Published in final edited form as:

Ann Neurol. 2014 May ; 75(5): 684–699. doi:10.1002/ana.24149.

Modulation of APP Expression Reduces A β Deposition in a Mouse Model

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Abstract

Objective—Proteolytic cleavage of the amyloid precursor protein (APP) generates β -amyloid (A β) peptides. Prolonged accumulation of A β in the brain underlies the pathogenesis of Alzheimer's disease (AD) and is regarded as a principal target for development of disease-modifying therapeutics.

Methods—Using CHO APP_{751SW} cells we identified and characterized effects of 2-[(pyridine-2-ylmethyl)-amino]-phenol (2-PMAP) on APP steady-state level and A β production. Outcomes of 2-PMAP treatment on A β accumulation and associated memory deficit were studied in APP_{SW}/PS1_{dE9} AD transgenic model mice.

Results—In CHO APP_{751SW} cells, 2-PMAP in a dose-response manner lowered the steady-state APP level and inhibited A β_{x-40} and A β_{x-42} production with minimum effective concentration 0.5 μ M. The inhibitory effect of 2-PMAP on translational efficiency of APP mRNA into protein was directly confirmed using 35S-methionine/cysteine metabolic labeling technique, while APP mRNA level remained unaltered. Administration of 2-PMAP to APP_{SW}/PS1_{dE9} mice reduced brain levels of full length APP and its C-terminal fragments along with lowering levels of soluble A β_{x-40} and A β_{x-42} . Four-month chronic treatment of APP_{SW}/PS1_{dE9} mice revealed no observable toxicity and improved animals' memory performance. 2-PMAP treatment also caused significant reduction in brain A β deposition determined by both unbiased quantification of A β plaque load and biochemical analysis of formic acid extracted A β_{x-40} and A β_{x-42} levels and the level of oligomeric A β .

Interpretation—We demonstrate the potential of modulating APP steady-state expression level as a safe and effective approach for reducing A β deposition in AD transgenic model mice.

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Authorship: M.J.S. designed research, A.A.A., M.G., J.E.P., S.S. Performed research, A.A.A. and M.J.S. interpreted data, and M.J.S. wrote the paper.

Potential Conflict of Interest: M.J.S. is a co-inventor on a U.S. Patent No. 8,658,677 "Pyridil-2-methylamino compounds, composition and uses thereof", which is related to the work described in this paper. M.J.S. has consulted with Phillips North America and received speakers' fees from Forest Laboratories Inc. outside the submitted work within the past 36 months.

Keywords

amyloid precursor protein (APP); β -amyloid ($A\beta$); behavioral testing; disease modifying therapy; transgenic mice; protein translation

Introduction

Neuropathological underpinning of Alzheimer's disease (AD) involves progressive accumulation of β -amyloid ($A\beta$) peptides in the brain, which oligomeric and fibrillar assemblies cause synaptic loss, set off local inflammatory response, and induce intraneuronal formation of neurofibrillary tangles¹. $A\beta$ peptides are generated during proteolysis of the amyloid precursor protein (APP), which is a type I transmembrane protein involved in synaptic plasticity². Mature APP undergoes trafficking between the plasma membrane and the endosomal compartment. En route APP is alternatively cleaved by enzymes with α -secretase activity localized to the plasma membrane, or by β -site APP-cleaving enzyme 1 (BACE1) in the endosomal compartment³. This first cleavage yields soluble APP (sAPP) fragments secreted to the extracellular space and intracellularly retained C-terminal fragments (CTF) α -CTF and β -CTF (also known as C99 and C83), resulting from α -secretase or BACE1 cleavage, respectively. Subsequently, γ -secretase complex (γ -SC) cleaves the N-terminus of β -CTF to produce $A\beta$ peptides of different lengths with $A\beta_{1-40}$ and $A\beta_{1-42}$ species being the most prevalent.

Accumulation of $A\beta$ peptides in the brain plays a pivotal role in the disease pathogenesis; hence it is considered a prime target for disease-modifying therapy of AD. Modulations of BACE1 and γ -SC APP processing have been extensively studied as potential therapeutic approaches⁴. As both BACE1 and γ -SC have multiple client proteins, undesired off-target effects are one of the main drawbacks to implementing their inhibitors in clinical practice^{5,6}. As an alternative to inhibiting APP processing, $A\beta$ production can also be reduced by modulation of APP expression levels^{7,8}. While development of APP secretases inhibitors are widely pursued, very few molecules capable of modulating APP expression and showing drug development potential have been identified thus far. Through a small molecule screening process conducted using Chinese hamster ovary (CHO) cells stably expressing human APP₇₅₁ with double "Swedish" familial mutation KM670/671NL⁹ (CHO APP_{751SW}), we discovered that 2-[(pyridine-2-ylmethyl)-amino]-phenol, dubbed 2-PMAP, in a dose-dependent manner, reduces concentrations of $A\beta_{x-40}$ and $A\beta_{x-42}$ in the conditioned media, along with lowering the steady-state APP level and intracellular levels of α -CTF and β -CTF. Since 2-PMAP showed no apparent toxicity and penetrates the blood-brain barrier (BBB), it was tested in APP_{SW}/PS1_{ΔE9} transgenic (Tg) mice where it ameliorated $A\beta$ pathology along with rescuing memory deficit.

Materials and Methods

Materials and reagents

2-PMAP (CAS 102212-26-0) was resynthesized from 2-aminophenol and 2-pyridine carboxaldehyde and purified by flash column chromatography by Princeton Global

Synthesis LLC (Bristol, PA) a commercial chemical manufacturer contractor. Purity of 2-PMAP used for the cell culture and animal studies was 98% as determined by HPLC/UV analysis. γ -SC inhibitor XXI (Compound E) was obtained from Sigma–Aldrich (St. Louis, MO) and EMD Millipore (Billerica, MA), respectively. Phenserine was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). A β ₁₋₄₀ and A β ₁₋₄₂ peptides were custom synthesized and purified in the WM Keck Proteomic Facility of Yale University (New Haven, CT) by the laboratory of Dr. James I. Elliott as previously described^{10,11}. PCR primers were custom synthesized by Gene Link (Hawthorne, NY). Cell culture media and supplements were obtained from Invitrogen Life Technologies (Carlsbad, CA) while cell culture plasticware was purchased from Corning Incorporated (Corning, NY). All other chemicals, reagents, and antibodies were obtained from Sigma–Aldrich (St. Louis, MO) unless otherwise specified.

Cell culture

SK-N-SH human neuroblastoma cell line # HTB-11 was obtained from the American Type Culture Collection (Manassas, VA) and cultured in minimal essential medium (MEM) supplemented with heat-inactivated 10% fetal bovine serum (FBS), penicillin (100 units / mL) and streptomycin (100 μ g / mL) at 37°C in 5% CO₂. A clone of Chinese hamster ovary (CHO) cells stably expressing human APP₇₅₁ with double “Swedish” familial mutation KM670/671NL (CHO APP_{751SW}) was characterized previously⁹ and kindly provided by Dr. E. H. Koo from Univ. of California San Diego. CHO APP_{751SW} cells were cultured in 10% FBS supplemented MEM with addition of penicillin (100 units / mL), streptomycin (100 μ g / mL) and geneticin (500 μ g / mL).

MTT cell viability assay

SK-N-SH and CHO APP_{751SW} cells were cultured in the presence of 0.1 μ M to 100 μ M of 2-PMAP for 72hr and then their viability was assessed using the (3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrasodium (MTT) metabolic assay kit (Roche Molecular Biochemicals, Indianapolis, IN) as previously described¹². To obtain positive toxicity controls, SK-N-SH cells were treated with 0.6 mg/L of CoCl₂.

Compound treatment of CHO APP_{751SW} cells and sample collection

Stock solutions (10mM) of 2-PMAP, Phenserine, and γ -SC inhibitor Compound E were prepared in sterile DMSO and added to the cells after making serial dilutions in DMSO so the final concentration of DMSO in the culture media always remained the same 0.1%. CHO APP_{751SW} cells were seeded on 6cm² Petri dishes in the amount of 2×10^5 cells per dish and grown for 24hr alone and then for the next 48hr in the presence of 2-PMAP (0.1 μ M to 50 μ M), Phenserine (0.1 μ M to 50 μ M), or Compound E (10 μ M). The vehicle control cells were treated with 0.1% DMSO only. At the conclusion of the experiment the conditioned media were carefully collected, and the cells were washed three times with PBS at 37°C and then harvested with 1 mL of the ice-cold cell lysis buffer containing 50mM Tris-HCl, pH 7.4, 150mM NaCl, 1mM ethylenediaminetetraacetic acid (EDTA), 1% nonidet-P40, 0.1% SDS, 0.2% diethylamine (DEA), 1mM phenylmethylsulfonyl fluoride (PMSF), 10 μ g/ml of a Complete Protease Inhibitor Cocktail (Roche Applied Science; Indianapolis, IN) supplemented with leupeptin, antipain, pepstatin (5 μ g/ml each)¹¹. Cell lysates were

collected into 2ml low adhesion tubes, and further homogenized with ten strokes of a Teflon pestle, and centrifuged at $10,000 \times g$ for 20min at 4 °C. The pellets containing cell debris were discarded while the total protein concentration in the supernatant was determined using bicinchoninic acid assay (BCA) kit (Pierce Biotechnology Inc.; Rockford, IL) according to the manufacturer's manual.

Quantification of A β_{x-40} and A β_{x-42} in the conditioned media by ELISA

Concentrations of A β_{x-40} and A β_{x-42} were quantified in the conditioned media of CHO APP_{751SW} cells using sandwich ELISA, which discriminates C-terminal configuration of A β peptides. Immulon 2 HB ELISA plates (Fisher Scientific; Pittsburgh, PA) were coated overnight with HJ2 (anti-A β_{35-40}) or HJ7.4 (anti-A β_{37-42}) monoclonal antibodies (mAbs)^{11,13} in the amount of 2 μ g/well. Non-specific binding was blocked with 4% bovine serum albumin (BSA) diluted in 0.01 phosphate buffered saline (PBS), for 60min at 37°C. Freshly harvested samples of the conditioned media were diluted 1:10 and applied overnight at 4°C together with samples of formic acid (FA) treated synthetic A β_{x-40} and A β_{x-42} serially diluted from 1,200 pg/mL to 1.65 pg/mL. Vigorous quadruple washing with PBS-T was applied between each step of ELISA. Captured A β peptides were detected using biotinylated 4G8 mAb (anti-A β_{17-24} ; Covance; Princeton, New Jersey), which was added for 90min and incubated at 37°C followed by the streptavidin–poly-horseradish peroxidase-40 (Research Diagnostics, Flanders, NJ) and then by Sigma SuperSlow TMB. Sulfuric acid (2M) was used to stop the color reaction. Absorbance was read on the Epoch Microplate Spectrophotometer (BioTek Instruments, Inc. Winooski, VT) at 450nm wavelength. Following background subtraction, absorbance values from serially diluted synthetic A β samples were used to generate a standard curve in GraphPad Prism v5.02 (GraphPad Software, Inc., San Diego) using a non-linear curve-fitting algorithm. A β_{x-40} and A β_{x-42} concentrations in conditioned media samples were determined by comparing their absorbance values against the standard curve and multiplied by the dilution factor of 10.

Immunoblot analysis

Aliquots of cell lysates containing 20 μ g of the total protein were boiled for 5min in reducing Laemmli buffer and then resolved on 10% SDS-PAGE and electroblotted onto nitrocellulose membranes (GE Healthcare Bio-Sciences Corp.; Piscataway, NJ). The membranes were blocked with 5% nonfat milk in TBS-T for 1hr at room temperature and then incubated with previously characterized, commercially available primary antibodies at the indicated dilutions: clone 22C11 that specifically reacts with the N terminus of APP (1:1,000; Millipore Corporation, Billerica, MA), clone 6E10 that reacts with the central fragment of APP¹⁴ (1:5,000; gift of Dr. R.J. Kascsak; NYS Institute for Basic Research, Staten Island, NY), A163 rabbit polyclonal antibody (pAb) to the C terminus of APP that detects full length APP and APP α -CTF and β -CTF (1:1,000 Leinco Technologies Inc. St. Louis, MO), rabbit pAb to amyloid precursor-like protein-2 (APLP-2) that detects full length APLP-2 (1:2,500; Abcam Cambridge, MA), and anti- β -actin mAb (1:1,000). The antigen-antibody complexes were detected using horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG antibodies both at 1:3,000 dilution (GE Healthcare Bio-Sciences Corp. Piscataway, NJ) and visualized using SuperSignal (Pierce Chemical; Rockford, IL) on X-Omat Blue XB-1 autoradiography films (Eastman Kodak Company; New Haven, CT). Autoradiographs were

digitized and subjected to densitometric analysis using NIH Image J software v 1.42 (Bethesda, MD) following our published protocols^{11,15,16}. Expression of the full length APP was densitometrically quantified on Western immunoblots developed using 6E10 mAb while expression of APP CTFs on those developed using A163 pAb. For each densitometrically analyzed protein all samples were electrophoresed and immunoblotted at the same time, rigorously maintaining the same experimental conditions.

For detection of sAPP in the conditioned media 50µL samples of the media were resolved on 10% SDS-PAGE, electroblotted onto nitrocellulose membranes and probed with 22C11 or 6E10 mAbs.

Immunocytochemistry

CHO APP_{751SW} cells were seeded on removable poly-L-lysine coated round coverslips and cultured for 48hr in the presence of 10µM of 2-PMAP or vehicle (DMSO 0.1%). At the conclusion of the experiment coverslips with attached cells were immersed three times in PBS at 37°C and then in 80% ice-cold methanol for 10min, and washed again three times in PBS. Non-specific bindings of the primary mAb and streptavidin was blocked using mouse on mouse (MOM) blocking mixture for 1hr followed by the Streptavidin/Biotin blocking kit for 30min. Both reagents were obtained from Vector Laboratories, Ltd. (Burlingame, CA). APP was detected using 22C11 mAb (1:1,000) followed by biotinylated secondary antibody (1:1,000) and Fluorescein isothiocyanate (FITC) conjugated streptavidin (1:500). Negative controls for immunocytochemistry included CHO APP_{751SW} cells immunostained using anti-mouse biotinylated secondary antibody and FITC conjugated streptavidin with omission of the primary antibody. Immunocytochemistry was analyzed using Zeiss LSM 510 confocal microscope (Carl Zeiss Microscopy GmbH, Jena, Germany).

APP mRNA

CHO APP_{751SW} cells were treated with 2-PMAP (25µM), γ -SC inhibitor Compound E (10µM) or vehicle (0.1% DMSO) as described above. At the conclusion of the experiment total RNAs were extracted from the cells using an RNAeasy kit (Qiagen; Germantown, MD). Four hundred ng of RNA were reverse transcribed into cDNA in 20µl final volume using the iScript reverse transcriptase kit (Bio-Rad; Hercules, CA). The polymerase chain reaction (PCR) conditions were optimized based on the various primer annealing temperatures. Reactions were performed in the MyiQ2 real-time PCR (RT-PCR) detection system (Bio-Rad) using iScript SYBR Green Master Mix (Bio-Rad), and RT-PCR efficiencies were calculated from the given slopes in the MyiQ software (Bio-Rad). The increase in fluorescence emission of the SYBR Green dye is proportional to the amount of full length as well as double stranded target product that has accumulated, which in turn is proportional to the starting target concentration. The following primer sequences were designed with Primer Express 4 software (Applied Biosystems; Foster City, CA) on the basis of the Genbank cDNA sequences as indicated: APP primer pair (NM_201414) 5'-CGA ACC CTA CGA AGA AGC CAC-3' and Reverse Primer 5'-GCT TTC TGG AAA TGG GCA TGT TC-3', β -actin primer pair (NM_007393.3) 5'-CCT GAA CCC TAA GGC CAA CC-3' and Reverse Primer 5'-CAG CTG TGG TGG TGA AGC TG-3' and mouse GAPDH primer pair (NM_008084) 5'-CCG GGG CTG GCA TTG CTC TC-3' and Reverse Primer 5'-TGT TGG

GGG CCG AGT TGG GA-3'. The specificity of PCR primers was determined by Blast¹⁷. The PCR protocol was carried out by 15s denaturation at 95°C, 30s annealing at 58°C, and 1min elongation at 72°C for 40 cycles. Fluorescence was detected at the end of every 72°C extension phase. To exclude the contamination of unspecific PCR products such as primers and dimers, melting curve analysis was applied to all final PCR products after the cycling protocol. Also, the PCR reactions without the reverse transcriptase reaction were performed for each sample in order to exclude genomic DNA contamination. Each sample was run in triplicate and threshold cycle (Ct) values were averaged from the triplicate. APP mRNA expression was expressed relative to β -actin or GAPDH Ct values in control vehicle treated cells. The final data were averaged from three separately conducted experiments.

Rate of APP synthesis

CHO APP_{751SW} cells were treated with 2-PMAP (10 μ M or 25 μ M) or vehicle (0.1% DMSO) as described above. After 24hr of 2-PMAP treatment the MEM medium was substituted for 30min with RPMI-1640 medium lacking methionine and cysteine supplemented with 10% FBS and then the cells were pulsed for 15min with 500 μ Ci/ml of 35S labeled methionine and cysteine mixture (TRAN35S-LABELTM; MP Biomedicals, Solon, OH), which was then replaced during the chase with "cold" MEM supplemented with 10% heat inactivated FBS. Vehicle and 2-PMAP were present in the media during pulse and chase periods. At the conclusion of the experiment the cells were washed three times with PBS at 37°C and then harvested with ice-cold lysis buffer as described above. The lysates were cleared of cell membrane debris by centrifugation at 10,000 $\times$ g for 20min at 4°C, and the total protein concentration in the supernatant was determined using BCA assay and diluted to 1 μ g/ μ L. APP was immunoprecipitated with C-terminal mAb (clone C1/6.1; Covance Inc., Princeton, NJ)¹⁸ (10 μ g mAb/200 μ L sample). Samples were also immunoprecipitated with mAbs against β -actin or connexin 43 (clone CXN-6). Protein/mAb complexes were isolated using Dynabeads M-280 sheep anti-mouse IgG magnetic beads (Invitrogen, Carlsbad, CA) following the manufacturer provided manual. The proteins were separated from the beads by boiling in sample buffer containing β -mercaptoethanol and resolved on 10% SDS-PAGE. The gels were dried under vacuum overnight and apposed to Amersham Hyperfilm MP films at -80°C (GE Healthcare Bio-Sciences Corp.; Piscataway, NJ). All autoradiographs were digitized and densitometrically analyzed using NIH Image J software v 1.42 (Bethesda, MD) following our established protocols^{11,15,16}.

Animals

All mouse care and experimental procedures were approved by Institutional Animal Care and Use Committee of the New York University School of Medicine. APP_{SW}/PS1_{dE9} mice^{19,20} were derived from our own animal colony where they were mated with wild-type (WT) C57BL/6 mice (Charles River Laboratories, Wilmington, MA) to maintain the line. Offsprings carrying APP_{SW}/PS1_{dE9} transgene were identified using MyiQ2 RT-PCR system (Bio-Rad Hercules, CA) as previously described^{11,21}. Given documented sex differences in the A β deposition in the APP_{SW}/PS1_{dE9} strain¹³, short-term and the long-term 2-PMAP treatment experiments were conducted only in female mice that were heterozygous for the APP_{SW}/PS1_{dE9} transgene.

Subacute treatment of APP_{SW}/PS1_{dE9} Tg mice

The subacute treatment experiment was performed in six-month old female APP_{SW}/PS1_{dE9} Tg mice that received 2-PMAP (50 mg/kg) or vehicle every 12hr for five days. For *in vivo* application the stock solution was prepared by dissolving 25mg of 2-PMAP powder in one mL of the stock solvent consisting of 60% Polyethylene glycol 300, 30% anhydrous ethanol and 10% Tween 80. Immediately prior to the administration of the compound to animals the 25 mg/mL 2-PMAP stock solution was further diluted 1:4 in sterile PBS and the pH of the solution was verified to be 7.4. Vehicle treated animals received PBS diluted stock solvent only. 2-PMAP and vehicle were given via ip injection using a 27 gauge needle. Four hours after the last dose of 2-PMAP or vehicle animals were killed by ip injection of sodium pentobarbital (150 mg/kg) and transcardially perfused. The brains were extracted from the skulls and the cortical mantle was dissected out and ultrasonically homogenized (1:10 w/v at 4°C) using Misonix XL-2000 ultrasonic homogenizer (Qsonica, LLC; Newtown, CT) in the brain homogenization buffer consisting of 20mM Tris-HCL (pH 7.4), 250mM sucrose, 1mM EDTA, 1mM PMSF, and 10µg/ml of a Complete Protease Inhibitor Cocktail (Roche Applied Science; Indianapolis, IN) supplemented with leupeptin, antipain, pepstatin (5 µg/ml each)^{21,22}. The total protein concentration in the brain homogenate was determined using BCA method as described above.

A set of samples from the brain cortex homogenate containing equal amount of total protein (5µg) was adjusted to equal volume by adding Tris-HCL buffer, boiled for 5min in reducing Laemmli buffer and then resolved on 10% SDS-PAGE and electroblotted onto nitrocellulose membranes. The membranes were probed with 22C11 mAb for detection of full length APP, A163 pAb for detection of full length APP and APP CTFs and anti-β-actin mAb as a loading control, as described above for immunoblotting of cell lysate. Autoradiographs resulting from these immunoblots were digitized and densitometrically analyzed using NIH Image J software v 1.42 (Bethesda, MD) following our published protocols^{11,15,16}.

A separate set of samples of the brain cortex homogenate was subjected to DEA extraction following our previously established protocols²². The DEA extraction releases from the brain tissue soluble Aβ, which is not associated with amyloid plaques and vascular deposits²³. Levels of DEA extractable soluble Aβ_{x-40} and Aβ_{x-42} in the brain cortex were quantified by sandwich ELISA following the protocol described above for determining concentration of Aβ peptides in the conditioned media. Results of the ELISA were presented as µg of Aβ per gram of wet brain accounting for dilutions made during preparation of brain homogenate and subsequent DEA extraction.

Long-term treatment of APP_{SW}/PS1_{dE9} Tg mice

Female APP_{SW}/PS1_{dE9} mice were treated with 2-PMAP (50mg/kg/day) or vehicle from the age of six to ten months during which time 2-PMAP or vehicle was administered once a day by ip injection using 27 gauge needles. 2-PMAP was prepared for the injections as described above for the subacute treatment experiment. During the long-term 2-PMAP treatment APP_{SW}/PS1_{dE9} mice were carefully observed for symptoms of toxicity. Evaluations included the following aspects of the animals' condition: body weight, general physical appearance, coat appearance, unprovoked behavior and response to external stimuli. At the

conclusion of the experiment the mice were killed with an ip overdose of sodium pentobarbital (150mg/kg) and transcardially perfused as described above. Their brains were extracted from the skulls, and the brain hemispheres were separated and processed for biochemical and morphological analyses. At the time of sacrifice, body organs were macroscopically evaluated for any irregular appearance. Samples of heart muscle, lungs, liver, kidneys and spleen were collected from six mice randomly selected from each treatment group. Organs samples were fixed in 10% buffered paraformaldehyde, embedded in paraffin blocks and cut into 12 μ m thick section. Hematoxylin and eosin stained sections of body organs were evaluated by a veterinary pathologist. Age matched female wild type littermates of APP_{SW}/PS1_{DE9} mice, which were of C57BL/6 background, were used as a reference control for body weight measurements and histopathological evaluation of the body organs.

During the last three weeks of the treatment the animals' memory performance was tested using object recognition and radial arm tests, which were conducted following previously published protocols^{22,24}. During the testing, APP_{SW}/PS1_{DE9} animals continued to receive daily injections of 2-PMAP or vehicle. A group of age matched female wild type (WT) littermates of APP_{SW}/PS1_{DE9} mice was included as a reference control in behavioral studies.

At the conclusion of 2-PMAP treatment the load of A β plaques (% of cross-sectional area occupied by A β plaques) was determined in the cerebral cortex of the right hemisphere in serial sections stained with Thioflavin-S for fibrillar A β deposits, or immunostained with anti-A β mAbs specific for C-terminal configuration of A β _{x-40} and A β _{x-42} peptides following our published protocols^{11,21}. To detect A β _{x-40} a mouse mAb (clone 11A50-B10; Covance Inc., Princeton, NJ) was used in 1:250 dilution followed by Texas Red conjugated goat anti-mouse IgG secondary antibody from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) diluted 1:200. For A β _{x-42} detection we used rabbit mAb (clone 1-11-3; Covance Inc., Princeton, NJ) at 1:1,00 dilution followed by Alexa Fluor® 488 conjugated goat anti-rabbit IgG (Invitrogen, Carlsbad, CA) secondary antibody diluted 1:250.

In addition we analyzed co-localization of A β _{x-40} and A β _{x-42} immunostaining in the same plaques on sections double immunostained for A β _{x-40} and A β _{x-42} using confocal microscopy. Serial Z stacks of 0.5 μ m-thick tomograms were collected simultaneously in Fluorescein and Texas Red channels using Zeiss LSM 510 confocal microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) and were further analyzed using Zeiss LSM Image Browser software (Jena, Germany) or NIH Image J software v 1.42 (Bethesda, MD).

Biochemical analysis of A β levels was conducted in the left hemisphere. The brain cortex including the hippocampus was dissected out, homogenized, and subjected to formic acid (FA) extraction^{21,22}. The levels of FA-extractable A β _{x-40} and A β _{x-42} were quantified by C-terminal specific sandwich ELISAs as described above. In addition, the level of oligomeric A β in the brain homogenate was measured using ELISA specifically detecting aggregates of multiple A β copies²¹. Results of all ELISAs were presented as μ g of A β per gram of wet brain accounting for all dilutions made during brain homogenization and subsequent processing of the homogenate.

Statistical analysis

Results of MTT cytotoxicity assay were analyzed using one-way analysis of variance (ANOVA) followed by Dunnett's Multiple Comparison Test for post hoc analysis. Effects of 2-PMAP and Phenserine dose-range treatment on $A\beta_{x-40}$ and $A\beta_{x-42}$ levels and the levels of APP, APP CTFs, and APLP-2 were analyzed using one-way ANOVA followed by Bonferroni's Multiple Comparison Test for post hoc comparison of each tested concentration against the vehicle control. Results of the radial arm maze behavioral testing were analyzed using one-way ANOVA followed by Newman-Keuls's Multiple Comparison Test. For all two-group comparisons in animal treatment experiments unpaired Student's *t* test was used.

Results

2-PMAP reduces secretion of $A\beta$ by CHO APP_{751SW} cells and simultaneously lowers the steady-state APP level and levels of APP α -CTF and β -CTF

2-PMAP (Fig 1) showed no apparent cellular toxicity up to the concentrations of 100 μ M as tested *in vitro* (Fig 2A, B). In CHO APP_{751SW} cells, 2-PMAP treatment caused concentration-dependent inhibition of $A\beta$ production (Fig 3A, B) with minimum effective concentration (MEC) on $A\beta_{x-40}$ and $A\beta_{x-42}$ secretions being 0.1 μ M (22.8% reduction) and 0.5 μ M (19.1% reduction), respectively. At 1 μ M concentration 2-PMAP reduced $A\beta_{x-40}$ and $A\beta_{x-42}$ levels in the conditioned media by 44.6% and 27.3%, respectively, while at the maximal tested concentration of 50 μ M by 88.6% and 84.9%, respectively.

Simultaneously, using densitometric analysis of Western immunoblots, we examined the effects of 2-PMAP on the level of full length APP and levels of α -CFT and β -CTF in lysates of CHO APP_{751SW} cells (Fig 4A-D). In parallel to inhibitory effect on $A\beta$ secretion, 2-PMAP in a concentration-dependent manner reduced levels of APP and those of its CTFs. The lowest concentration of 2-PMAP producing changes in APP steady-state level detectable by Western immunoblot densitometry was 2.5 μ M (22.1% reduction for mature and immature APP bands combined), while reduction in APP CTFs was clearly seen at concentration of 0.5 μ M (33.9% reduction for α -CFT and β -CTF combined). At 10 μ M concentration 2-PMAP reduced levels of APP and APP CTFs by 50.2% and 83.5%, respectively; while 50 μ M concentration of 2-PMAP reduced levels of APP and APP CTFs by 75.5% and 92.0%, respectively. In parallel to concentration-dependent reduction in APP and APP CTFs levels in the cell lysates by 2-PMAP, we also noticed concentration dependent reduction in the level of soluble APP secreted to the conditioned media (Fig 4A). There were no significant changes in the steady-state level of β -actin seen in cells treated with 2-PMAP at concentrations ranging from 0.1 μ M to 50 μ M.

Furthermore, we have also analyzed the steady-state expression level of APLP-2, which is a close APP homolog, exhibiting the same domain structure, and undergoing proteolytic cleavage by the same secretases (reviewed in²⁵). We wanted to determine whether 2-PMAP down-regulates APLP-2 steady-state level in a similar way as it affects that of APP. At concentration of 25 μ M, 2-PMAP reduced APLP-2 steady state level by 33.7% comparing to the vehicle treated cells (difference not statistically significant), while at 50 μ M

concentration it caused significant reduction in APLP-2 level by 59.8% (Fig 4A, D). Hence, 2-PMAP exerts its effect on the steady-state level of both APP and APLP-2, but its effect on the latter homolog requires higher concentration and is less pronounced.

Our experiments also included a well characterized γ -SC inhibitor Compound E, which was used as a positive treatment control. The treatment of CHO APP_{751SW} cells with 10 μ M of Compound E reduced A β _{x-40} and A β _{x-42} concentrations in the conditioned media by 99.3% and 98.5%, respectively; but in contrast to 2-PMAP, Compound E increased levels of full length of APP, APP CTFs and APLP-2 to 138%, 366%, and 152% of those in vehicle treated cells, respectively.

In addition, we employed confocal microscopy to compare expression and distribution of APP in CHO APP_{751SW} cells treated with vehicle and 10 μ M of 2-PMAP. Anti-APP immunostaining of vehicle treated cells revealed a granular staining pattern in the cytoplasm and also immunoreactivity within the cellular membrane. Cells treated with 2-PMAP demonstrated reduced anti-APP immunostaining, without evidence of APP redistribution or compartmentalization (Fig 4E).

Since the inhibitory effect of 2-PMAP on A β secretion was associated with reduction of the APP steady-state expression level, we compared 2-PMAP to Phenserine, which is an established APP post-transcriptional modulator, using the same CHO APP_{751SW} cell model (Fig 5). MEC of Phenserine on A β _{x-40} production in CHO APP_{751SW} cells was 5 μ M, and at this concentration it reduced A β _{x-40} in the conditioned media by 19.1% compared to vehicle treated control cells. MEC of Phenserine on A β _{x-42} production was 25 μ M and it diminished A β _{x-42} concentration in the conditioned media by 36.7%. At concentration of 50 μ M Phenserine reduced the secretion of A β _{x-40} and A β _{x-42} by 72.2% and 64.7%, respectively. Densitometric analysis of APP steady-state level in Phenserine treated CHO APP_{751SW} cell showed 18.6% reduction in cells treated with 10 μ M of Phenserine comparing to the vehicle control (difference not statistically significant), while 25 μ M concentration of Phenserine reduced APP level by 51.4%.

2-PMAP inhibits APP translation but does not affect APP mRNA level

To elucidate the mechanism through which 2-PMAP reduces cellular APP steady-state level, we examined levels of APP mRNA and the rate of APP synthesis under 2-PMAP treatment. In CHO APP_{751SW} cells treated with 25 μ M of 2-PMAP we found no changes in APP mRNA level relative to the mRNA level of β -actin or GAPDH, which served as housekeeping genes in two separate sets of experiments (Fig 6A). In control experiments CHO APP_{751SW} cells treated with 10 μ M of Compound E also showed no changes in APP mRNA level (Fig 6A).

Effect of 2-PMAP on APP translation rate was evaluated using 35S-methionine/cysteine metabolic labeling technique. Radiolabeled APP was immunoprecipitated using C-terminal mAb, which allowed visualization of both mature (mAPP) and immature (imAPP) APP forms on the autoradiographs. Treatment of CHO APP_{751SW} cells with 10 μ M of 2-PMAP reduced levels of mAPP and imAPP generated during the 15min pulse by 24.1% and 20.8%, respectively; 25 μ M of 2-PMAP reduced mAPP and imAPP levels by 44.1% and 28.1%,

respectively (Fig 6B, C). For comparison, immunoprecipitation of β -actin and connexin 43, which like APP is a transmembrane protein with comparable half-life^{26,27}, performed during the same experiments showed no significant effect of 2-PMAP on their biosynthesis (Fig 6B, D). Detection of both mAPP and imAPP forms at the conclusion of the pulse-labeling experiment indicates that 2-PMAP does not interfere with APP maturation. We also assessed the effect of 2-PMAP on APP's half-life by analyzing density of full length APP bands immunoprecipitated from CHO APP_{751SW} cells after 60min chase (approximated APP half-life), and compared them to density of bands immunoprecipitated immediately after concluding 15min pulse for matching treatment conditions (Fig 6E). In vehicle treated control cells the combined optical density for mAPP and imAPP bands following the chase was reduced by 40.0% compared to those after the pulse, while in cells treated with 10 μ M and 25 μ M 2-PMAP APP bands were reduced by 34.6% and 42.9% relative to their levels at the conclusion of the pulse for matching 2-PMAP concentrations, respectively. Differences in the degree of APP reduction across all three treatment conditions were not statistically significant (Fig 6F).

Subacute 2-PMAP treatment lowers brain levels of APP and APP α -CTF and β -CTF and reduces brain concentration of soluble A β in APP_{SW}/PS1_{dE9} Tg mice

2-PMAP is BBB permeable. We administered 2-PMAP for five days to six-month old female APP_{SW}/PS1_{dE9} mice, which have minimal A β deposits at this age²⁰. 2-PMAP treatment reduced the level of full length APP by 22.5% relative to the APP steady-state expression level in vehicle treated mice (Fig 7A, B). The significant reduction was also seen in jointly quantified α -CTF and β -CTF levels, which were reduced by 29.7%. Brain concentrations of soluble A β _{x-40} and A β _{x-42}, measured in DEA brain extracts^{22,23} were reduced in 2-PMAP treated mice by 84.0% and 67.6% relative to the vehicle treated controls, respectively (Fig 7C).

Long-term treatment with 2-PMAP is well tolerated, prevents memory deficit and reduces A β accumulation in APP_{SW}/PS1_{dE9} Tg mice

The long-term treatment of APP_{SW}/PS1_{dE9} mice with 2-PMAP was conducted between the age of six and ten months and did not reveal any evidence of observable toxicity including histopathological analysis of selected body organs. Behavioral testing conducted towards the end of the treatment included the object recognition and the radial arm maze tests, which both assess animals' memory. While vehicle treated APP_{SW}/PS1_{dE9} mice could not distinguish between the novel and the familiar objects during the retention session of object recognition testing, 2-PMAP treated animals showed proper exploratory behavior toward the novel object (Fig 8A). During the first four days of radial arm maze testing both 2-PMAP and vehicle treated APP_{SW}/PS1_{dE9} mice and their wild type littermates made comparable number of errors per session while showing gradually improved performance from day to day. After day five 2-PMAP treated APP_{SW}/PS1_{dE9} mice and their wild type littermates continued to improve their performance navigating through the maze with significantly less errors than vehicle treated APP_{SW}/PS1_{dE9} mice, which showed no further performance improvement after day five (Fig 8B).

Analysis of Thioflavin-S stained brain sections revealed 49.9% reduction in fibrillar A β plaque load and 38.2% reduction in their numerical density in 2-PMAP treated APP_{SW}/PS1_{DE9} mice comparing to vehicle controls (Fig 9A-C). The reduction in A β plaque load was also observed on sections immunostained against A β C-terminal configuration. The load of A β plaques positive for A β _{x-40} and A β _{x-42} was reduced in 2-PMAP treated mice by 41.9% and 35.3%, respectively (Fig 9D, E). Confocal microscopy analysis of A β plaques composition revealed presence of both A β _{x-40} and A β _{x-42} in plaques in both vehicle and 2-PMAP treated animals (Fig 9F). In 2-PMAP treated animals the plaques were smaller and contained somewhat greater relative A β _{x-42} component.

Biochemical analysis performed on FA extracted brain homogenates, which releases A β associated with fibrillar A β deposits^{22,23}, also revealed significant reduction in A β _{x-40} and A β _{x-42} levels in 2-PMAP treated animals by 82.3% and 56.0%, respectively (Fig 10A). Quantification of A β oligomer level showed 20.5% reduction in 2-PMAP treated mice (Fig 10B).

Discussion

Progressive accumulation of A β in the brain is a critical upstream event in AD pathogenesis^{1,4}. One mechanism of A β accumulation may result from elevated APP expression giving rise to increased A β production, as in Down's syndrome (Trisomy 21)²⁸ and familial AD caused by isolated APP locus duplication²⁹. There is also evidence that certain genetic variations in the APP promoter region may affect APP transcription leading to an increase in its steady-state level, and those promoter variations have been associated with increased risk for sporadic AD^{30,31}. Conversely APP steady-state level can be reduced in order to lower A β production. Our experiments demonstrated that 2-PMAP in a dose-dependent manner lowers APP steady-state level along with lowering A β secretion. APP gene expression analysis showed unaltered mRNA level in 2-PMAP treated cells, providing evidence that 2-PMAP operates as a post-transcriptional modulator of the APP expression, which was directly confirmed using 35S-methionine/cysteine pulse labeling technique. Furthermore, 2-PMAP appears to be a selective inhibitor of APP translation as it had no effect on translation of β -actin and connexin 43, and caused reduction of the level of APLP-2, which belongs to the same family as APP, at concentrations greater than 25 μ M.

Post-transcriptional regulation of APP expression level is complex. Regulatory factors include inhibitory micro RNA species³², proteins controlling APP mRNA half-life³³, and mRNA binding proteins which may act as direct repressors or promoters of APP mRNA translation^{7,34}. Although experimental evidence consistently indicates that 2-PMAP reduces translational efficiency of APP mRNA into protein, its exact modus operandi remains to be elucidated. Given the selectivity of its effect, two most likely mechanisms of 2-PMAP interaction with the APP post-transcriptional regulatory system are either direct binding to coding or noncoding APP mRNA sequences or interference between APP mRNA and one of its binding proteins, acting as a specific translational activator. Few other compounds have been reported to reduce APP level through interference with its translation process. Examples include Phenserine and its chiral isomer Posiphen with reduced anti-cholinesterase activity⁸ that inhibit APP translation through interaction with 5'-untranslated

region of APP mRNA³⁵. In direct comparison, we found that 2-PMAP reduces A β_{x-40} and A β_{x-42} secretion with MEC of 0.1 μ M and 0.5 μ M, respectively; in contrast, MEC of Phenserine in the same cell culture model was 5 μ M and 25 μ M, respectively, indicating that 2-PMAP is a more potent inhibitor.

Furthermore, our data provide no evidence to indicate that 2-PMAP's effect on A β production could be associated with modulation of APP secretases activities. In CHO APP_{751SW} cells studies 2-PMAP was directly compared with a γ -SC inhibitor Compound E. Compound E treatment was associated with modest elevation of APP levels and nearly four-fold increase in APP CTFs. It has been recognized that intracellular accumulation of APP CTFs due to γ -SC inhibition can result in cytotoxicity creating one of the potential side-effects of γ -SC inhibitors³⁶. In contrast, 2-PMAP not only lowered APP level but also reduced levels of α -CTFs and β -CTFs, suggesting an additional advantage of modulation of APP expression over γ -SC inhibition. Furthermore, there is no evidence to suggest that 2-PMAP acts as a BACE1 inhibitor, the effects of which are typically associated with selective lowering β -CTF level while steady-state APP level remains unaltered³⁷. Potentially enhanced α -secretase processing could also reduce APP level along with diminishing A β production. The possibility that 2-PMAP is an α -secretase activator was excluded by showing that 2-PMAP treatment was associated with reduction of both sAPP level in the conditioned media and α -CTF level in the cell lysate and brain homogenate; an α -secretase activator would have the opposite effect on both sAPP and α -CTF³⁸. Furthermore, several experimental approaches targeting APP trafficking or effecting changes in its cellular distribution can lower A β production, but such approaches either had no effect on the APP steady-state level^{39,40} or even effected its increase, as shown with overexpression of the sorting protein-related receptor⁴¹. Similarly approaches promoting autophagy and directing the content of endosomal compartments for lysosomal degradation were associated with reduced A β production but did not alter APP steady-state level^{42,43}. To examine whether 2-PMAP alters APP trafficking and its cellular distribution we immunostained 2-PMAP treated CHO APP_{751SW} cells against APP and examined them under confocal microscope. Although intensity of anti-APP immunoreactivity in those cells was markedly reduced we noticed no evidences of APP redistribution. Furthermore, we have experimentally confirmed that 2-PMAP does not affect APP maturation and does not promote APP degradation.

2-PMAP is a BBB permeable molecule. Although in APP_{SW}/PS1_{dE9} mice, 2-PMAP caused relatively modest reduction in APP level (22.5%), associated reduction in the levels of soluble A β_{x-40} and A β_{x-42} were substantially greater: 84.0% and 67.6%, respectively. The discrepancy between the magnitude of reduction in APP steady-state level and reduction in A β secretion was also seen in CHO APP_{751SW} cells. These observations suggest that 2-PMAP could exert a meaningful therapeutic effect on A β production, while its effect on the steady-state APP level remains modest, which inherently reduces risk of toxicity. In fact, long-term 2-PMAP treatment was well tolerated by APP_{SW}/PS1_{dE9} mice and ameliorated memory deficit along with reducing A β pathology. Notably, greater reduction in deposition of A β_{x-40} than that of A β_{x-42} , was demonstrated by both ELISA following FA A β extraction and by morphometric quantification of A β_{x-40} and A β_{x-42} positive plaque load. An explanation of this phenomenon is likely associated with different brain clearance capacities of A β_{x-40} and A β_{x-42} . Although both humans and APP_{SW}/PS1_{dE9} Tg mice produce more

$A\beta_{x-40}$ than $A\beta_{x-42}$, $A\beta_{x-40}$ is also more readily cleared from the brain than $A\beta_{x-42}$ ⁴⁴. Clearance of $A\beta$ peptides from the brain can be improved by passive immunization with anti- $A\beta$ mAbs, which enhance efflux of $A\beta$ across the BBB⁴⁵. It has already been shown that use of anti- $A\beta$ passive immunization in mice with conditional APP knock-out augmented the effect of passive immunization and that the passive immunization was able to clear $A\beta$ deposits formed prior to the conditional knock-out onset⁴⁶. This observation implies potential benefit of combining post-translational APP modulators with passive immunization approaches.

Recent clinical studies in AD subjects provided encouraging evidence that passive immunization using anti- $A\beta$ mAbs can reduce brain $A\beta$ load^{47,48} and lower CSF levels of total and phosphorylated tau⁴⁹. Unfortunately effects on cognitive metrics were seen only in a sample-limited cohort of early AD patients in Solanuzemab Phase III trial⁵⁰. The disparity between effects on biological target and modest efficacy on disease symptoms, limited to least affected patients, revealed in clinical trials of passive immunization support the notion that $A\beta$ -centered approaches should be used as a preventive strategy rather than for symptomatic therapy. Studies on the natural course of AD pathology in Down's syndrome subjects⁵¹ and disease models built on recent biomarkers studies⁵² indicate buildup of $A\beta$ in the brain may take over 20 years before first signs of memory impairment herald clinical onset of the disease. The protracted preclinical phase of AD, means deployment of $A\beta$ -targeting therapeutics for disease prevention need to be continued safely on a long-term basis and would favor agents administered orally. Several orally available BACE1 inhibitors and γ -SC modulators are under development^{53,54}. However, given multiple targets cleaved by BACE1 and γ -SC, these drugs would still need to demonstrate satisfactory effect in humans, without producing significant off-target effects. Modulation of APP expression is unlikely to evoke the off-target effects of APP secretase inhibitors. Results of 2-PMAP long-term treatment in APP_{SW}/PS1_{ΔE9} mice provide strong support for the validity of partial reduction of APP steady-state expression level as a preventive approach to reduce $A\beta$ deposition.

Acknowledgments

We thank Dr. Richard R. Kascak from the New York State Institute for Basic Research in Developmental Disabilities (Staten Island, NY) for providing 6E10 anti- $A\beta$ mAb and Dr. E. H. Koo from Univ. of California San Diego for providing CHO APP751SW cells. We would like to acknowledge the following individuals affiliated with Aria Neurosciences Inc. Dr. Bijan Almassian for providing 2-PMAP and its pharmacokinetic characterization, Dr. Kevin X. Lin for analysis of 2-PMAP physicochemical properties, and Dr. Margaret Harbison for histopathological analysis body organs from 2-PMAP treated APP_{SW}/PS1_{ΔE9} mice. This work was supported by NIH/NIA grants: R01 AG31221 and K02 AG34176 to M. J. S. and by in kind effort of Aria Neurosciences Inc. as described above.

References

1. Querfurth HW, LaFerla FM. Alzheimer's disease. *N Engl J Med*. 2010; 362:329–344. [PubMed: 20107219]
2. Cirrito JR, Yamada KA, Finn MB, et al. Synaptic activity regulates interstitial fluid amyloid-beta levels in vivo. *Neuron*. 2005; 48:913–922. [PubMed: 16364896]
3. Haass C, Kaether C, Thinakaran G, et al. Trafficking and proteolytic processing of APP. *Cold Spring Harb Perspect Med*. 2012; 2:a006270. [PubMed: 22553493]

4. Selkoe DJ. Resolving controversies on the path to Alzheimer's therapeutics. *Nat Med.* 2011; 17:1060–1065. [PubMed: 21900936]
5. Marks N, Berg MJ. Neurosecretases provide strategies to treat sporadic and familial Alzheimer disorders. *Neurochem Int.* 2008; 52:184–215. [PubMed: 17719698]
6. Extance A. Alzheimer's failure raises questions about disease-modifying strategies. *Nat Rev Drug Discov.* 2010; 9:749–751. [PubMed: 20885394]
7. Lee EK, Kim HH, Kuwano Y, et al. hnRNP C promotes APP translation by competing with FMRP for APP mRNA recruitment to P bodies. *Nat Struct Mol Biol.* 2010; 17:732–739. [PubMed: 20473314]
8. Lahiri DK, Chen D, Maloney B, et al. The experimental Alzheimer's disease drug posiphen [(+)-phenserine] lowers amyloid-beta peptide levels in cell culture and mice. *J Pharmacol Exp Ther.* 2007; 320:386–396. [PubMed: 17003227]
9. Weggen S, Eriksen JL, Das P, et al. A subset of NSAIDs lower amyloidogenic Abeta42 independently of cyclooxygenase activity. *Nature.* 2001; 414:212–216. [PubMed: 11700559]
10. Sadowski M, Pankiewicz J, Scholtzova H, et al. A synthetic peptide blocking the apolipoprotein E/ beta-amyloid binding mitigates beta-amyloid toxicity and fibril formation in vitro and reduces beta-amyloid plaques in transgenic mice. *Am J Pathol.* 2004; 165:937–948. [PubMed: 15331417]
11. Kuszczyk MA, Sanchez S, Pankiewicz J, et al. Blocking the Interaction between Apolipoprotein E and Abeta Reduces Intraneuronal Accumulation of Abeta and Inhibits Synaptic Degeneration. *Am J Pathol.* 2013; 182:1750–1768. [PubMed: 23499462]
12. Pankiewicz J, Prelli F, Sy MS, et al. Clearance and prevention of prion infection in cell culture by anti-PrP antibodies. *Eur J Neurosci.* 2006; 23:2635–2647. [PubMed: 16817866]
13. Kim J, Castellano JM, Jiang H, et al. Overexpression of low-density lipoprotein receptor in the brain markedly inhibits amyloid deposition and increases extracellular A beta clearance. *Neuron.* 2009; 64:632–644. [PubMed: 20005821]
14. Wisniewski HM, Sadowski M, Jakubowska-Sadowska K, et al. Diffuse, lake-like amyloid-beta deposits in the paraventricular layer of the presubiculum in Alzheimer disease. *J Neuropath Exp Neurol.* 1998; 57:674–683. [PubMed: 9690671]
15. Sadowski MJ, Pankiewicz J, Prelli F, et al. Anti-PrP Mab 6D11 Suppresses PrP^{Sc} Replication in Prion Infected Myeloid Precursor LineFDC-P1/22L and in the Lymphoreticular System In Vivo. *Neurobiol Dis.* 2009; 34:267–278. [PubMed: 19385058]
16. Asuni AA, Pankiewicz JE, Sadowski MJ. Differential molecular chaperone response associated with various mouse adapted scrapie strains. *Neurosci Lett.* 2013; 538:26–31. [PubMed: 23370284]
17. Altschul SF, Gish W, Miller W, et al. Basic local alignment search tool. *J Mol Biol.* 1990; 215:403–410. [PubMed: 2231712]
18. Jiang Y, Mullaney KA, Peterhoff CM, et al. Alzheimer's-related endosome dysfunction in Down syndrome is Abeta-independent but requires APP and is reversed by BACE-1 inhibition. *Proc Natl Acad Sci U S A.* 2010; 107:1630–1635. [PubMed: 20080541]
19. Jankowsky JL, Fadale DJ, Anderson J, et al. Mutant presenilins specifically elevate the levels of the 42 residue beta-amyloid peptide in vivo: evidence for augmentation of a 42-specific gamma secretase. *Hum Mol Genet.* 2004; 13:159–170. [PubMed: 14645205]
20. Garcia-Alloza M, Robbins EM, Zhang-Nunes SX, et al. Characterization of amyloid deposition in the APP^{swe}/PS1^{dE9} mouse model of Alzheimer disease. *Neurobiol Dis.* 2006; 24:516–524. [PubMed: 17029828]
21. Duszczyk M, Kuszczyk M, Guridi M, et al. In vivo hippocampal microdialysis reveals impairment of NMDA receptor-cGMP signaling in APP(SW) and APP(SW)/PS1(L166P) Alzheimer's transgenic mice. *Neurochem Int.* 2012; 61:976–980. [PubMed: 22841892]
22. Sadowski MJ, Pankiewicz J, Scholtzova H, et al. Blocking the apolipoprotein E/amyloid-beta interaction as a potential therapeutic approach for Alzheimer's disease. *Proc Natl Acad Sci U S A.* 2006; 103:18787–18792. [PubMed: 17116874]
23. Schmidt SD, Jiang Y, Nixon RA, et al. Tissue processing prior to protein analysis and amyloid-beta quantification. *Methods in Molecular Biology.* 2005; 299:267–278. [PubMed: 15980611]

24. Dodart JC, Bales KR, Gannon KS, et al. Immunization reverses memory deficits without reducing brain A beta burden in Alzheimer's disease model. *Nature Neurosci.* 2002; 5:452–457. [PubMed: 11941374]
25. Aydin D, Weyer SW, Muller UC. Functions of the APP gene family in the nervous system: insights from mouse models. *Exp Brain Res.* 2012; 217:423–434. [PubMed: 21931985]
26. Berthoud VM, Minogue PJ, Laing JG, et al. Pathways for degradation of connexins and gap junctions. *Cardiovasc Res.* 2004; 62:256–267. [PubMed: 15094346]
27. Laing JG, Beyer EC. The gap junction protein connexin43 is degraded via the ubiquitin proteasome pathway. *J Biol Chem.* 1995; 270:26399–26403. [PubMed: 7592854]
28. Rumble B, Retallack R, Hilbich C, et al. Amyloid A4 protein and its precursor in Down's syndrome and Alzheimer's disease. *N Engl J Med.* 1989; 320:1446–1452. [PubMed: 2566117]
29. Sleegers K, Brouwers N, Gijselink I, et al. APP duplication is sufficient to cause early onset Alzheimer's dementia with cerebral amyloid angiopathy. *Brain.* 2006; 129:2977–2983. [PubMed: 16921174]
30. Brouwers N, Sleegers K, Engelborghs S, et al. Genetic risk and transcriptional variability of amyloid precursor protein in Alzheimer's disease. *Brain.* 2006; 129:2984–2991. [PubMed: 16931535]
31. Theuns J, Brouwers N, Engelborghs S, et al. Promoter mutations that increase amyloid precursor-protein expression are associated with Alzheimer disease. *Am J Hum Genet.* 2006; 78:936–946. [PubMed: 16685645]
32. Patel N, Hoang D, Miller N, et al. MicroRNAs can regulate human APP levels. *Mol Neurodegener.* 2008; 3:10. [PubMed: 18684319]
33. Rajagopalan LE, Malter JS. Growth factor-mediated stabilization of amyloid precursor protein mRNA is mediated by a conserved 29-nucleotide sequence in the 3'-untranslated region. *J Neurochem.* 2000; 74:52–59. [PubMed: 10617105]
34. Cho HH, Cahill CM, Vanderburg CR, et al. Selective translational control of the Alzheimer amyloid precursor protein transcript by iron regulatory protein-1. *J Biol Chem.* 2010; 285:31217–31232. [PubMed: 20558735]
35. Shaw KT, Utsuki T, Rogers J, et al. Phenserine regulates translation of beta -amyloid precursor protein mRNA by a putative interleukin-1 responsive element, a target for drug development. *Proc Natl Acad Sci U S A.* 2001; 98:7605–7610. [PubMed: 11404470]
36. Dewachter I, Reverse D, Caluwaerts N, et al. Neuronal deficiency of presenilin 1 inhibits amyloid plaque formation and corrects hippocampal long-term potentiation but not a cognitive defect of amyloid precursor protein [V717I] transgenic mice. *J Neurosci.* 2002; 22:3445–3453. [PubMed: 11978821]
37. Fukumoto H, Takahashi H, Tarui N, et al. A noncompetitive BACE1 inhibitor TAK-070 ameliorates Abeta pathology and behavioral deficits in a mouse model of Alzheimer's disease. *J Neurosci.* 2010; 30:11157–11166. [PubMed: 20720123]
38. Postina R. Activation of alpha-secretase cleavage. *J Neurochem.* 2012; 120(Suppl 1):46–54. [PubMed: 21883223]
39. Chaufy J, Sullivan SE, Ho A. Intracellular amyloid precursor protein sorting and amyloid-beta secretion are regulated by Src-mediated phosphorylation of Mint2. *J Neurosci.* 2012; 32:9613–9625. [PubMed: 22787047]
40. Xie Z, Dong Y, Maeda U, et al. RNA interference silencing of the adaptor molecules ShcC and Fe65 differentially affect amyloid precursor protein processing and Abeta generation. *J Biol Chem.* 2007; 282:4318–4325. [PubMed: 17170108]
41. Andersen OM, Reiche J, Schmidt V, et al. Neuronal sorting protein-related receptor sorLA/LR11 regulates processing of the amyloid precursor protein. *Proc Natl Acad Sci U S A.* 2005; 102:13461–13466. [PubMed: 16174740]
42. Shinohara M, Sato N, Kurinami H, et al. Reduction of brain beta-amyloid (Abeta) by fluvastatin, a hydroxymethylglutaryl-CoA reductase inhibitor, through increase in degradation of amyloid precursor protein C-terminal fragments (APP-CTFs) and Abeta clearance. *J Biol Chem.* 2010; 285:22091–22102. [PubMed: 20472556]

43. Spilman P, Podlitskaya N, Hart MJ, et al. Inhibition of mTOR by rapamycin abolishes cognitive deficits and reduces amyloid-beta levels in a mouse model of Alzheimer's disease. *PLoS One*. 2010; 5:e9979. [PubMed: 20376313]
44. Bell RD, Sagare AP, Friedman AE, et al. Transport pathways for clearance of human Alzheimer's amyloid beta-peptide and apolipoproteins E and J in the mouse central nervous system. *J Cereb Blood Flow Metab*. 2007; 27:909–918. [PubMed: 17077814]
45. DeMattos RB, Bales KR, Cummins DJ, et al. Peripheral anti-A beta antibody alters CNS and plasma A beta clearance and decreases brain A beta burden in a mouse model of Alzheimer's disease. *Proc Natl Acad Sci USA*. 2001; 98:8850–8855. [PubMed: 11438712]
46. Wang A, Das P, Switzer RC III, et al. Robust amyloid clearance in a mouse model of Alzheimer's disease provides novel insights into the mechanism of amyloid-beta immunotherapy. *J Neurosci*. 2011; 31:4124–4136. [PubMed: 21411653]
47. Ostrowitzki S, Deptula D, Thurfjell L, et al. Mechanism of amyloid removal in patients with Alzheimer disease treated with gantenerumab. *Arch Neurol*. 2012; 69:198–207. [PubMed: 21987394]
48. Rinne JO, Brooks DJ, Rossor MN, et al. 11C-PiB PET assessment of change in fibrillar amyloid-beta load in patients with Alzheimer's disease treated with bapineuzumab: a phase 2, double-blind, placebo-controlled, ascending-dose study. *Lancet Neurol*. 2010; 9:363–372. [PubMed: 20189881]
49. Blennow K, Zetterberg H, Rinne JO, et al. Effect of immunotherapy with bapineuzumab on cerebrospinal fluid biomarker levels in patients with mild to moderate Alzheimer disease. *Arch Neurol*. 2012; 69:1002–1010. [PubMed: 22473769]
50. Tayeb HO, Murray ED, Price BH, et al. Bapineuzumab and solanezumab for Alzheimer's disease: is the 'amyloid cascade hypothesis' still alive? *Expert Opin Biol Ther*. 2013; 13:1075–1084. [PubMed: 23574434]
51. Wisniewski KE, Wisniewski HM, Wen GY. Occurrence of Neuropathological Changes and Dementia of Alzheimers-Disease in Down Syndrome. *Ann Neurol*. 1985; 17:278–282. [PubMed: 3158266]
52. Villemagne VL, Burnham S, Bourgeat P, et al. Amyloid beta deposition, neurodegeneration, and cognitive decline in sporadic Alzheimer's disease: a prospective cohort study. *Lancet Neurol*. 2013; 12:357–367. [PubMed: 23477989]
53. Basi GS, Hemphill S, Brigham EF, et al. Amyloid precursor protein selective gamma-secretase inhibitors for treatment of Alzheimer's disease. *Alzheimers Res Ther*. 2010; 2:36. [PubMed: 21190552]
54. May PC, Dean RA, Lowe SL, et al. Robust central reduction of amyloid-beta in humans with an orally available, non-peptidic beta-secretase inhibitor. *J Neurosci*. 2011; 31:16507–16516. [PubMed: 22090477]

Figure 1. Chemical structure of 2-PMAP

2-PMAP chemical formula is C₁₂H₁₂N₂O and its molecular mass as determined by MS is 200.2 Da.

Figure 2. 2-PMAP demonstrates no cytotoxicity in cell culture

Shown are results of MTT assays conducted in SK-N-SH human neuroblastoma cells (**A**) and in CHO APP_{751SW} cells (**B**), which were cultured in the presence of increasing concentrations of 2-PMAP for 72hr. CoCl₂ was used as a positive control to induce toxicity. Mean percentage values relative to the vehicle control and SEM in three independent experiments are shown. ****p* < 0.001 compared with the vehicle control (Dunnett's post-hoc test).

Figure 3. 2-PMAP shows dose-dependent effect on reducing secretion of Aβ peptides by CHO APP_{751SW} cells

Shown are concentrations of Aβ_{x-40} (**A**) and Aβ_{x-42} (**B**) in the conditioned media of CHO APP_{751SW} cells treated with increasing concentrations of 2-PMAP for 48hr. Compound (Cpd.) E in concentration of 10 μM was used as a positive treatment control. Mean values and SEM in four independent experiments are shown. ANOVA $p < 0.0001$ in (A) and (B), *** $p < 0.001$ versus the vehicle control (Bonferroni's post-hoc test).

Figure 4. 2-PMAP effects reduction of the APP steady-state level and those of APP α-CTF and β-CTF in CHO APP_{751SW} cells in dose-dependent manner

A: Shown is immunoblot analysis of the protein levels of soluble APP (sAPP) in the conditioned media, and full length APP including its mature (mAPP) and immature (imAPP) forms, APP α-CTF and β-CTF, and APLP-2 in the cell lysate of CHO APP_{751SW} cells treated with increasing concentrations of 2-PMAP or 10μM of Compound (Cpd.) E for 48hr. Included is also immunoblot for β-actin, which was used as a loading control. Shown are results of densitometric quantification of protein band optic densities for full length APP (mAPP + imAPP) (**B**), APP CTFs (α-CTF + β-CTF) (**C**), and APLP-2 (**D**). Density of full length APP and APP CTFs protein bands becomes noticeably diminished at 2.5μM and 0.5μM 2-PMAP concentrations, respectively; and shows further reduction with increasing concentrations of 2-PMAP. Both mAPP and imAPP protein bands show dose-dependent reduction with 2-PMAP treatment. For comparison, significant effect of 2-PMAP on the APLP-2 level was detected only at concentration of 50μM, what indicates relatively high selectivity of 2-PMAP toward APP. All protein band optic densities are expressed relative to those in vehicle control and presented as mean values (+ SEM) from at least four independent experiments. ANOVA $p < 0.0001$ in B and C and $p = 0.0014$ in D. Post-hoc * p

< 0.05 , $**p < 0.01$, $***p < 0.001$ versus the vehicle control (Bonferroni's post-hoc test). **E:** Shown are representative confocal microscopy images of CHO APP_{751SW} cells treated with vehicle (0.1% DMSO) and 10 μ M of 2-PMAP stained against APP. There is marked reduction in the intensity of anti-APP immunoreactivity without evidence of protein redistribution or compartmental accumulation in 2-PMAP treated cells compared to vehicle control.

Figure 5. Effect of Phenserine on secretion of Aβ peptides and steady-state level of APP in CHO APP_{751SW} cells

Shown are concentrations of Aβ_{x-40} (**A**) and Aβ_{x-42} (**B**) in the conditioned media of CHO APP_{751SW} cells treated with increasing concentrations of Phenserine. **C**: Immunoblot analysis of APP level in Phenserine treated CHO APP_{751SW} cells. Shown are (left) representative immunoblots for APP and β-actin used as a loading control. Values in the graph (right) show the mean percentage of the APP band optic densities relative to those in vehicle control. Values in A-C are shown as mean and SEM from three independent experiments. ANOVA $p < 0.0001$ (A), $p = 0.0006$ (B) and $p = 0.0159$ (C), * $p < 0.05$, *** $p < 0.001$ versus the vehicle control (Bonferroni's post-hoc test).

Figure 6. 2-PMAP shows no effect on APP mRNA level but in dose-dependent manner reduces translational efficiency of APP mRNA into protein

A: Effect of 2-PMAP on APP mRNA level was analyzed in CHO APP_{751SW} cells following 48hr treatment with vehicle (0.1% DMSO), 25 μ M of 2-PMAP, or 10 μ M of Compound (Cpd.) E. Values in the graph are mean (+ SEM) of RT-PCR threshold cycles (Ct) for APP mRNA normalized to those of mRNA for β -actin or GAPDH in three separate experiments for each housekeeping gene and demonstrate no significant differences across treatment conditions. **B:** Shown are autoradiographs of 35S labeled APP, β -actin, and connexin 43 immunoprecipitated from CHO APP_{751SW} cells treated with vehicle (0.1% DMSO) or 2-

PMAP (10 μ M or 25 μ M) immediately after the conclusion of the 15min long pulse. APP was immunoprecipitated using C-terminal specific antibody, which revealed two separate bands representing full length mature (mAPP) and immature (imAPP) APP forms. Optical density of mAPP and imAPP bands was markedly reduced in 2-PMAP treated cells, compared to those treated with vehicle (**C**), while the optical density of bands for β -actin and connexin 43, showed no apparent difference across treatment conditions (**D**). Values in the graphs (**C**) and (**D**) show the mean percentage (+ SEM) of band intensities relative to those in vehicle control analyzed by densitometry in three independent experiments. **E**: Shown are autoradiographs of 35S labeled APP immunoprecipitated from CHO APP_{751S}W cells treated with vehicle (0.1% DMSO) or 2-PMAP (10 μ M or 25 μ M) immediately after the conclusion of 15min pulse and following 60min chase. Included are also autoradiographs of 35S labeled β -actin, which was used as the internal control. **F**: Values in the graph express the mean percentage (+ SEM) of APP bands intensity (mAPP + imAPP) in chase autoradiographs relative to those in pulse autoradiographs for matching treatment conditions from three independent experiments and show no significant differences. Images of APP and β -actin autoradiographs following 15min pulse in (**B**) are intentionally the same as the ones included in (**E**) to indicate “Chase 0min”. ANOVA values shown above each graph. Post-hoc * p < 0.05, ** p < 0.01 versus the vehicle control (Bonferroni’s post-hoc test).

Figure 7. Subacute 2-PMAP treatment reduces brain levels of full length APP and those of APP CTFs, and levels of soluble Aβ peptides in APP_{SW}/PS1_{DE9} mice

A: Immunoblot analysis of full length APP (APP FL) and APP α-CTF and β-CTF from six-month old female APP_{SW}/PS1_{DE9} animals, which were systemically treated with 2-PMAP (100mg/kg/d) for five days, demonstrates significant reduction in full length APP and APP CTFs band densities compared to vehicle treated controls. Included are also Western immunoblots for β-actin, which was used as a loading control. **B:** Shown are the percentage of APP FL and APP CTFs (α-CTF + β-CTF) band optic densities analyzed by densitometry relative to those in the brains of vehicle treated animals. **C:** Brain levels of soluble Aβ_{x-40} and Aβ_{x-42} obtained using DEAE extraction in 2-PMAP treated animals were significantly lower than in vehicle treated controls. All quantitative results in (B) and (C) represent the mean and SEM from 5-7 animals per group. **p* < 0.05, *****p* < 0.0001 versus vehicle treated APP_{SW}/PS1_{DE9} mice (Student's *t* test).

Figure 8. Long-term 2-PMAP treatment rescues memory deficit in APP_{SW}/PS1_{dE9} Tg mice
 Scores of object recognition test (A) and radial arm maze test (B) demonstrate memory improvement in 2-PMAP treated APP_{SW}/PS1_{dE9} Tg mice compared to those treated with vehicle. The two behavioral tests were conducted during the last three weeks of the long-term 2-PMAP treatment experiment, where 2-PMAP or vehicle was systemically administered to APP_{SW}/PS1_{dE9} mice between the age of 6 and 10 months. Values in the graph (A) represent mean (+SEM) of time spent by vehicle or 2-PMAP treated APP_{SW}/PS1_{dE9} Tg mice and their wild-type (WT) littermates of matched age and sex exploring familiar and novel objects presented concurrently during the retention session of the object recognition test. * $p < 0.05$, ** $p < 0.01$, versus familiar object (Student's t test). Values in the graph (B) represent the number of errors ($\pm$ SEM) per session of the radial arm maze plotted against testing days. ANOVA $p = 0.0057$ for days 4 through 10, ** $p < 0.01$, *** $p < 0.001$, versus vehicle treated APP_{SW}/PS1_{dE9} mice (Newman-Keuls's post-hoc test). $n=10-13$ /group for either test.

Figure 9. Long-term 2-PMAP treatment reduces Aβ plaque load in APP_{SW}/PS1_{DE9} Tg mice
A: Representative microphotographs of Thioflavin-S stained coronal brain sections at the level of the anterior commissure (upper row) and the dorsal hippocampus (lower row) from 10 months old APP_{SW}/PS1_{DE9} mice treated with vehicle or 2-PMAP from the age of six months. **B, C:** Unbiased quantification of Thioflavin-S positive plaques in the cerebral necortices. Mean values and SEM of Thioflavin-S positive plaque load (% area covered by Aβ plaques) (B) and the plaque density (number per mm²) (C) are shown; vehicle (n=13) and 2-PMAP (n=10). **D:** Anti-Aβ_{x-40} and Aβ_{x-42} immunohistochemistry. Shown are representative microphotographs of the cerebral necortices from APP_{SW}/PS1_{DE9} mice treated with vehicle or 2-PMAP. **E:** Quantification of Aβ_{x-40} and Aβ_{x-42} positive Aβ plaque load. Values are given as mean and SEM. **F:** Confocal microscopy analysis of representative Aβ plaques double immunostained with anti-Aβ_{x-40} and anti-Aβ_{x-42} antibodies demonstrates reduction in both Aβ_{x-40} and Aβ_{x-42} components. ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001 versus vehicle treated APP_{SW}/PS1_{DE9} mice (Student's *t* test).

Figure 10. Long-term 2-PMAP treatment reduces brain levels of insoluble Aβ_{x-40} and Aβ_{x-42} and those of Aβ oligomers in APP_{SW}/PS1_{dE9} Tg mice

Shown are means (and SEM) of FA extracted Aβ_{x-40} and Aβ_{x-42} levels (**A**) and those of Aβ oligomers (**B**) in brains 10 months old APP_{SW}/PS1_{dE9} Tg mice treated with vehicle (n=12) or 2-PMAP (n=7) since the age of 6 months. **p* < 0.05, ****p* < 0.001 versus vehicle treated APP_{SW}/PS1_{dE9} mice (Student's *t* test).