secretase activity assays are not necessarily specific for BACE1 if used on tissue extracts instead of purified BACE1 protein. However, of the alternatives, BACE1 is the primary β -secretase [29] and is likely to be responsible for most of the measured activity and changes in this activity.

We did note apparently dramatic changes between postnatal days 5 to 20 in levels of mouse SP1 and APP mRNA, in mouse APP and in mouse A β . While these results may help illuminate regulation of these genes in mouse, we have previously published in greater detail on developmental expression of such genes in rodents [26, 52, 53].

Given the differences we found in A β PP mRNA and protein levels between mouse and monkey, we examined homology at the genomic level. Rhesus monkey and human A β PP promoter sequences up to 4kb have previously been shown to have high (>90%) homology. [54]. The full-length (~300kb) monkey genomic sequence preserved approximately 90% homology with the human reference A β PP genomic sequence, while mouse had no more than 3% homological regions. Respective Sp1 genomic sequences' homology comparison with human showed that monkey sequence had up to 63% homology coverage vs. 19% for mouse vs. human. The majority of non-homologous regions were intronic. Intronic regulation of mRNA levels has been well demonstrated [55, 56]. The large differences between mouse and monkey respective genomic Sp1 homologies to human likely contribute to differences in mRNA levels for this transcription factor, although greater differences in A β PP homologies do not seem to alter tight transcriptional control of A β PP by Sp1.

We also examined the promoters of monkey and mouse Sp1 genes for structural differences vs. the human Sp1 gene and determined very high homology (>90%) between monkey and human over most of 4kb upstream of the +1 TSS, while mouse and human homology remained around 75% for most of this length. In addition, the primate Sp1 promoter sequences were predicted to have several transcription factor binding sites absent in the mouse sequence. Of particular interest is the presence of AP2 and HIF1 sites. AP2 has been implicated in regulation of the BACE1 gene promoter [57]. HIF1 activity has been linked to neuroprotection against A β [58] but paradoxically upregulates BACE1 and results in greater generation of A β [59]. In addition, the presence of RBPJ κ sites may be pathogenically

secretase pathway declined due to aging, and, while β -secretase activity was increased, levels of A β 40 and A β 42 did not change with age. Since they only measured the insoluble form of A β , this result did not represent the whole A β pool. Aging is also a crucial factor resulting in reduced A β clearance by degrading enzymes. Aging rats showed much less elimination of A β 1–40 from brain to peripheral circulation than young rats [73].

In addition to aging-related factors, species-intrinsic differences also need to be considered, such as those we have recently demonstrated in our analysis of the human apolipoprotein E (APOE) gene promoter in human-origin vs. rodent-origin cell lines and nuclear extracts [74].

Regarding the aforementioned results and their interpretations, the overall mouse mRNA data is similar to that performed in an independent set of rats previously published [26]. An mRNA analysis of monkeys produced similar readings, as previously published by us for aged monkeys [75]. Confidence is also raised in the rodent protein data through measurement at multiple intermediate ages. The data points on the curves presented were derived from 3–4 animals. When considering these profiles, one may keep in mind that the overall signature profiles are more important than individual points on the curve. Similarity and contrast of the lifespan expression of amyloid pathway genes and their products between amyloid-producing species (cynomolgus monkey) and non-amyloid species (mouse) are the major focus of the present work.

Alternative explanations may suffice for the observed differences between mouse and monkey in A β PP expression and A β metabolism. While both populations were maintained in laboratory conditions, these conditions could not, of course, have been identical. Each species has different nutritional requirements, requiring that their feed differ from each other. This introduces the potential for dietary effects on A β PP expression, similar to those seen for the PSEN1 gene [76]. Epigenetic effects also cannot be ruled out [77]. Likewise, the great divergence in overall lifespan raises the possibility of a fundamental difference of aging programs that is not limited to SP1, A β PP, or BACE1. That is to say, differences seen in the expression and activity of these genes' products may actually reflect a deeper and more profound difference between the two species, altogether, one that raises the question of applicability of results from one model to another.

In a broader context, the present work underscores the importance of studying the regulation of gene expression in relation to the disease process. Structural similarity of gene coding sequences is often taken as a strong indicator of the suitability of a model system, especially when a model is transgenic, since it has the exact human coding sequence. Experience has shown that such models have limits. Notably, a natural model considered as close as 99% homologous to humans, such as the chimpanzee, does not afford a 99% level of gene-based pathogenic functional similarity. Fundamental differences in gene regulation and interaction may play a more important role in determining differences than primary DNA sequence [78]. These regulatory differences could likewise be significantly influenced by environmental factors, such as diet and metals, at the very early stage of development and, importantly, could be manifested later in the life after prolonged latency as explained in a

recently proposed ‘LEARN’ model (Latent Early-life Associated Regulation) [74] and discussed for the somatic epitype [79].

In summary, we have examined the lifetime profiles of intermediates in the amyloid cascade pathway in both non plaque-forming rodents and plaque-forming primates (Table 5). We found that the expression pattern of A β PP mRNA mirrors that of the transcription factor Sp1, and thus SP1 may be a lifelong regulator of the A β PP gene. We also noted that in both species, there is a complex relationship between A β PP mRNA expression and A β PP protein levels. Furthermore, in non plaque-forming rodents, A β does not accumulate in the brain with aging; however, in plaque-forming primates, both A β PP protein and A β accumulated. These findings are summarized diagrammatically in Fig. 9. Proteolytic enzyme activity does not match the production levels of A β . The discrepancy between A β PP mRNA and A β PP protein as well as A β in both species may be due to different nucleic acid structure-based mechanisms, as our comparisons of genomic and mRNA sequences suggest. In mice, posttranslational pathways related to A β clearance or degradation appears to prevent any accumulation A β levels. On the other hand, enhanced translational efficiency or decreased A β PP degradation may lead to an accumulation of A β PP and A β in non-human primates. Posttranslational regulation and A β PP protein metabolism may also play a crucial role in amyloidogenesis.

Our results point toward the need to carefully and rationally select animal models, even transgenic models, to understand fundamental molecular mechanisms of human disease. Differential regulation of “pathogenic” genes points toward the need for models that preserve a similar regulatory milieu to the ultimate target species (in the case of AD, human). Transgenic models may be able to fulfill this need, but each level of regulation (transcriptional, translational, posttranslational) requires more transgenes be combined, and raises the possibility that the transgenes, themselves, may be subject to potentially important species-differentiated regulation.

The current prospect for mouse models of AD is mixed, with much work done on animals transgenic for mutant A β PP, mutant PSEN1, and the two combined. Likewise, there are also knockouts for A β PP, BACE1, MAPT, and other genes implicated in AD. Most of the transgenic lines use a “strong” promoter specifically to ensure high production of the protein of interest. This practice has also meant that studying possible contributions of gene regulation to etiology may have been sidestepped, entirely. Recently, mouse lines have begun to be developed that are transgenic for both the coding sequence and promoter of AD-related genes of interest, such as tau. It should be noted that work with the promoters of the AD-associated gene APOE for mouse and human have revealed that the human promoter can have different responses in rodent-origin cell lines and nuclear extracts than in cells and extracts of human origin [74]. Thus, even though improvements are likely in rodent models, there may always be an inherent limitation to the practice. The authors do not wish this cautionary statement regarding mouse models to be taken as an indictment of animal modeling of AD, altogether. This work raises the question of the applicability of a particular model species toward translation of AD research to humans. Even though there are stark contrasts between mouse and primate expression patterns, other rodent species,

such as guinea pig [80], could serve as useful non-transgenic models for APP processing and expression.

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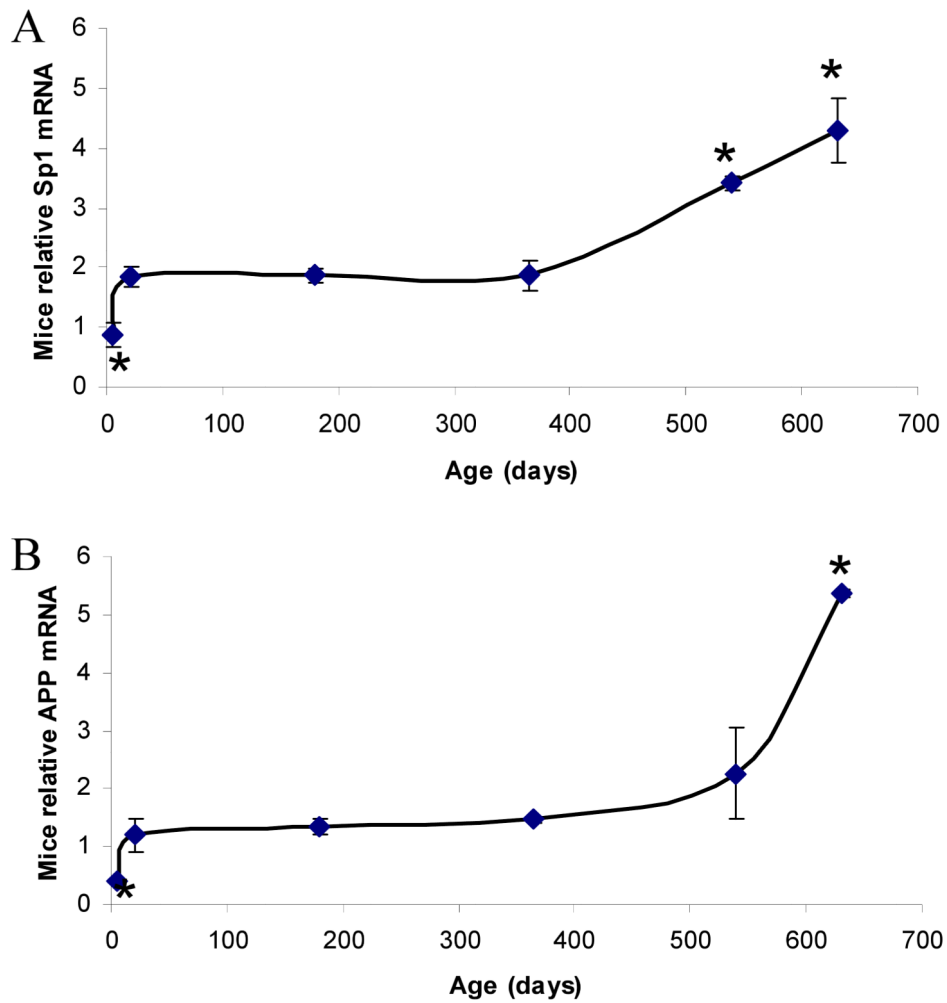


Fig. 1. Lifetime mRNA expression changes in AD related genes (Sp1, AβPP) in the mouse brain cortex

Relative quantification was measured by using 7500 Real-Time PCR System (Applied Biosystems, CA) with β-actin serving as the endogenous control. A) Relative Sp1 mRNA signal. B) Relative AβPP mRNA signal. Each data point in the curve is the mean + SEM (n=3-4 animals). “*” denotes significant difference for SP1 or AβPP mRNA time point when compared to that of PND 365.

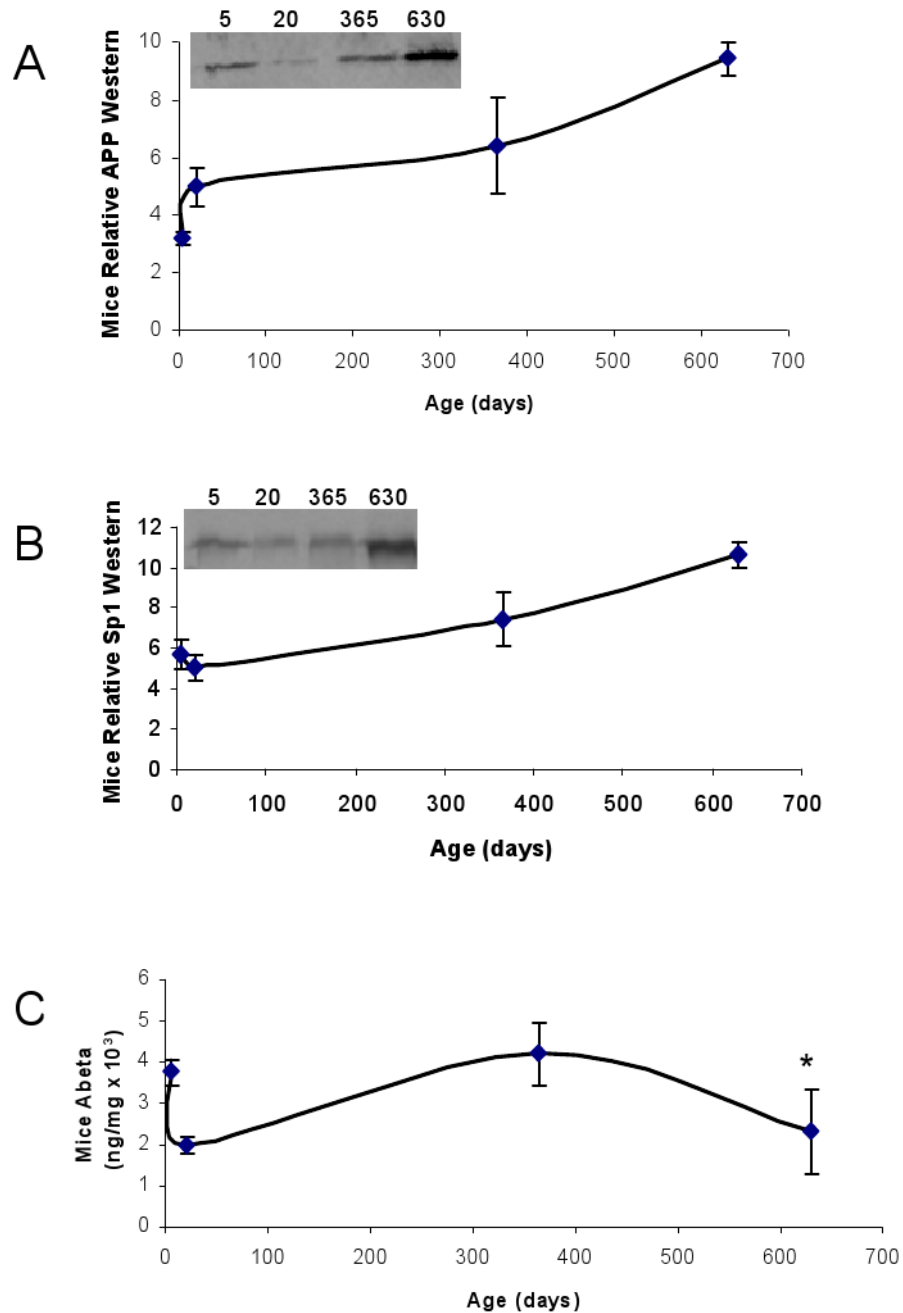


Fig. 2. Lifetime profile of AD related proteins (AβPP, SP1, Aβ) in the mouse brain
 Protein from cortical tissue of wild-type mice was isolated, equal protein amounts were loaded and probed with 22C11 AβPP N-terminus antibody with SDS-PAGE western blot analysis as described in the methods section. The SP1 antibody used is mentioned in the methods section. Protein was also used to measure Aβ 1–40 by a sandwich ELISA assay (IBL, Japan). Six time points are shown. Representative blots are shown in insets for western analysis. A) Relative AβPP levels; B) Relative SP1 levels; C) Aβ40 levels. Each data point in the curve is the mean + SEM (n=3 animals). “*” denotes significant difference

between A β levels at PND 630 as compared to PND 365. Western blot results were normalized to β -actin signal.

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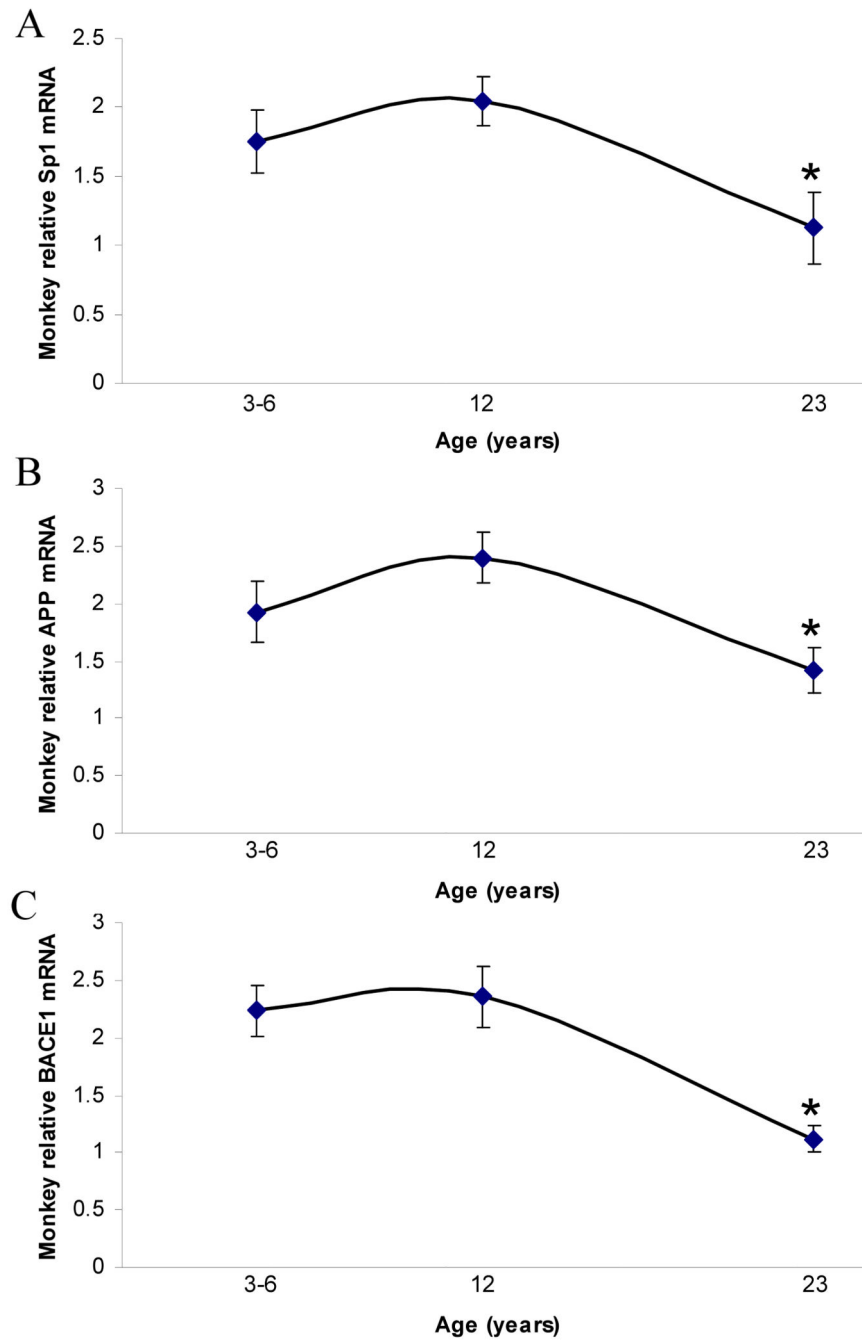


Fig. 3. Lifespan mRNA expression changes in AD related genes (Sp1, A β PP, BACE1) in the primate brain cortex
 Relative quantification was measured by using 7500 Real-Time PCR System (Applied Biosystems, CA) with GAPDH serving as the endogenous control. Three time points are shown. Representative blots are shown in insets for western analysis. A) Relative A β PP mRNA. B) Relative SP1 mRNA. C) Relative BACE1 mRNA. Each data point in the curve is the mean + SEM (n=3 monkeys). “*” denotes significant difference for SP1 or A β PP mRNA time point when compared to 12 years age.

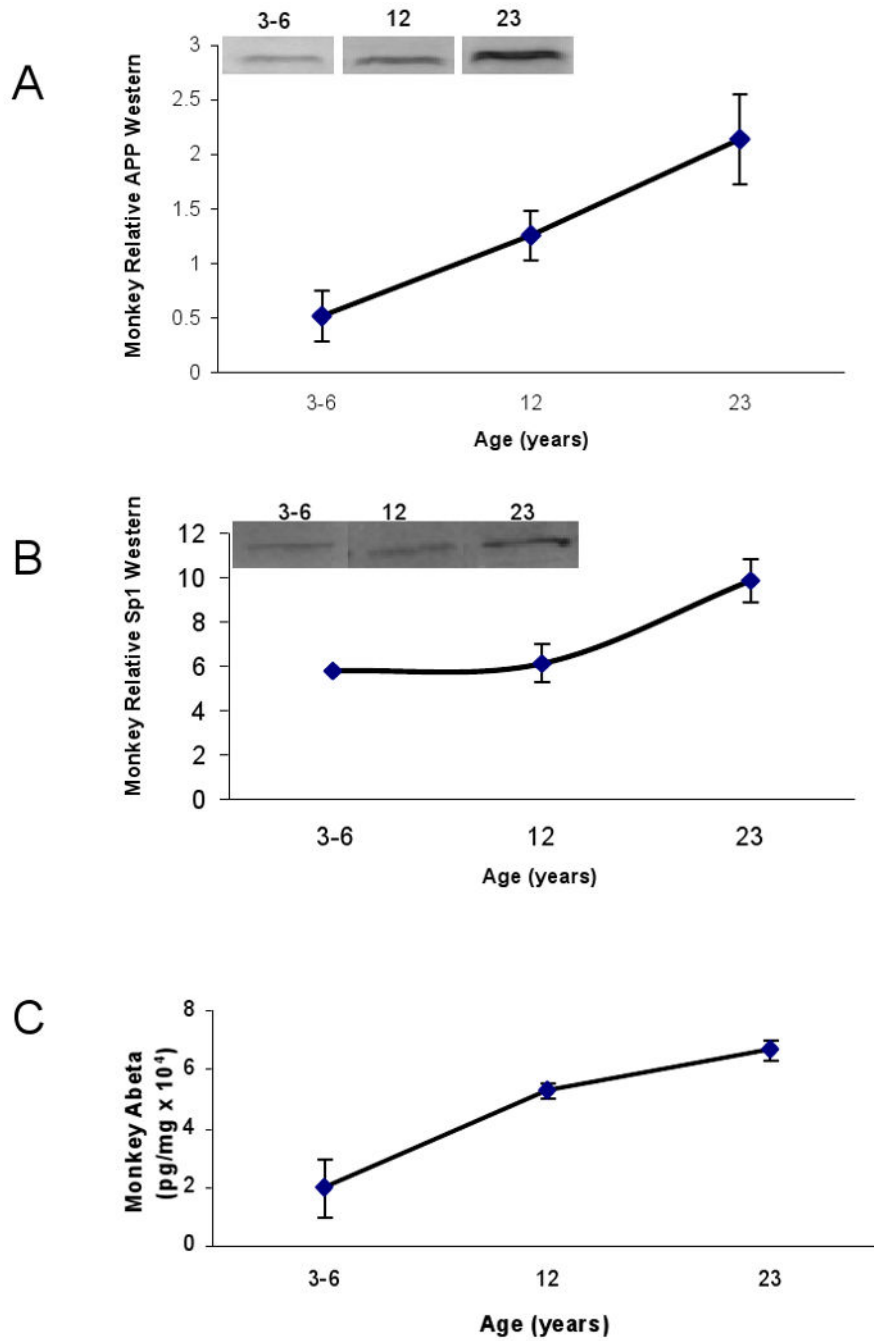


Fig. 4. Lifespan profile of AD related proteins (AβPP, SP1, and Aβ) in the primate brain
 Protein from cortical tissue of Cynomolgus primate was isolated and probed with 22C11 AβPP N-terminus antibody with SDS-PAGE western blot analysis as described in the text. The SP1 antibody used is mentioned in the methods section. Protein was also used to measure Beta amyloid (Aβ) 1–40 using an ELISA assay (IBL, Japan). A) Relative AβPP protein signal; B) Relative SP1 signal; C. Aβ40 levels. Each data point in the curve is the mean + SEM (n=3 monkeys). Western blot results were normalized to β-actin signal.

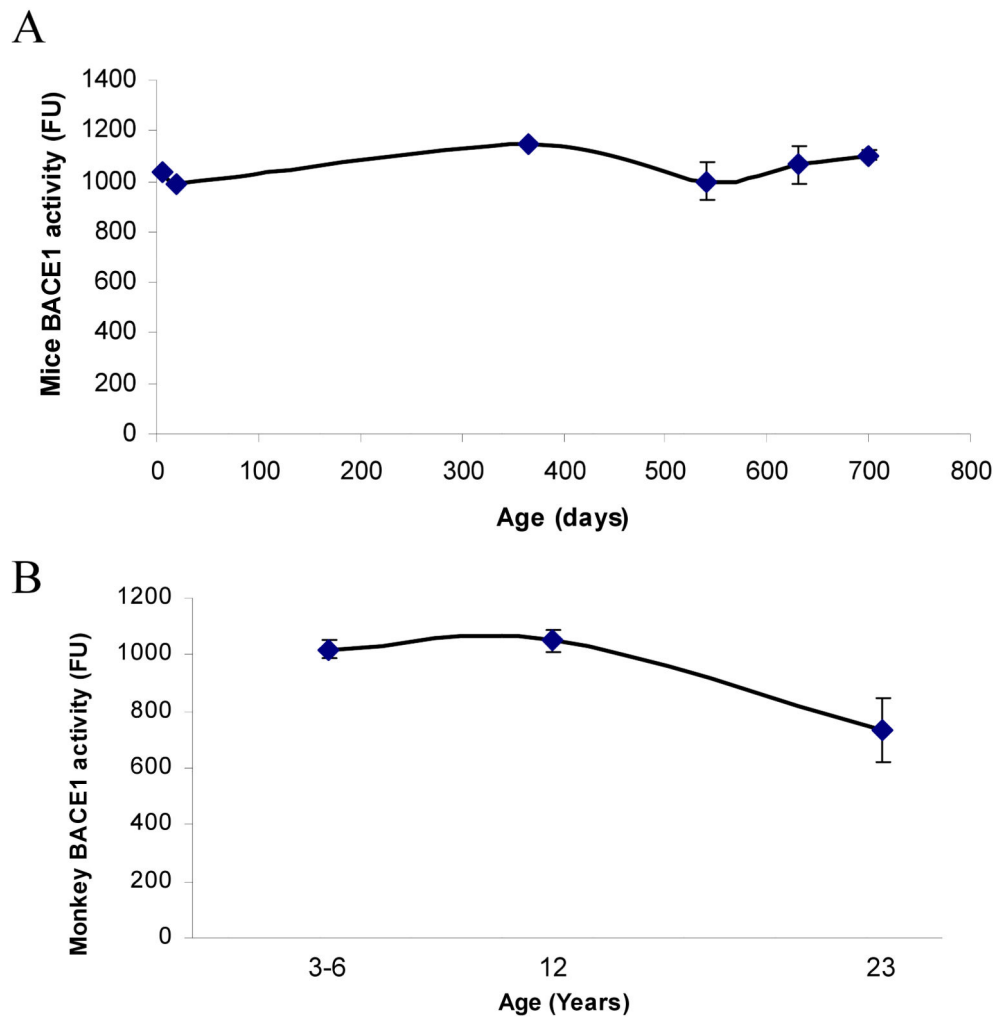


Fig. 5. Lifespan profile of BACE1 secretase activity in the mouse and primate brain
 Protein from cortical tissue of mouse and Cynomolgus primate was isolated and BACE1 activity measured using the BACE1 activity assay kit (R&D Systems, Inc., MN). The y-axis reports raw intensity numbers from fluorescence spectrophotometry. A) BACE1 activity in mice at six time points. B) BACE1 activity in monkeys at three time points. Each data point in the curve is the mean + SEM (n=3 animals). “*” denotes significant difference for SP1 or A β PP mRNA time point when compared to PND 365 for mouse or 12 years age for monkey.

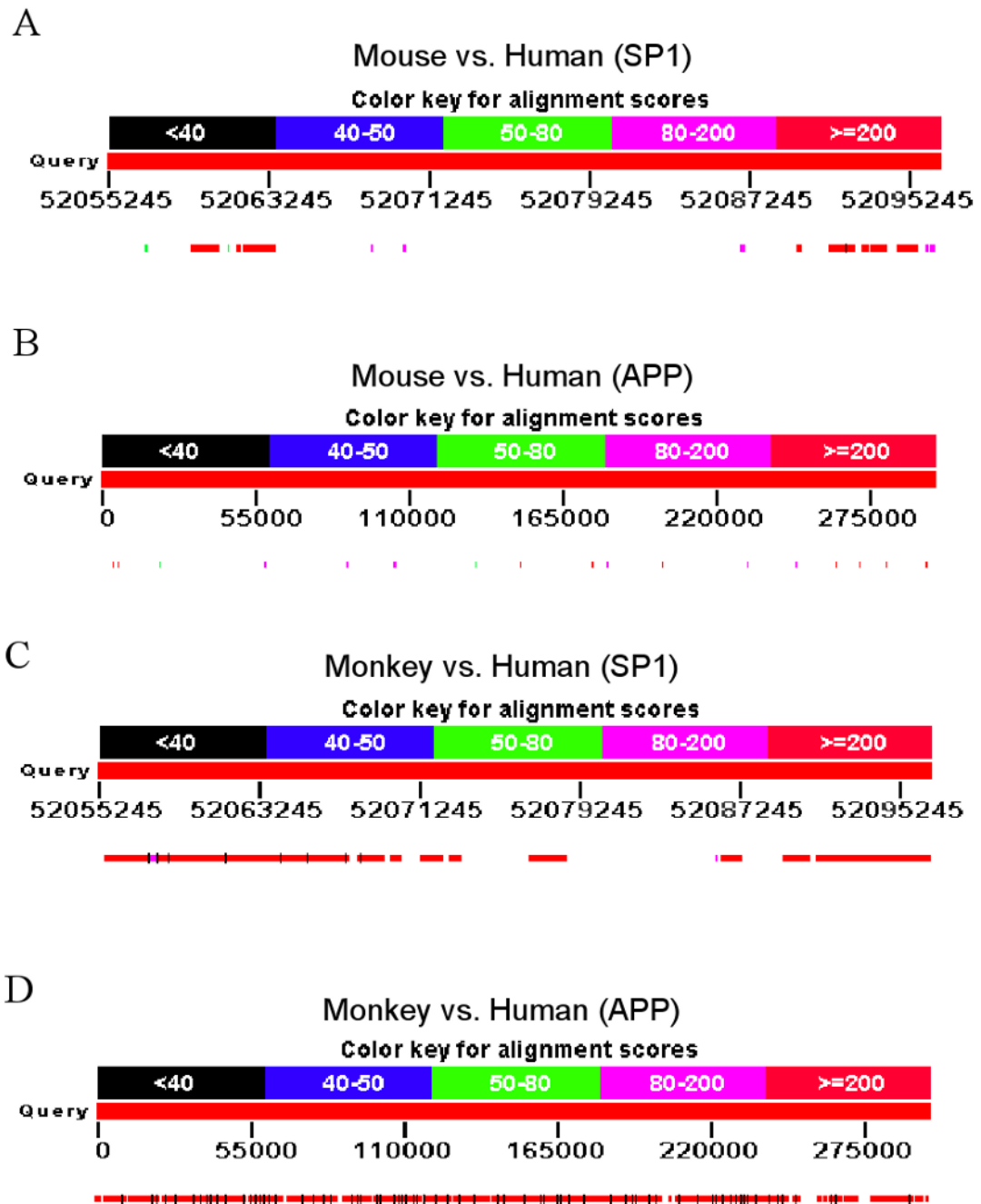


Fig. 6. Genomic homology of mouse Sp1 and AβPP with human Sp1 and AβPP
 Genomic reference sequences for human Sp1 and AβPP were used to BLAST the mouse reference genomic assembly as described in the text. Diagram represents coverage of homological regions over entire genomic sequences. Colors indicate percent homology as indicated by keys on figure. A) Homology of mouse Sp1 vs. human Sp1. Numbers along alignment indicate position in human chromosomal genomic sequence. B) Homology of mouse AβPP vs. human AβPP. Numbers along alignment indicate position in human reference gene sequence [81]. C) Homology of monkey Sp1 vs. human Sp1. Numbers along

alignment indicate position in human chromosomal genomic sequence. D) Homology of monkey A β PP vs. human A β PP. Numbers along alignment indicate position in human reference gene sequence [81].

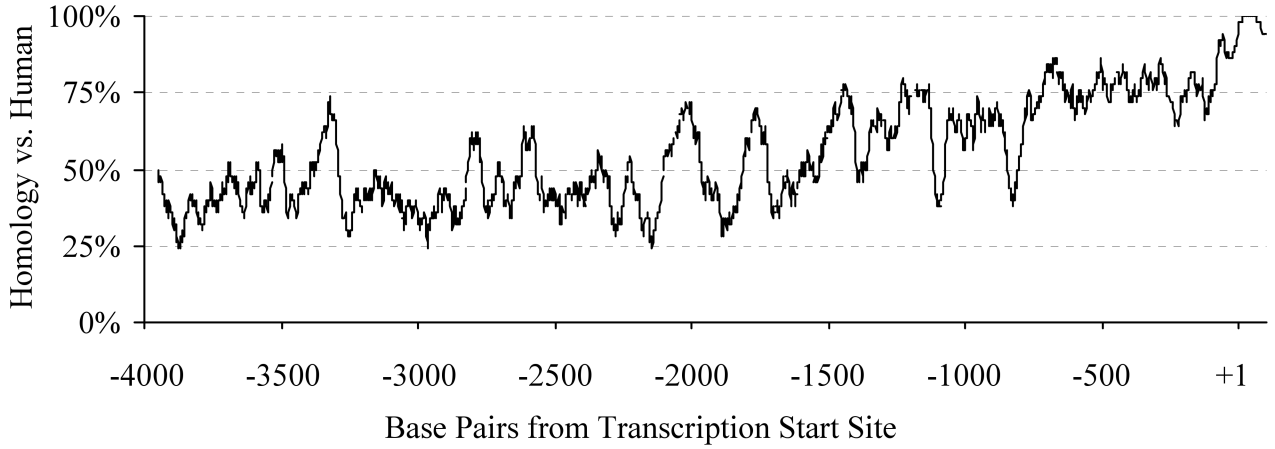
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A. Mouse vs. Human (SP1 promoter)



B. Monkey vs. Human (SP1 promoter)

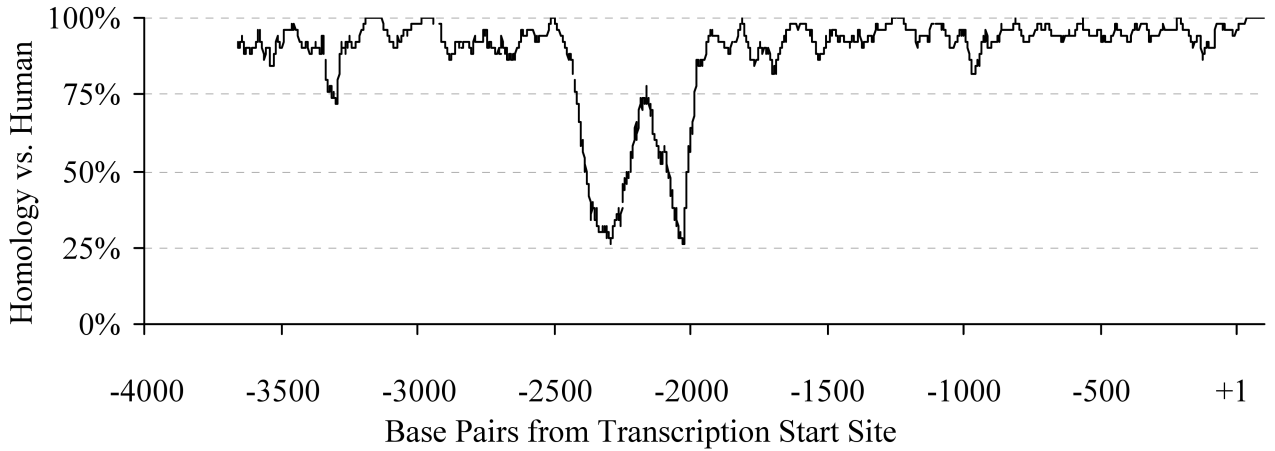


Fig. 7. Homology of mouse and monkey Sp1 promoter sequences with human Sp1 sequence
Sequences of 4kb length upstream of the +1 TSS were taken from genomic reference sequences for mouse, rhesus monkey, and human Sp1, and ClustalX was used to align each of the monkey and mouse sequences to the human. Diagram represents percent homology of each non-human sequence with human sequence in a 50-bp window. A) Homology of mouse Sp1 promoter vs. human Sp1 promoter. B) Homology of monkey Sp1 promoter vs. human Sp1 promoter.

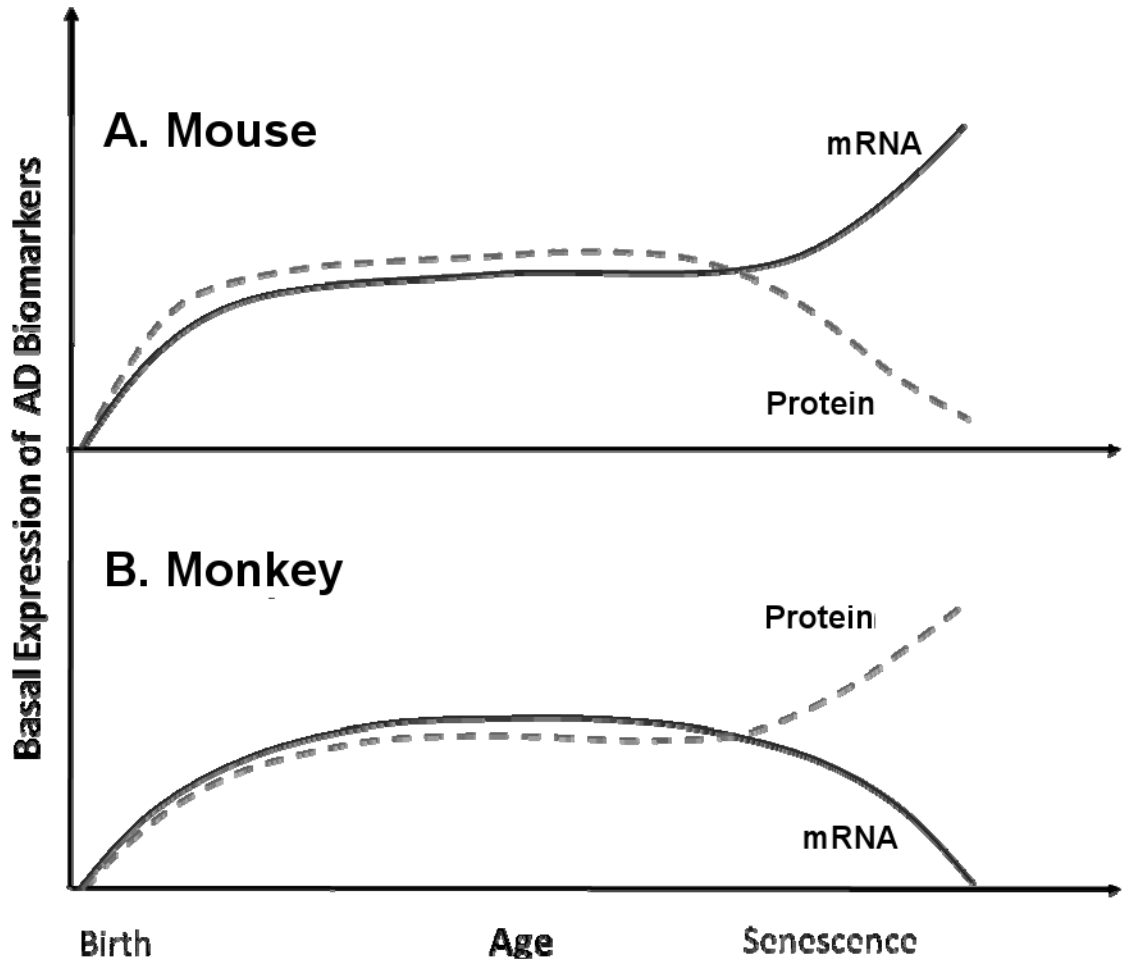


Fig. 8. Relationship between mRNA and protein product levels of selected AD-related genes in mouse vs. monkey

Diagram illustrates levels of mRNA vs. protein product levels of SP1 and APP in animal models studied. Solid line indicates mRNA levels over lifespan while dashed line indicates protein levels over lifespan. For each species, mRNA and protein levels diverge from each other in later life, albeit in opposite directions when comparing species to species. A) Mouse mRNA and protein levels. B) Monkey mRNA and protein levels.

Table 1

Oligomers for real-time PCR of mouse and monkey mRNA.

Gene	Organism	Orientation	Sequence	Reference	Location
AβPP	mouse	sense	5'-TGC AGC AGA ACG GAT ATG AG-3'	NM_007471.2	2175-2194
AβPP	mouse	antisense	5'-ACA CCG ATG GGT AGT GAA GC-3'	NM_007471.2	2285-2304
SP1	mouse	sense	5'-CCT GAA TGG GAA CAT CAC CT-3'	NM_013672.2	874-893
SP1	mouse	antisense	5'-ACC AAG CTG GCA GAA CTG AT-3'	NM_013672.2	1010-1029
β-actin	mouse	sense	5'-TGT TAC CAA CTG GGA CGA CA-3'	NM_007393.3	304-323
β-actin	mouse	antisense	5'-TCT CAG CTG TGG TGA AG-3'	NM_007393.3	676-695
AβPP	monkey	sense	5'-GCT GGC TGA ACC CCA GAT-3'	M58727.1	227-244
AβPP	monkey	antisense	5'-CCC ACT TCC CAT TCT GGA CAT-3'	M58727.1	280-300
BACE1	monkey	sense	5'-TTT GTG GAG ATG GTG GAC AA-3'	XM_001093566.1 ^a	624-643
BACE1	monkey	antisense	5'-CAG CAC CCA CTG CAA AGT TA-3'	XM_001093566.1 ^a	737-756
Sp1	monkey	sense	5'-CAA GCC CAA ACA ATC ACC TT-3'	XM_001104877.1 ^a	1575-1594 ^b
Sp1	monkey	antisense	5'-CAA TGG GTG TGA GAG TGG TG-3'	XM_001104877.1 ^a	1640-1659 ^b
GAPDH	monkey	sense	5'-TGA AGC AGG CGT CGG AGG G-3'	DQ46411.1	115-133
GAPDH	monkey	antisense	5'-CGA AGG TGG AAG AGT GGG TG-3'	DQ46411.1	197-216

^a Sequence from rhesus monkey.

^b A single-base mismatch exists (underlined) between rhesus sequence and oligomer.

Table 2

Unique transcription factor family sites in Sp1 promoters of human and monkey vs. mouse.

Factor	Family	Human	Monkey	Mouse
AARE	AARE binding factors	-3830, -2743, -1833	-3050, -2132	
AP2	Activator protein 2			-1232, -1232
AIRE	Autoimmune regulatory element binding factor			-3472, -3308, -2044, -1476
BARBIE	Barbiturate-inducible element box from pro+eukaryotic genes			-2272, -1707, -732, -671
TBX5	Brachyury gene, mesoderm developmental factor	-3920, -823	-806	
KAISO	BTB/POZ (broad complex, TramTrack, Bric à brac/pox viruses and zinc fingers) transcription factor	-1970	-2270	
DICE	Downstream Immunoglobulin Control Element, critical for B cell activity and specificity			-1636
GTF3R4	GTF2I repeat domain-containing factors			-58
NUDR	Homolog to deformed epidermal autoregulatory factor-1 from D. melanogaster			-872, -766
FLI	Human and murine ETS1 factors	-313	-294	
HIF1	Hypoxia inducible factor, bHLH/PAS protein family	-1877, -330	-3910	
HIC1	Krueppel-like C2H2 zinc finger factors hypermethylated in cancer	-482	-463	
TCF11	MAF and AP1 related factors	-364	-345	
MEF3	MEF3 binding sites	-2731	-3834, -3204	
NRSE	Neuron-restrictive silencer factor	-2820, 48	67	
PTF1	Pancreas transcription factor 1, heterotrimeric transcription factor	-1281	-1258	
RBPJ κ	Notch-associated RBPJ- κ	-3977	-3646, -3635	
THAP1	THAP domain containing protein	-2799	-3106	

Table 3

Presence of selected motifs in A β PP 5'-UTR mRNA from human, monkey, and mouse.

Motif	Human	Monkey	Mouse
TSS ^a	1	1	1
AUG	148	144	146
TOP ^b	6, 52, 131	5, 51, 127	5
hsa ^c -miR-324-3p	<i>54</i>	<i>44</i>	
hsa-miR-328			29
hsa-miR-346	<i>69</i>	<i>62</i>	
hsa-miR-502	<i>71</i>	<i>70</i>	
hsa-miR-504	<i>123</i>	<i>119</i>	
mmu ^d -miR-296	33	35	35
mmu-miR-324-3p	<i>54</i>	<i>44</i>	
mmu-miR-328			29
mmu-miR-346	<i>63</i>	<i>62</i>	

Sites unique to mouse are in boldface. Sites found in monkey and human but not mouse are italic.

^a +1 Transcription Start Site

^b Terminal Oligopyrimidine Tract

^c *Homo sapiens*, human

^d *Mus musculus*, mouse

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Table 4

Presence of selected motifs in A β PP 3'-UTR mRNA from human, monkey, and mouse.

Motif	Human	Monkey	Mouse
stop codon	2458 ^a	2229 ^a	2231 ^b
A β PP SCE ^c	2659	2435	2445
CPE ^d			2931, 2941
GAIT ^e	2704, 2787, 3013, 3096, 3130, 3392, 3397	2291, 2562, 2788, 2901, 2938	2580, 2638
SECIS ^f	3478, 3479	2361, 2362, 2365	2567
hsa-miR-17-5p	<i>3155</i>	<i>2926</i>	
hsa-miR-20b	<i>3156</i>	<i>2927</i>	
hsa-miR-99b			2960
hsa-miR-106b	<i>3158</i>	<i>2929</i>	
hsa-miR-483	<i>3181</i>	<i>2952</i>	
hsa-miR-485-5p	<i>2464</i>	<i>2235</i>	
mmu-miR-17-5p	<i>3155</i>	<i>2926</i>	
mmu-miR-20a	<i>3156</i>	<i>2927</i>	
mmu-miR-99b			2960
mmu-miR-106a	<i>3157</i>	<i>2928</i>	
mmu-miR-106b	<i>3158</i>	<i>2929</i>	
mmu-miR-485-5p	<i>2464</i>	<i>2235</i>	

Sites unique to mouse are in boldface. Sites found in monkey and human but not mouse are italic.

^a“UAG”

^b“UAA”

^c Amyloid- β Precursor Protein mRNA Stability Control Element

^d Cytoplasmic Polyadenylation Element

^e Gamma Interferon Activated Inhibitor of *Ceruloplasmin* mRNA Translation

^f Selenocysteine Insertion Sequence-type 1

Table 5

Summary of mRNA expression, protein or peptide levels, and enzymatic activity, monkey vs. mouse.

Gene	Monkey		Mouse	
	mRNA	Protein/Peptide/Activity	mRNA	Protein/Peptide/Activity
SP1	↓	↑	↑	↑
AβPP	↓	↑	↑	↑
BACE1	↓	↓	nc ^a	↑
Aβ		↑		↓

^aNo change

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