

# Selective Antihypertensive Dihydropyridines Lower A $\beta$ Accumulation by Targeting both the Production and the Clearance of A $\beta$ across the Blood-Brain Barrier

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Several large population-based or clinical trial studies have suggested that certain dihydropyridine (DHP) L-type calcium channel blockers (CCBs) used for the treatment of hypertension may confer protection against the development of Alzheimer disease (AD). However, other studies with drugs of the same class have shown no beneficial clinical effects. To determine whether certain DHPs are able to impact underlying disease processes in AD (specifically the accumulation of the Alzheimer A $\beta$  peptide), we investigated the effect of several antihypertensive DHPs and non-DHP CCBs on A $\beta$  production. Among the antihypertensive DHPs tested, a few, including nilvadipine, nitrendipine and amlodipine inhibited A $\beta$  production *in vitro*, whereas others had no effect or raised A $\beta$  levels. *In vivo*, nilvadipine and nitrendipine acutely reduced brain A $\beta$  levels in a transgenic mouse model of AD (Tg PS1/APPsw) and improved A $\beta$  clearance across the blood-brain barrier (BBB), whereas amlodipine and nifedipine were ineffective showing that the A $\beta$ -lowering activity of the DHPs is independent of their antihypertensive activity. Chronic oral treatment with nilvadipine decreased A $\beta$  burden in the brains of Tg APPsw (Tg2576) and Tg PS1/APPsw mice, and also improved learning abilities and spatial memory. Our data suggest that the clinical benefit conferred by certain antihypertensive DHPs against AD is unrelated to their antihypertensive activity, but rely on their ability to lower brain A $\beta$  accumulation by affecting both A $\beta$  production and A $\beta$  clearance across the BBB.

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## INTRODUCTION

Alzheimer disease (AD) is the major cause of dementia in the elderly in Western countries and is characterized by the progressive accumulation of intracellular neurofibrillary tangles, extracellular parenchymal senile plaques and cerebrovascular deposits (1). The principal component of senile plaques and cerebrovascular deposits is the 39–43 amino acid  $\beta$ -amyloid peptide (A $\beta$ ), which is proteolytically derived from the amyloid precursor protein (APP) (2). Although the central role of A $\beta$  in AD remains to be proven in clinical trials, data accumulated during the past two decades place A $\beta$  peptides at center

stage as the main culprit in initiating the pathological cascade that eventually leads to AD (3–5). A $\beta$  peptides are derived from the sequential proteolysis of APP by  $\beta$ - and  $\gamma$ -secretases. The major  $\beta$ -secretase is an aspartyl protease termed BACE-1 ( $\beta$ -site APP cleaving enzyme) (6). BACE-1 cleaves APP within the extracellular domain of APP, resulting in the secretion of the large ectodomain (sAPP $\beta$ ) and generating a membrane-tethered C-terminal fragment CTF $\beta$  or C99 which serves as a substrate for  $\gamma$ -secretase. The multimeric  $\gamma$ -secretase complex cleaves at multiple sites within the transmembranous CTF $\beta$ , generating C-terminally heteroge-

neous A $\beta$  peptides ranging between 38 to 43 amino-acid residues in length that are secreted (2). In addition to BACE-1 and  $\gamma$ -secretase, APP can be cleaved by  $\alpha$ -secretase within the A $\beta$  domain between Lys<sup>16</sup> and Leu<sup>17</sup>, releasing APPs $\alpha$  and generating CTF $\alpha$  (C83) which is further cleaved by  $\gamma$ -secretase to generate an N-terminally truncated A $\beta$  termed p3.

During the course of examining risk factors for AD, such as hypertension, it has become clear that certain antihypertensive compounds may be protective, not just against stroke-related dementia, but also independently against AD. For instance, in the Syst-Eur trial, which involved active treatment with the dihydropyridine (DHP) calcium channel blocker (CCB) nitrendipine in over 2,400 patients, there was a 55% reduction in the incidence of AD (7–9). Although nitrendipine is an antihypertensive and would therefore be expected to lower

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the incidence of stroke-related dementia, the protection against AD is unexplained by the known mechanism of action of nifedipine. Small clinical studies of another DHP, nilvadipine, have shown stabilization of cognitive decline and reduced incidence of AD in hypertensive MCI patients, whereas the closely related antihypertensive DHP amlodipine failed to provide any benefit (10–12), suggesting that the beneficial effect of nilvadipine is not related to its antihypertensive activity. Although only a small number of study cases were taking DHPs in the Cache County antihypertensive study (13), a trend also was reported toward a lowered risk for AD. This was not observed for non-DHP calcium channel blockers. The Baltimore longitudinal study of aging (14) also showed a strong trend toward reduced relative risk of AD in DHP calcium channel blocker users, whereas no lowered risk was observed in the non-DHP calcium channel blocker using group. Additional evidence suggests that antihypertensive activity *per se* is not responsible for the protective effects against AD. For instance, several studies with antihypertensive medications have shown no evidence of prophylactic activity against AD. For example, although use of angiotensin converting enzyme (ACE) inhibitors has been shown to decrease the incidence of stroke-related dementia, no positive effect was demonstrated for AD in several studies (9,15–17). In addition, antihypertensive treatment with a thiazide did not protect against dementia in the Systolic Hypertension in the Elderly Program trial which again argues against the prevention of dementia by solely lowering blood pressure (18). Moreover, some studies have even suggested that certain antihypertensive DHPs may be detrimental, as far as risk for AD is concerned. For instance, older people who had a history of hypertension and who were taking the DHP nifedipine were more likely than subjects taking other antihypertensive agents to experience cognitive decline during the 5-year fol-

low-up period of the Canadian Study of Health and Aging (19). Overall then, it is clear that any clinical signal from DHPs in the protection against AD is not a drug class effect and is not related to the antihypertensive effects of these drugs. However, specific DHPs like nitrendipine and nilvadipine do suggest clinical protective signals against AD whereas amlodipine and nifedipine do not. Given that central nervous system (CNS) accumulation of A $\beta$  peptides are thought to be central to the AD process and that certain DHPs protect against AD, we investigated the effect of DHPs as well as non-DHP CCBs on the production of A $\beta$  peptides.

## MATERIALS AND METHODS

### Cell Culture Experiments

7W CHO (20) cells stably transfected with human APP751 were maintained in DMEM (ATCC, Manassas, VA, USA) medium containing 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA), 1 $\times$  mixture of penicillin/streptomycin/fungizone mixture (Cambrex, Charles City, IA, USA) and 0.3% geneticin (Invitrogen) as a selecting agent. Cells were cultured in 96-well culture plates and treated for 24 h with different calcium channel blockers as indicated in the figure legend. All test compounds were diluted in dimethyl sulfoxide (DMSO) before being exposed to confluent Chinese hamster ovary (CHO) cells. Control wells received the same volume of DMSO, and the final DMSO concentrations in the culture medium for all treatment conditions were lower than 1%. Potential cytotoxicity of the different calcium channel blockers was evaluated using the Cytotoxicity Detection Kit (Roche Diagnostics, Mannheim, Germany) and no significant toxicity of the different calcium channel blockers was observed for the dose range studied (data not shown). A $\beta_{1-40}$  and A $\beta_{1-42}$  were analyzed in the culture medium by using commercially available sandwich enzyme-linked immunosorbent assays (ELISAs) (Invitrogen). All experiments were repeated 3–4 $\times$ .

### Western Blots

The impact of nilvadipine and amlodipine on APP processing was evaluated using 7W CHO cells as we published previously (21). Briefly, confluent 7W CHO cells were treated for 24 h with different doses of nilvadipine and amlodipine in 6-well plates. Cellular proteins were extracted with 150  $\mu$ L of ice cold M-PER Reagent (Pierce Biotechnology, Rockford, IL, USA) containing 1 mmol/L phenylmethanesulfonyl fluoride, 1 $\times$  of protease cocktail inhibitor (Roche Diagnostic Corporation, Indianapolis, IN, USA) and 1 mmol/L sodium orthovanadate. Samples were sonicated, denatured by boiling in Laemmli buffer (Bio-Rad, Hercules, CA, USA) and resolved onto 4% to 20% gradient polyacrylamide gels (Bio-Rad). After electrotransferring onto polyvinylidene difluoride membranes, Western blots were immunoprobed with an anti-APP C-terminal (751–770) antibody (EMD Chemicals Inc., Gibbstown, NJ, USA), with an antiactin antibody (Chemicon, Temecula, CA, USA) used as a reference antibody to ensure that an equal amount of proteins were electrotransferred. Additionally, sAPP $\alpha$  was detected by Western-blot in the culture medium surrounding 7W CHO cells using the antibody 6E10 (Signet Laboratories Inc., Dedham, MA, USA) which recognizes the amino acids 1–17 of A $\beta$ , and sAPP $\beta$  was detected in the culture medium using an antihuman sAPP $\beta$  antibody (Immuno-Biological Laboratories Co. Ltd., Gunma, Japan).

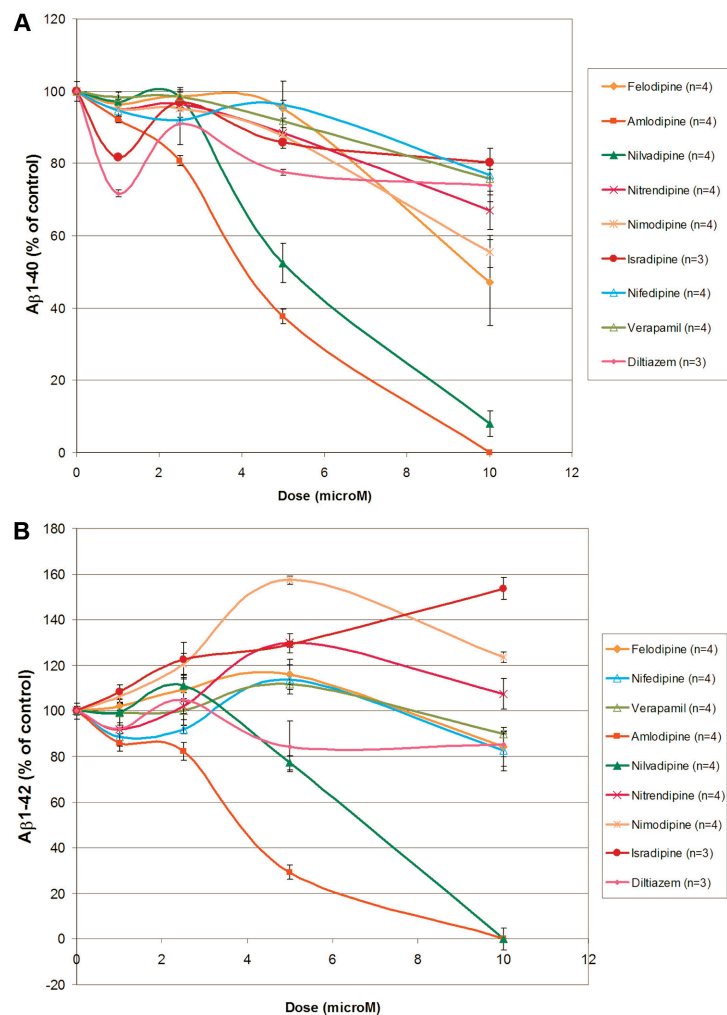
### $\beta$ -Secretase Activity Measurements

$\beta$ -Secretase activity was measured using human recombinant BACE-1 (Calbiochem, San Diego, CA, USA) with two commercially available kits, a FRET-based assay (Biovision Inc, Mountain View, CA, USA) and a chemoluminescent assay (DiscoverX, Fremont, CA, USA) following the recommendations of the manufacturers. The  $\beta$ -secretase inhibitor IV (BACE IV) inhibitor was used as a positive control in both assays and was purchased from EMD Chemicals Inc.

FRET and chemoluminescent signals were quantified on a HTS Synergy multi-plate reader from Biotek (Winooski, VT, USA).

### Acute Effect of Antihypertensive Dihydropyridines on Brain Soluble A $\beta$ Levels in Tg PS1/APPsw Mice

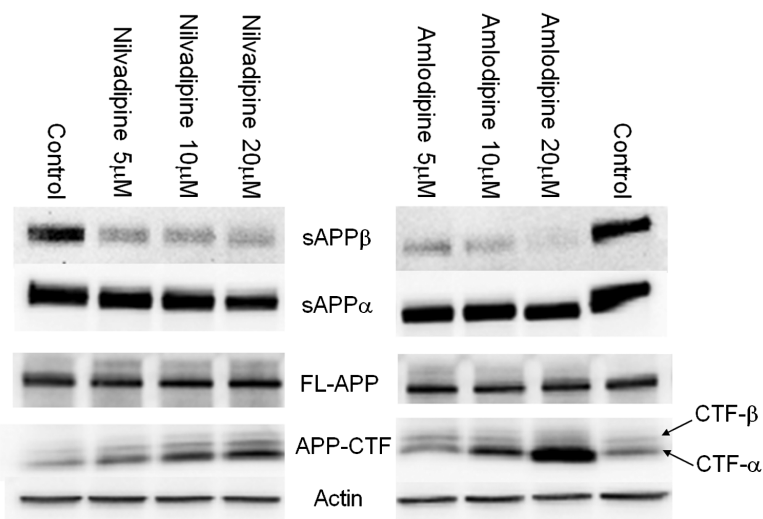
DAPT (10 mg/kg of body weight), nilvadipine (2 mg/kg), amlodipine (2 mg/kg), nitrendipine and nifedipine (2 mg/kg) were administered daily to 6-month-old Tg PS1/APPsw (22) mice intraperitoneally for 4 d. Vehicle-treated Tg PS1/APPsw mice received an i.p. injection of 50% DMSO in PBS for 4 d. Within 1 h after the last injection, animals were humanely euthanized, their brains and plasma were immediately frozen in liquid nitrogen until being analyzed for A $\beta_{1-40}$  and A $\beta_{1-42}$  levels using commercially available ELISA kits (Invitrogen). Briefly, brains were homogenized at 4°C in mammalian protein extraction reagent (MPER) containing 1 mmol/L PMSF and 1 $\times$  protease inhibitor cocktail (protein research product from Pierce) and centrifuged at 15,000g for 30 min at 4°C. The supernatant containing soluble A $\beta$  was collected and further diluted with the sample diluent provided in the A $\beta$  ELISA kits and assessed for A $\beta$  according to the manufacturer's protocol. Protein concentrations were measured in the supernatant using the BCA method (Pierce) and results of brain soluble A $\beta_{1-40}$  and A $\beta_{1-42}$  were calculated in pg/mg of protein and expressed as a percentage of the values obtained in the vehicle treated animals for both A $\beta_{1-40}$  and A $\beta_{1-42}$ . Plasma samples were diluted with the diluent provided in the ELISA kit and assayed according to the manufacturer's recommendation. Plasma A $\beta_{1-40}$  and A $\beta_{1-42}$  values in the different treatment groups were expressed as a percentage of the values obtained in the vehicle treatment group. All experiments using animals were performed under protocols approved by the Institutional Animal Care and Use Committee of the Roskamp Institute.



**Figure 1.** Dose-dependent effect of antihypertensive calcium channel blockers on A $\beta$  production by 7W CHO cells overexpressing APP. (A) Effect of a 24 h treatment with different calcium channel blockers on A $\beta_{1-40}$  production. A $\beta_{1-40}$  values were expressed as a percentage of the A $\beta_{1-40}$  values obtained in the vehicle treatment conditions  $\pm$  S.E.M. ANOVA reveals statistically significant main effect of amlodipine ( $P < 0.001$ ), nilvadipine ( $P < 0.001$ ), nitrendipine ( $P < 0.05$ ) and nimodipine ( $P < 0.002$ ), but no statistically significant main effect of felodipine ( $P = 0.1$ ), isradipine ( $P = 0.055$ ), nifedipine ( $P = 0.245$ ), diltiazem ( $P = 0.09$ ) or verapamil ( $P = 0.173$ ) on A $\beta_{1-40}$  production. *Post hoc* analyses show significant differences between A $\beta_{1-40}$  values in untreated cells and cells treated with amlodipine at 1 ( $P < 0.02$ ), 2.5 ( $P < 0.001$ ), 5 ( $P < 0.001$ ) and 10  $\mu\text{mol/L}$  ( $P < 0.001$ ), with nilvadipine at 5 and 10  $\mu\text{mol/L}$  ( $P < 0.001$ ), with nitrendipine at 5 and 10  $\mu\text{mol/L}$  ( $P < 0.001$ ) and with nimodipine at 5 and 10  $\mu\text{mol/L}$  ( $P < 0.001$ ). (B) Effect of a 24 h treatment with different calcium channel blockers on A $\beta_{1-42}$  production. A $\beta_{1-42}$  values were expressed as a percentage of the A $\beta_{1-42}$  values obtained in the vehicle treatment conditions  $\pm$  S.E.M. ANOVA reveals statistically significant main effect of amlodipine ( $P < 0.001$ ), nilvadipine ( $P < 0.001$ ), isradipine ( $P < 0.001$ ) and nimodipine ( $P < 0.001$ ), but no statistically significant main effect of felodipine ( $P = 0.577$ ), nitrendipine ( $P = 0.293$ ), nifedipine ( $P = 0.663$ ), verapamil ( $P = 0.968$ ) and diltiazem ( $P = 0.308$ ) on A $\beta_{1-42}$  production. *Post hoc* analyses show significant differences between A $\beta_{1-42}$  values in untreated cells and cells treated with amlodipine at 1, 2.5, 5 and 10  $\mu\text{mol/L}$  ( $P < 0.005$ ), with nilvadipine at 2.5, 5 and 10  $\mu\text{mol/L}$  ( $P < 0.01$ ), with nimodipine at 2.5, 5 and 10  $\mu\text{mol/L}$  ( $P < 0.002$ ) and with isradipine at 2.5, 5 and 10  $\mu\text{mol/L}$  ( $P < 0.001$ ). (Average A $\beta_{1-40}$  values observed in the control conditions were 2,092.3 pg/mL whereas A $\beta_{1-42}$  values were 189.5 pg/mL).

### Effects of Dihydropyridines on A $\beta$ Transcytosis *In Vitro*

Human brain microvascular endothelial cells (HBMEC), endothelial cell media (ECM), fetal bovine serum, penicillin/streptomycin solution, and endothelial cell growth supplement (ECGS) were purchased from Sciencell Research Laboratories (Carlsbad, CA, USA). Fibronectin solution was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Fluorescein-A $\beta_{1-42}$  (FLA $\beta_{1-42}$ ) was purchased from rPeptide (Bogart, GA, USA). The lyophilized peptide was dissolved to 1 mg/mL in 1,1,1,3,3,3-hexafluoro-2-propanol at 4°C to minimize the formation of  $\beta$ -sheet structures and promote  $\alpha$ -helical secondary structure and monomerize the peptide. The peptide was allowed to air dry in a chemical fume hood for 1 h at room temperature, followed by further drying in a speedvac (Thermo-Savant, Manassquan, NJ, USA) for 30 min. The resulting clear film was resuspended in 100% dimethylsulfoxide (DMSO) to a concentration of 1 mmol/L, followed by aliquoting and storage at -80°C. The 24-well membrane inserts (translucent, 0.4  $\mu$ m pore) and 24-well companion plates were purchased from Fisher Scientific (St. Louis, MO, USA). A $\beta$  transcytosis *in vitro* was quantified as we described previously using HBMEC (23). Briefly, HBMEC were seeded at 50,000 cells/cm<sup>2</sup> onto fibronectin-coated (4  $\mu$ g/cm<sup>2</sup>), 24-well, 0.4  $\mu$ m-pore, translucent membrane inserts (0.3 cm<sup>2</sup>/insert) to establish a polarized HBMEC monolayer representative of the BBB. The layer of HBMEC separates this system into apical ("blood") and basolateral ("brain") compartments. ECM containing 2- $\mu$ mol/L FLA $\beta_{1-42}$  was placed in the basolateral (donor) compartment. The apical (receiver) side of the membrane was exposed to various concentrations of nilvadipine, amlodipine and nitrendipine (1, 5, and 10  $\mu$ mol/L) in ECM. The donor compartment was sampled at time 0 to establish the initial concentration of FLA $\beta_{1-42}$  in each group. Following exposure of the insert to the well containing



**Figure 2.** Effect of nilvadipine and amlodipine on APP processing. Western-blot depicting the effect of a 24-h treatment with 5, 10 and 20  $\mu$ mol/L of amlodipine and nilvadipine on the secretion of APPs $\alpha$  and APPs $\beta$  in the culture medium of 7W CHO cells overexpressing APP, showing an inhibition of extracellular sAPP $\beta$  production by nilvadipine and amlodipine. In cell extracts, no alteration of full-length APP (FL-APP) level was observed with nilvadipine and amlodipine treatments, whereas an accumulation of APP-CTF $\alpha$  was observed with amlodipine. Actin was used as a loading control.

FLA $\beta_{1-42}$  samples were collected from the apical compartment at various time points up to 90 min to assess the movement of FLA $\beta_{1-42}$  across the HBMEC monolayer (basolateral-to-apical). The samples were analyzed ( $\lambda_{ex}$  = 485 nm and  $\lambda_{em}$  = 516 nm) for FLA $\beta_{1-42}$  using a BioTek Synergy HT multi-detection microplate reader. The apparent permeability ( $P_{app}$ ) of FLA $\beta_{1-42}$  was determined using the equation  $P_{app} = 1/AC_0 * (dQ/dt)$ , where A represents the surface area of the membrane, C<sub>0</sub> is the initial concentration of FLA $\beta_{1-42}$  in the basolateral compartment, and dQ/dt is the amount of FLA $\beta_{1-42}$  appearing in the apical compartment in the given time period. The  $P_{app}$  of FLA $\beta_{1-42}$  in the presence of drug was compared with control (that is, no drug exposure) and expressed as a percentage. We corrected for permeability resistance associated with the blank membrane as reported recently (24).

### Effects of Dihydropyridines on A $\beta$ Clearance across the BBB *In Vivo*

Wild-type B6/SJL F1 mice (12 month-old, Jackson Laboratories, Bar Harbor,

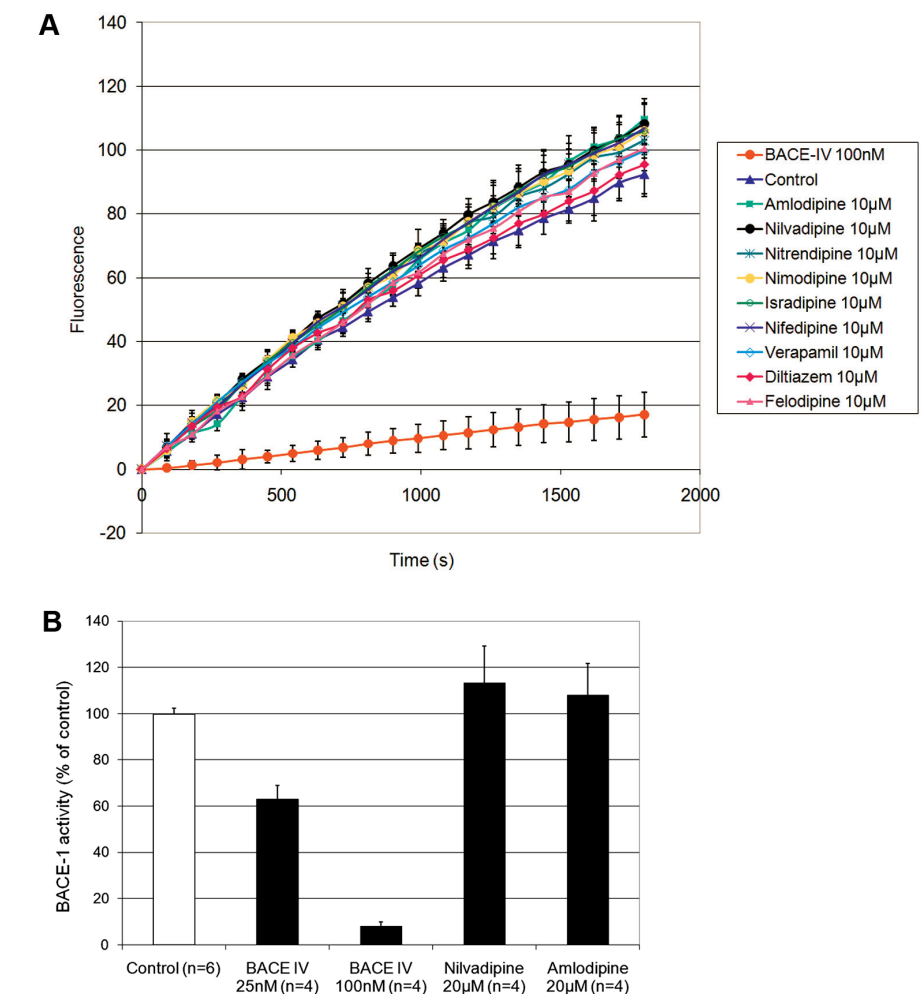
ME, USA) were anesthetized via inhalation using a 3% isoflurane/oxygen mix and maintained at 37°C using a homeothermic blanket system (Harvard Apparatus, Holliston, MA, USA). While under anesthesia, the mice were injected intraperitoneally with the vehicle only (50% DMSO in PBS) or with nilvadipine, nitrendipine and amlodipine at a dosage of 2 mg/kg. Five min after the intraperitoneal injection, the mice were stereotaxically injected with 3 nmol of human A $\beta_{1-42}$  (Invitrogen) into the caudate putamen of the brain (3 mm posterior to the eye line and 2.0 mm lateral to the midline and 3 mm below the surface of the brain) using a sterile 27-gauge needle connected to a 10- $\mu$ l luer-tip Hamilton syringe (Fisher Scientific). (In separate studies, we confirmed the site of delivery was in the caudate putamen by injecting Evans blue.) Ten min after the intracranial administration of human A $\beta_{1-42}$ , plasma samples were collected and analyzed for human A $\beta_{1-42}$  by ELISA. The level of human A $\beta_{1-42}$  in the plasma samples collected from the DHP-treated mice was compared with



those receiving the vehicle alone. Control experiments were performed to determine whether the intracranial injection procedure and the administration of nilvadipine could affect the permeability of the BBB. Vascular permeability/BBB leakage was evaluated by measuring the extravasation of the Evans blue dye in the brain as described previously (25,26). Briefly, B6/SJL F1 mice were intraperitoneally injected with 100  $\mu$ L of 50% DMSO/PBS (vehicle), with 2 mg/kg of nilvadipine or were injected intracranially as described above with 3  $\mu$ L of vehicle, with 3 nmol of A $\beta_{1-42}$  or went untreated (control mice). A group of mice also was subjected to a cold injury procedure to induce BBB leakage (positive control). A cylinder of aluminum (3 mm diameter) cooled in liquid nitrogen was applied for 15 s bilaterally at the surface of the skull (after removing the periosteum) in the occipital area of anesthetized mice. Five min after subjecting the animals to these different procedures, the Evans blue dye (Sigma) was injected intraperitoneally (400 mg/kg of body weight) and mice were euthanized 1 h later. The brains of the animals were collected, weighed and incubated at 37°C for 72 h in 2 mL of pure formamide (Sigma) to extract the Evans blue dye. Optical density of the extracted dye was measured at 620 nm, and values were reported per gram of brain. All experiments using animals were performed under protocols approved by the Institutional Animal Care and Use Committee of the Roskamp Institute.

### Chronic Nilvadipine Treatment of Tg PS1/APPsw and Tg 2576 Mice

Ten-month-old transgenic mice Tg PS1/APPsw (22) overexpressing APP695 containing the "Swedish" mutation and a mutant presenilin-1 (M146L) were fed with an irradiated powder diet (18% protein, Harlan Teklad, Madison, WI, USA) containing 0.03% (weight to weight) of nilvadipine (n = 8) formulation for oral dosage (which corresponds to an oral drug intake in mice of ap-



**Figure 3.** Effect of antihypertensive calcium channel blockers on human BACE-1 activity using cell free assays. (A) BACE-1 mediated proteolytic process of a FRET peptide substrate containing the Swedish peptide sequence (EVNLDAEFK) as a function of time. The inhibitor BACE-IV was used as a reference inhibitor for BACE-1. (B) Quantification of BACE-1 activity using the chemoluminescent HitHunter assay. Data show that calcium channel blockers do not inhibit BACE-1 activity directly and, in particular nilvadipine and amlodipine (no statistically significant main effect of nilvadipine and amlodipine doses was observed by ANOVA ( $P > 0.05$ )) do not have any impact on BACE-1 activity for the dose range tested.

proximately 50 mg/kg/day, equivalent to a 2 mg/kg/day intraperitoneal dosage of nilvadipine according to pharmacokinetic data [data not shown] or a placebo (0.03% of solid dispersion without nilvadipine) (n = 7) for 10 months. Tg PS1 control littermates were fed for 10 months as indicated above (n = 10 for nilvadipine treatment and n = 13 for placebo treatment). Additionally, 3-month-old Tg APPsw (27) were fed

with the same formulation of nilvadipine (n = 10) or placebo (n = 12) for a period of 17 months. Solid dispersion formulations of nilvadipine and placebo were provided by Astellas Pharma Inc. (Osaka, Japan). Twenty-month-old mice were humanely euthanized and their brains were fixed in 4% paraformaldehyde for 24 h at 4°C before embedding in paraffin blocks using a Tissue-Tek (Sakura, Torrance, CA, USA). All experi-

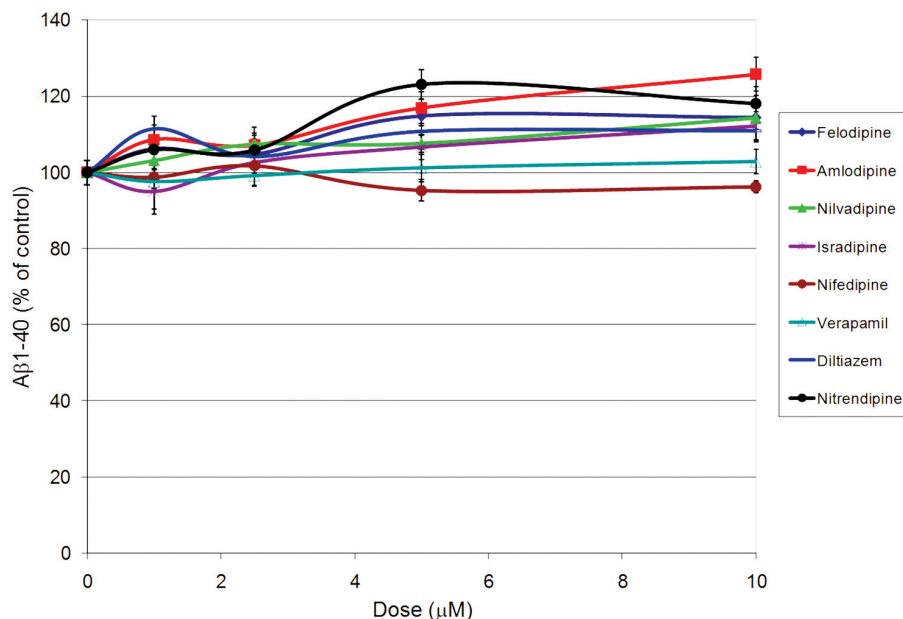
ments using animals were performed under protocols approved by the Institutional Animal Care and Use Committee of the Roskamp Institute.

### Measurement of $\beta$ -Amyloid ( $A\beta$ ) Burden

Brains were cut sagittally into 6- $\mu$ m-thick sections with a microtome (2030 Biocut, Reichert/Leica, Wetzlar, Germany) and  $A\beta$  burden determined as we published previously (21). Briefly, sections were mounted on slides and deparaffinized in xylene (2  $\times$  5 min) and hydrated in graded ethanol (2  $\times$  5 min in 100%, 5 min in 85%, 5 min 70%) to water. The endogenous peroxidase activity was quenched with a 20-min  $H_2O_2$  treatment (0.3% in water) and after being rinsed, sections were incubated with blocking buffer (Protein Block Serum-free, Dako-Cytomation, Carpinteria, CA, USA) for 20 min. The monoclonal antibody 4G8 (Signet Laboratories) recognizing human  $A\beta$  (diluted 1:750) was applied onto the sections overnight at 4°C and was detected using the Vectastain ABC (avidin-biotin-peroxidase complex) Elite kit (Vector Laboratories, Burlingame, CA, USA). For each brain, 4 to 5 nonconsecutive and randomly selected sections containing the hippocampus were used to perform the quantification of  $A\beta$  burden. The stained area within particular regions (hippocampus, cortex or subfields of cortex) was quantified using the Image-Pro Plus software (Media Cybernetics, Bethesda, MD, USA). An average value was calculated for each area from individual mice. These averages were used to estimate the overall staining for each treatment group. Results of the  $A\beta$  burden were expressed as a percentage of  $A\beta$  area stained/total area examined.

### Behavioral Assessment of the Mice

After 10 months of treatment with nilvadipine or a placebo, 20-month-old Tg PS1/APPsw and their control littermates Tg PS1 mice were subjected to the following behavioral tests. A general test of activity and exploratory behavior was conducted by placing each animal in a

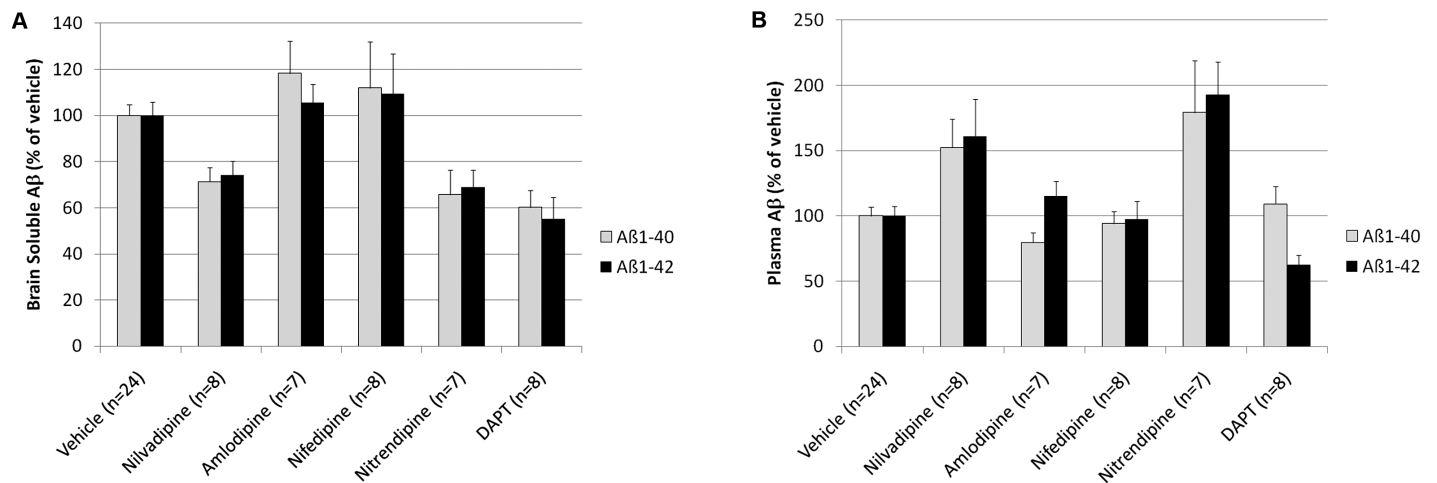


**Figure 4.** Effect of antihypertensive DHPs on  $A\beta$  production in neuroblastoma SHSY cells overexpressing the C99 C-terminal fragment of APP. C99 overexpressing SHSY cells were treated for 24 h with different DHPs. Results were expressed as a percentage of the  $A\beta_{1-40}$  values observed in the vehicle treatment conditions  $\pm$  S.E.M. ANOVA reveals no statistically significant main effect for the different doses of the DHPs tested on  $A\beta_{1-40}$  production ( $P > 0.05$ ) showing that antihypertensive DHPs do not inhibit the production of  $A\beta_{1-40}$  by C99-overexpressing cells. (Average  $A\beta_{1-40}$  values observed in the control conditions were 770.1 pg/mL.)

large open field arena for 30 min. Exploratory behavior was monitored via an overhead video camera and the video signal was sent to a computer for analysis using EthoVision (Noldus Information Technology, Sterling, VA, USA) tracking software. Key dependent measures were total distance traveled in the entire arena, distance traveled along the outer 10-cm perimeter of the arena, and average distance from the center arena point. Dependent measures were calculated across time blocks and rates were compared across the duration of the open field session.

A mouse version of the Morris water maze (28) was used to assess spatial learning and memory. A 2-m polypropylene pool, filled with opacified water (24°C) and located in an approximately 3.5 m<sup>2</sup> room rich in extra-maze cues was used. Testing began with a standard hidden platform reference memory protocol, where the pool contained a 20-cm

diameter platform hidden 1.5 cm below the water surface in the center of one pool quadrant (NE, SE, SW or NW). Mice were placed in the pool from 1 of 4 entrance points (N, E, S or W) and allowed 90 s to locate the hidden platform. Four consecutive acquisition trials were given per day across 9 d. Mice were allowed to remain on the platform 30 s prior to subsequent trials, and any mouse that did not locate the hidden platform within 90 s was guided to the platform using an extraction tool as a beacon. During the acquisition trials the platform location remained constant for a given mouse. During all water maze trials, an overhead video camera captured the image of the mouse in the water maze and it was digitally tracked using the EthoVision software. All experiments using animals were performed under protocols approved by the Institutional Animal Care and Use Committee of the Roskamp Institute.



**Figure 5.** (A) Effect of an acute treatment with nilvadipine, amlodipine, nifedipine, nitrendipine and DAPT on brain soluble A $\beta$  levels in Tg PS1/APPsw mice. DAPT (10 mg/kg of body weight), nilvadipine (2 mg/kg), amlodipine (2 mg/kg) and nifedipine (2 mg/kg) were dissolved in 50% DMSO/PBS and administered to Tg PS1/APPsw mice (6-month-old) intraperitoneally for 4 d. Vehicle treated Tg PS1/APPsw mice received an i.p. injection of the vehicle for 4 d. Within 1 h after the last injection, brains and plasma samples were collected. Brain soluble A $\beta$  levels and plasma A $\beta$  levels were quantified by ELISAs. ANOVA reveals statistically significant main effect of the acute treatment with nilvadipine ( $P < 0.05$ ), nitrendipine ( $P < 0.01$ ) and DAPT ( $P < 0.004$ ) on brain A $\beta$  level but no main effect for amlodipine ( $P = 0.184$ ) and nifedipine ( $P = 0.36$ ) treatments. *Post hoc* analysis show statistically significant differences in brain A $\beta_{1-40}$  levels between control and nilvadipine treated mice ( $P < 0.05$ ) as well as significant differences between brain A $\beta_{1-42}$  and A $\beta_{1-40}$  levels between vehicle and nitrendipine-treated mice ( $P < 0.01$ ) and between vehicle- and DAPT-treated mice ( $P < 0.004$ ). (Average brain soluble A $\beta_{1-40}$  and A $\beta_{1-42}$  values observed in vehicle-treated mice were respectively 10249.1 and 8770.8 pg/mg of protein). (B) Effect of an acute treatment with nilvadipine, amlodipine, nifedipine, nitrendipine and DAPT on plasma A $\beta$  levels in Tg PS1/APPsw mice. An elevation of plasma A $\beta_{1-40}$  and A $\beta_{1-42}$  was observed following nilvadipine and nitrendipine treatments, whereas a decreased plasma A $\beta_{1-42}$  level was observed following DAPT treatment. ANOVA reveals a statistically significant main effect of the acute treatment with nilvadipine ( $P < 0.05$ ) and nitrendipine ( $P < 0.01$ ) on plasma A $\beta_{1-40}$  level and significant main effect of nilvadipine ( $P < 0.05$ ), nitrendipine ( $P < 0.01$ ) and DAPT ( $P < 0.04$ ) on plasma A $\beta_{1-42}$ , but no main effect for amlodipine and nifedipine treatments ( $P > 0.05$ ). *Post hoc* comparisons shows significant differences in plasma A $\beta_{1-40}$  values between vehicle, nilvadipine and nitrendipine treated mice ( $P < 0.05$ ) and significant differences in plasma A $\beta_{1-42}$  values between vehicle, nilvadipine, nitrendipine and DAPT treatments ( $P < 0.05$ ). (Average plasma A $\beta_{1-40}$  and A $\beta_{1-42}$  values observed in vehicle treated mice were respectively 1384.9 and 293.9 pg/mL.)

### Statistical Analysis

Statistical analysis were performed by analysis of variance (ANOVA) using SPSS Version 12.0 for Windows and *post hoc* analysis were carried out using Bonferroni correction.  $P < 0.05$  was considered significant.

### RESULTS

#### Effects of DHPs and Non-DHP L-Type Calcium Channel Blockers on A $\beta$ Production in Cell Culture

In this study, we compared the effects of eight commonly used antihypertensive compounds known to inhibit L-type calcium channels on A $\beta_{1-40}$  and A $\beta_{1-42}$  production using 7W CHO cells overexpressing human APP751 (20), including seven

clinically used antihypertensive DHPs (felodipine, nifedipine, amlodipine, nilvadipine, nitrendipine, nimodipine and isradipine) and two non-DHPs (verapamil and diltiazem). Among the L-type calcium channel blockers tested, only nilvadipine and amlodipine lowered both A $\beta_{1-40}$  and A $\beta_{1-42}$  for doses between 1 and 10  $\mu\text{mol/L}$  significantly (Figure 1). Nitrendipine appears to marginally inhibit A $\beta_{1-40}$  at 5 and 10  $\mu\text{mol/L}$  but does not affect A $\beta_{1-42}$  production significantly. At higher doses, nitrendipine inhibits both A $\beta_{1-40}$  and A $\beta_{1-42}$  production (data not shown). Felodipine, nifedipine, diltiazem and verapamil have no significant impact on A $\beta_{1-40}$  and A $\beta_{1-42}$  production for the dose range studied. Nimodipine appears to dose dependently stimulate both A $\beta_{1-40}$

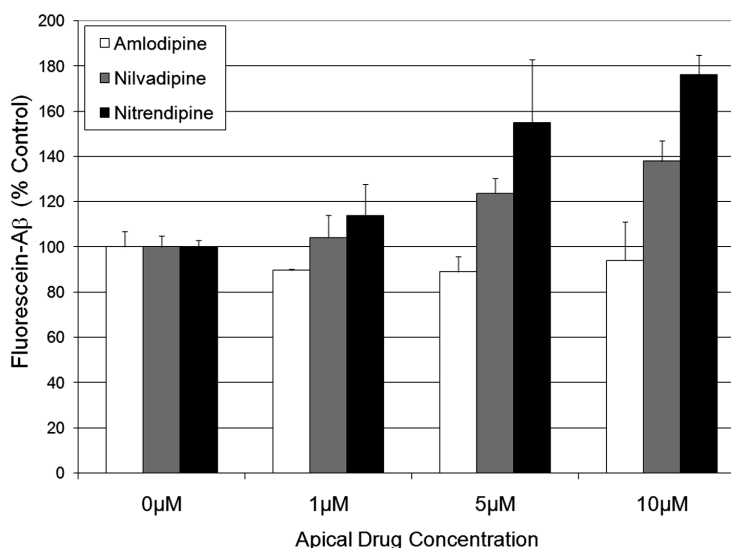
and A $\beta_{1-42}$  levels whereas isradipine stimulates A $\beta_{1-42}$  levels without significantly affecting A $\beta_{1-40}$  values (see Figure 1).

As L-type calcium channel blockers also may impact other types of calcium channels (for instance amlodipine significantly blocks N-type and P/Q-type calcium channels [29]), we also investigated the effect of different toxins (agatoxin TK, agatoxin IVa, conotoxin GVIA and conotoxin MVIIC) known to selectively block N, P and Q-type calcium channels. Blockade of N, P or Q-type calcium channels with these toxins did not significantly impact A $\beta_{1-40}$  or A $\beta_{1-42}$  production (data not shown) in 7W cells. In addition, blockade of potassium, sodium and chloride channels with glyburide, lidocaine N-ethyl bromide (QX-314), protopine, 4'-([1-(2-[6-

Methyl-2-pyridinyl] ethyl)-4-piperidinyl] carbonyl) methanesulfonanilide (E4031), 5-Nitro-2-(3-phenylpropylamino) benzoic Acid (NPPB) or 4,4'-Diisothiocyanostilbene-2,2'-disulfonic Acid, 2Na (DIDS) did not affect A $\beta$  production (data not shown). Also, neither FPL64176 (methyl 2,5 dimethyl-4[2-(phenylmethyl)benzoyl]-1H-pyrrole-3-carboxylate) nor Bay-K 8664, two potent agonists of L-type calcium channels, significantly affected A $\beta_{1-40}$  or A $\beta_{1-42}$  levels (data not shown). Altogether these data suggest that selective DHPs are able to lower A $\beta$  production *in vitro* independently of their inhibitory action on L-type calcium channels.

### Effect of Nilvadipine and Amlodipine on APP Processing

The effects of nilvadipine and amlodipine (the two most potent A $\beta$ -lowering DHP identified *in vitro* among the CCB tested) on APP-CTF, APP $\alpha$  and APP $\beta$  secretion using 7W CHO cells were investigated. In whole cell lysate, neither amlodipine nor nilvadipine modified the level of full length APP (Figure 2). Following 24 h of treatment with amlodipine, a dose-dependent increase in APP-CTF $\alpha$  and decrease in CTF $\beta$  levels were observed, whereas a slight stimulation of APP-CTF $\alpha$  was noted for nilvadipine (see Figure 2). However, no significant increase in sAPP $\alpha$  production was observed in the culture media following nilvadipine or amlodipine treatments suggesting that these compounds do not stimulate the  $\alpha$ -secretase cleavage of APP. A decreased APP $\beta$  production was observed following 24 h of treatment with amlodipine and nilvadipine suggesting that these compounds impact the  $\beta$ -cleavage of APP (see Figure 2). A decreased APP $\beta$  secretion also was observed following 6 h of treatment with nilvadipine and amlodipine (data not shown). BACE-1 ( $\beta$ -secretase) specific FRET and chemoluminescent assays were implemented using recombinant human BACE-1 to determine a possible direct inhibition of BACE-1 activity by nilvadipine and amlodipine. No noticeable inhibition of



**Figure 6.** Effects of amlodipine, nitrendipine and nilvadipine on A $\beta$  transcytosis across an *in vitro* model of the BBB. Fluorescein labeled human A $\beta_{1-42}$  was added to the basolateral compartment ("brain" side) whereas different doses of amlodipine, nitrendipine and nilvadipine were added to the apical side ("blood" side) of the *in vitro* BBB model. The amount of fluorescein-A $\beta_{1-42}$  was quantified in the apical side over a period of 90 min to calculate the apparent permeability of A $\beta_{1-42}$  for the different treatments conditions. ANOVA shows a statistically significant effect of nilvadipine ( $P < 0.05$ ), of nitrendipine ( $P < 0.001$ ) but not of amlodipine ( $P = 0.910$ ) on A $\beta$  transcytosis *in vitro* across the BBB layer of human brain microvascular endothelial cells. *Post hoc* analysis reveals a significant effect of nilvadipine at 10  $\mu$ mol/L ( $P < 0.05$ ) and a significant effect of nitrendipine at 5  $\mu$ mol/L ( $P < 0.01$ ) and 10  $\mu$ mol/L ( $P < 0.001$ ), showing that nilvadipine and nitrendipine stimulate the transport of A $\beta$  from the brain to the periphery in an *in vitro* model of the BBB, whereas amlodipine is inefficient.

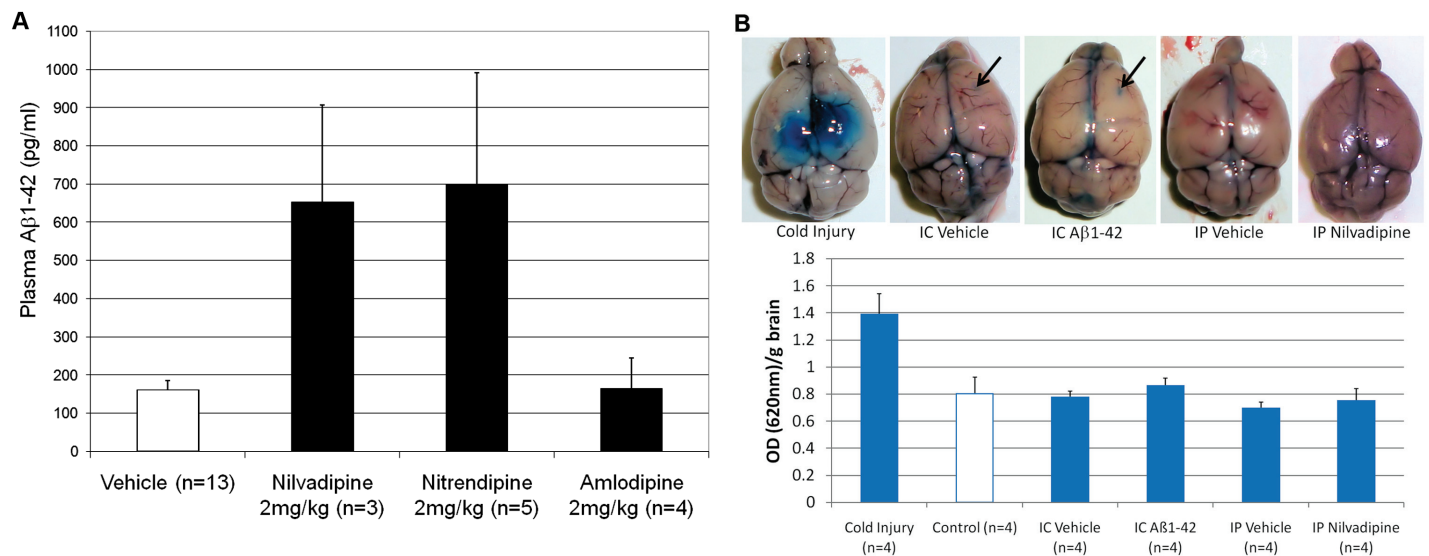
BACE-1 activity was observed with either nilvadipine or amlodipine in either assays ruling out the possibility that amlodipine and nilvadipine are direct BACE-1 inhibitors for the dose range studied (Figure 3).

The impact of nilvadipine and amlodipine on the  $\gamma$ -secretase pathway also was tested by investigating A $\beta$  production in SHSY cells overexpressing the APP-CTF $\beta$  (C99), the sole substrate of  $\gamma$ -secretase leading to A $\beta$  production. Neither nilvadipine nor amlodipine inhibited A $\beta$  production in C99 overexpressing cells (Figure 4). In addition, no alteration of Notch cleavage (data not shown) was observed after treatment with nilvadipine or amlodipine, further suggesting that these compounds do not impact the  $\gamma$ -secretase pathway significantly.

### In Vivo Effects of Antihypertensive DHPs in Transgenic Mouse Models of AD

We tested nilvadipine, amlodipine, nifedipine, nitrendipine and DAPT for their ability to acutely affect A $\beta_{1-40}$  and A $\beta_{1-42}$  levels in the brains of Tg PS1/APPsw mice. The known functional  $\gamma$ -secretase inhibitor DAPT was used as a positive control. Nifedipine, which was unable to lower A $\beta$  production *in vitro*, was used as a negative control. Although nitrendipine shows only a weak inhibition of A $\beta$  production *in vitro*, this compound was included because it shows a clear prophylactic activity against AD (7). Nilvadipine, amlodipine, nitrendipine and nifedipine were dosed at 2 mg/kg/d (a physiologically relevant dose conferring antihypertensive activity) whereas DAPT was used at a dosage of 10 mg/kg/d for a period of 4 d. An anal-





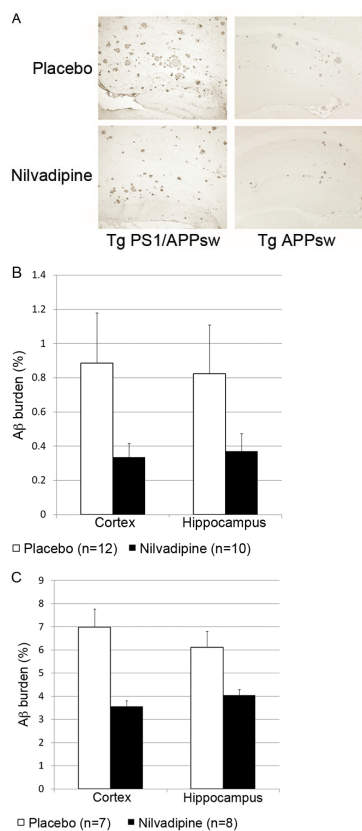
**Figure 7.** (A) Effect of amlodipine, nitrendipine and nilvadipine on Aβ clearance across the BBB *in vivo*. Following an i.p. injection with the vehicle alone, amlodipine, nitrendipine and nilvadipine (2 mg/kg), wild-type B6/SJL F1 mice were injected stereotaxically with human Aβ<sub>1-42</sub>. Plasma human Aβ<sub>1-42</sub> levels were quantified by ELISA following the intracranial injection of Aβ to reveal the amount of human Aβ<sub>1-42</sub> clearance across the BBB. ANOVA shows a significant main effect of nilvadipine ( $P < 0.001$ ), of nitrendipine ( $P < 0.001$ ), but not of amlodipine ( $P = 0.969$ ) on plasma Aβ<sub>1-42</sub> level. *Post hoc* comparisons reveal statistically significant differences between plasma Aβ<sub>1-42</sub> for the vehicle and the nilvadipine treatment groups ( $P < 0.004$ ), as well as significant differences between the vehicle and nitrendipine treatment groups ( $P < 0.001$ ), showing that nitrendipine and nilvadipine stimulate the clearance of Aβ across the BBB whereas amlodipine is inefficient. (B) Effect of the nilvadipine treatment and of the intracranial Aβ injection on BBB leakiness. Mice were subjected either to cold injury of the cortex to induce a breakdown of the BBB (positive control), were untreated (control mice), were injected intraperitoneally with 100 μL of the vehicle (50% DMSO in PBS) (IP vehicle) or with 2 mg/kg of nilvadipine (IP nilvadipine) or injected intracranially with 3 μL of the vehicle (IC vehicle) or with 3 nmol of Aβ<sub>1-42</sub> (IC Aβ<sub>1-42</sub>). Potential BBB leakage was evaluated by injecting the Evans blue dye intraperitoneally and measuring its extravasation in the brain 1 h later. Representative pictures of the brains for the different treatments are shown. Leakage of the Evans blue dye at the sites of the cold injury is evident, the site of the needle puncture created by the intracranial injection is indicated by an arrow. The histogram represents the amount of Evans blue dye extravasated in the brain of the animals for the different treatment groups. ANOVA reveals a significant main effect of the treatments on brain levels of Evans blue ( $P < 0.001$ ). *Post hoc* analyses show a statistically significant elevation of Evans blue in the brains of the mice subjected to the cold injury ( $P < 0.005$ ) compared with all the other groups but no statistically significant difference between untreated mice and IC vehicle injected animals ( $P = 0.999$ ), IC Aβ<sub>1-42</sub> injected mice ( $P = 0.999$ ), IP vehicle injected mice ( $P = 0.982$ ) and nilvadipine treated mice ( $P = 0.999$ ) showing that none of these treatments increased the leakiness of the BBB to Evans blue.

ysis of brain Aβ levels following 4 d of treatment shows that DAPT, nilvadipine and nitrendipine significantly reduce soluble Aβ levels in the brains of Tg PS1/APPsw mice whereas amlodipine and nifedipine were ineffective (Figure 5). The failure of amlodipine to lower Aβ levels *in vivo* despite its ability to lower Aβ *in vitro* may be related to its inability to cross the blood-brain barrier (BBB) compared with highly lipophilic DHPs such as nilvadipine and nitrendipine which accumulate in the brain (30). In addition, these data further confirm that the Aβ-lowering activity of nilvadipine and nitrendipine *in vivo* is not related to their antihypertensive activity, since both am-

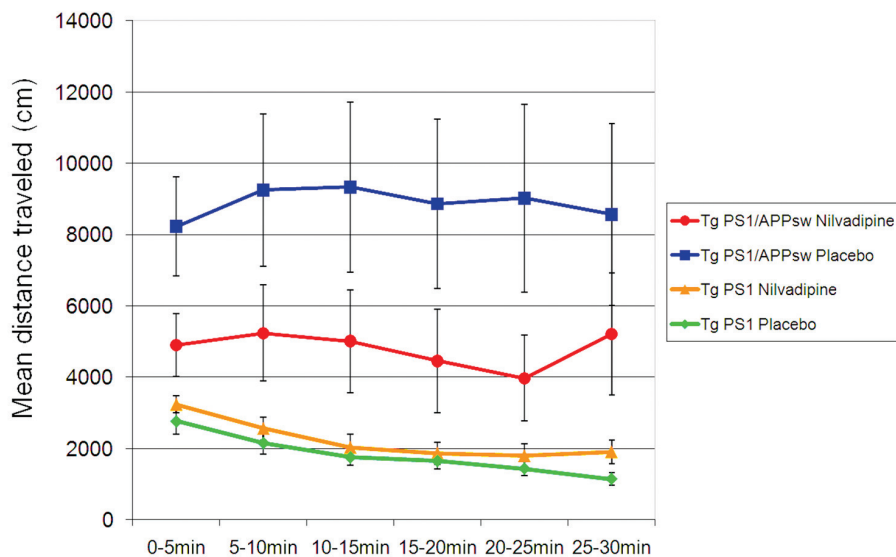
lodipine and nifedipine were unable to reduce brain Aβ levels.

Surprisingly, an increased plasma Aβ level was observed in animals that received the acute treatment with nilvadipine and nitrendipine despite a reduction in brain Aβ levels (see Figure 5), suggesting that these compounds may facilitate the clearance of Aβ across the BBB or may prevent Aβ degradation/elimination in the periphery. We therefore investigated the effect of nilvadipine, nitrendipine and amlodipine in an *in vitro* model of the BBB, employing human brain microvascular endothelial cells. Both nilvadipine and nitrendipine increased Aβ clearance from the brain to

the peripheral side of the *in vitro* BBB model, whereas amlodipine was ineffective (Figure 6). In addition, nilvadipine and nitrendipine appear to facilitate the clearance of human Aβ across the BBB *in vivo* in wild-type mice that were injected intracranially with human Aβ, whereas amlodipine was ineffective (Figure 7), further confirming that the elevation in plasma Aβ observed in Tg PS1/APPsw are likely reflective of an increased clearance of Aβ across the BBB. We performed additional control experiments to determine whether nilvadipine or the intracranial Aβ injection procedure were impacting the leakiness of the BBB using Evans blue injections to monitor BBB



**Figure 8.** Effect of a chronic oral treatment with nilvadipine on Aβ burden in Tg APPsw and Tg PS1/APPsw mice. Three-month-old Tg APPsw were fed for 17 months with a powder diet containing 0.03% (weight/weight) of nilvadipine formulation of oral dosage (n = 10) or a placebo (n = 12), whereas 10-month-old Tg PS1/APPsw were fed for 10 months with nilvadipine (n = 8) or a placebo (n = 7). (A) Representative pictures (20 × objective) showing the amount of Aβ deposits in the hippocampus of Tg APPsw and Tg PS1/APPsw following treatment with a placebo or nilvadipine. Histograms representing the amount of Aβ burden in the cortex and hippocampus of Tg APPsw mice (B) and Tg PS1/APPsw mice (C) treated with nilvadipine and the placebo. ANOVA reveals a statistically significant main effect of the nilvadipine treatment in Tg APPsw ( $P < 0.003$ ) and in Tg PS1/APPsw mice ( $P < 0.001$ ). *Post hoc* analyses reveals a statistically significant difference in Aβ burden for the cortex and hippocampus of placebo and nilvadipine treated Tg APPsw ( $P < 0.003$ ) and Tg PS1/APPsw mice ( $P < 0.001$ ) showing that nilvadipine is reducing Aβ accumulation in the brains of Tg APPsw and Tg PS1/APPsw mice.



**Figure 9.** Effect of nilvadipine on the exploratory activity of Tg PS1 and Tg PS1/APPsw mice in the open field apparatus. (The distance traveled in a 1 meter open field over 30 min is represented). Tg PS1/APPsw mice show a hyperactivity behavior in the open field compared with Tg PS1 littermate controls. The nilvadipine treatment appears to reduce the hyperactivity of Tg PS1/APPsw mice. Repeated ANOVA measures followed by *post hoc* analysis show statistically significant differences for the total distance traveled in the open field arena between Tg PS1/APPsw treated with nilvadipine and Tg PS1/APPsw treated with a placebo ( $P < 0.001$ ), between Tg PS1/APPsw treated with nilvadipine and Tg PS1 treated with nilvadipine ( $P < 0.001$ ) and between Tg PS1/APPsw treated with a placebo and Tg PS1 treated with a placebo ( $P < 0.001$ ).

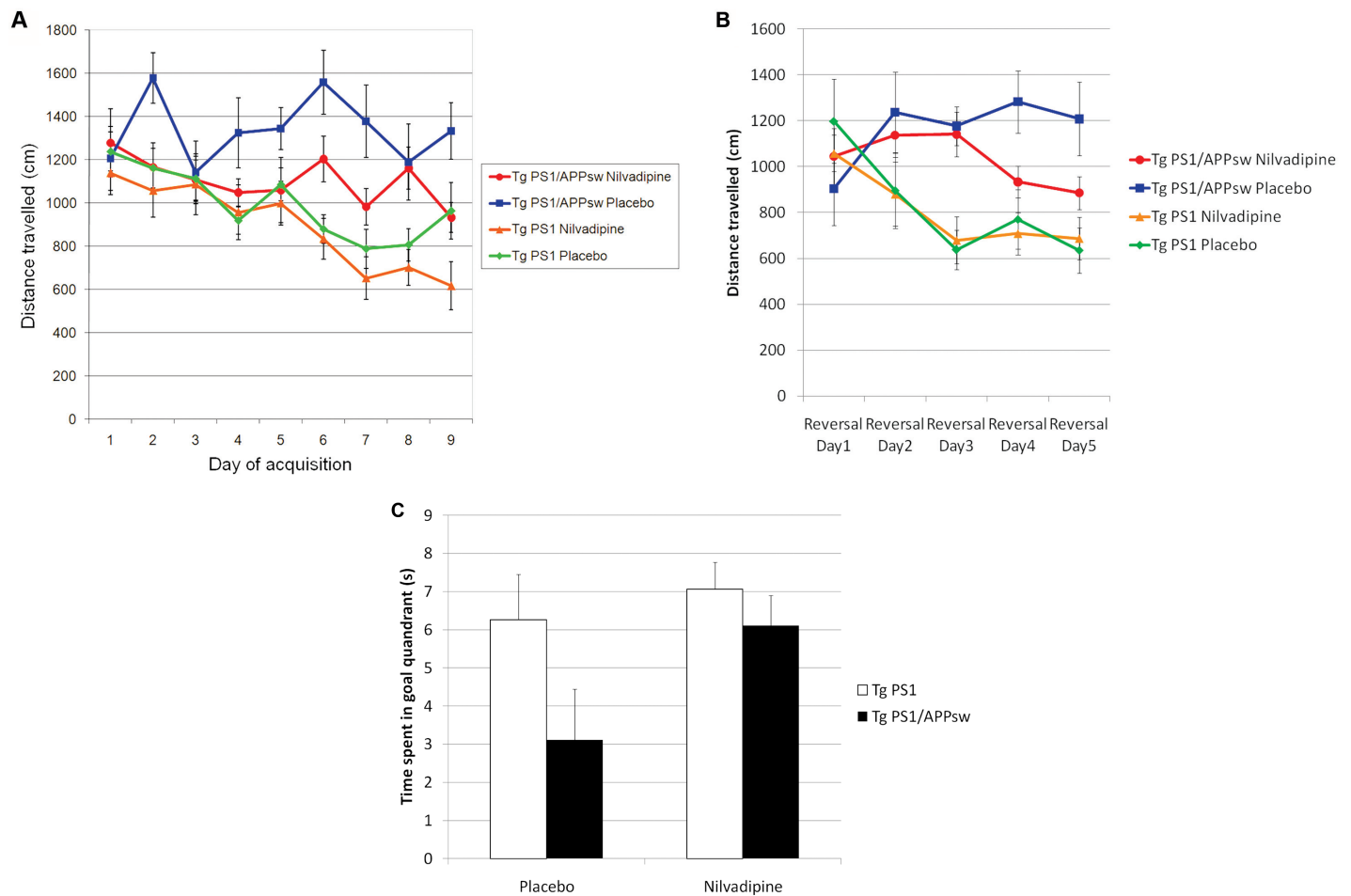
permeability (25,26). The cold injury procedure which induces a rapid breakdown of the BBB (31) was used as a positive control and led to a significant extravasation of the Evans blue dye in the brain (see Figure 7). We did not observe a significant effect of nilvadipine or of the intracranial injection procedure on the leakiness of the BBB (see Figure 7), suggesting that nilvadipine is affecting the transport of Aβ across the BBB and is not increasing the permeability of the BBB nonspecifically.

### Chronic Effects of Nilvadipine in Transgenic Mouse Models of AD

We also investigated the effect of chronic treatment with nilvadipine on β-amyloid burden in Tg APPsw and Tg PS1/APPsw mice. Ten-month-old Tg PS1/APPsw mice (already presenting a β-amyloid pathology) and their control littermates were fed a powder diet con-

taining 0.03% of nilvadipine (solid dispersion formulation for oral dosage) or a placebo (solid dispersion formulation without nilvadipine) for 10 months. In addition, 3-month-old Tg APPsw (Tg2576; prophylactic treatment initiated prior to Aβ deposits) were fed with nilvadipine or a placebo as indicated above until 20 months of age. Data show that chronic nilvadipine treatments reduced plaque burden by approximately 40% for different areas of the brains of Tg PS1/APPsw mice and by approximately 50% in the brains of Tg APPsw mice (Figure 8).

Behavioral testing was conducted in Tg PS1/APPsw mice and control littermates (Tg PS1; these mice do not show cognitive impairment and perform similarly to wild-type mice in the open field and the Morris water maze [32]) fed with nilvadipine and the placebo. In the open field test (Figure 9), Tg PS1/APPsw mice



**Figure 10.** Effect of nilvadipine on cognitive behavior of Tg PS1 and Tg PS1/APPsw mice in the Morris water maze. (A) Learning curve for Morris water maze acquisition trials across a period of 9 d. (B) Learning curve for Morris water maze during the reversal period (hidden platform moved to a new location). (C) Effect of nilvadipine on memory retention in the Morris water maze (probe trials). Placebo-treated Tg PS1/APPsw mice have the worst learning performance in the Morris water maze compared with placebo-treated Tg PS1 littermate controls ( $P < 0.001$ ) during the acquisition trials and the reversal. Nilvadipine treated Tg PS1/APPsw mice locate the hidden platform more efficiently than placebo-treated Tg PS1/APPsw mice ( $P < 0.04$ ) during the acquisition trials and the reversal. Pooled probe trials for the Morris water maze show a significant increase in retention for Tg PS1 control littermates compared with Tg PS1/APPsw treated with the placebo ( $P < 0.01$ ) as well as an improved memory retention for nilvadipine-treated Tg PS1/APPsw mice compared with placebo treated Tg PS1/APPsw ( $P < 0.02$ ).

show a hyperactivity and do not habituate to the arena compared with their Tg PS1 littermate controls. Nilvadipine treatment significantly increases the habituation of Tg PS1/APPsw to the arena, suggesting an improvement in memory (see Figure 9). In the Morris water maze, the acquisition of place learning was impaired in Tg PS1/APPsw mice compared with Tg PS1 mice (Figure 10). Nilvadipine treatment improved the learning functions of Tg PS1/APPsw mice in the Morris water maze to values comparable to

their control littermates during the acquisition trials, showing improvement in learning (see Figure 10). The reversal training was used to distinguish goal-directed navigation from search strategy. With the platform moved to the opposite quadrant of the pool, path length to the platform during the reversal trials was reduced for Tg PS1, Tg PS1 treated with nilvadipine and Tg PS1/APPsw treated with nilvadipine compared with placebo treated Tg PS1/APPsw (see Figure 10), showing that nilvadipine improved spa-

tial learning in Tg PS1/APPsw mice. Probe trials (in which the platform was removed) were conducted after a 5-day period of rest to assess the impact of nilvadipine on long-term memory. Memory of the platform localization (retention) was reduced significantly in Tg PS1/APPsw mice compared with their control littermates (Tg PS1 mice) (see Figure 10). Nilvadipine significantly improved retention in Tg PS1 mice and Tg PS1/APPsw compared respectively to Tg PS1 and Tg PS1/APPsw mice of the

placebo group. Overall, nilvadipine significantly reduced the impaired learning and memory deficits which characterize the cognitive dysfunction observed in transgenic mouse models of AD.

## DISCUSSION

The use of particular antihypertensive medications, specifically the DHPs nitrendipine and nilvadipine, have been associated with a reduced risk of developing dementia, including AD (7,8,10,16,33). We therefore investigated the effects of several clinically used antihypertensive DHPs as well as non-DHP CCBs on A $\beta$  production as this peptide is believed to be central to the disease process. Our data reveal that the DHPs amlodipine and nilvadipine are the most potent of the DHPs that we tested for inhibiting A $\beta_{1-40}$  and A $\beta_{1-42}$  production *in vitro* whereas other DHPs and non-DHP CCBs appear inefficient or even raise the levels of A $\beta_{1-40}$  and A $\beta_{1-42}$  *in vitro* significantly. Nilvadipine and amlodipine were unable to inhibit A $\beta$  production in a cell line overexpressing the APP-CTF $\beta$  fragment and did not impact the  $\gamma$ -secretase cleavage of Notch, suggesting that these compounds do not significantly impact the  $\gamma$ -secretase pathway. The *in vitro* A $\beta$ -lowering activity of nilvadipine and amlodipine appear to be mediated by an inhibition of the  $\beta$ -cleavage of APP since a reduction in sAPP $\beta$  production was observed. However, no direct effect of nilvadipine and amlodipine was observed on  $\beta$ -secretase activity in two different cell free assays, suggesting that these drugs are indirect inhibitors of BACE-1 activity. The effect of nilvadipine and amlodipine on A $\beta$  levels is independent of their calcium channel blocking activity, as other antihypertensive DHPs that display similar activity toward L-type calcium channels do not display the same ability to inhibit A $\beta$  production.

*In vivo* acute dosage studies in Tg PS1/APPsw mice reveal that nilvadipine and nitrendipine reduce brain levels of soluble A $\beta$ , whereas other antihypertensive DHPs such as amlodipine and felodipine are inactive, further confirm-

ing that the *in vivo* A $\beta$ -lowering activity of nilvadipine and nitrendipine is independent of their antihypertensive activity. Surprisingly, an increased level of A $\beta$  in the plasma was observed following an acute dosage with nilvadipine and nitrendipine, which prompted us to explore whether these drugs were facilitating the clearance of A $\beta$  across the BBB. We observed, both *in vitro* and *in vivo*, that nilvadipine and nitrendipine effectively improved the clearance of A $\beta$  across the BBB whereas amlodipine was ineffective. We used the Evans blue methodology to determine whether nilvadipine was impacting the overall permeability of the BBB. Compared with peripheral organs such as the liver, kidney and heart, we observed that the extravasation of the Evans blue dye was approximately ten times lower in the brain (data not shown), illustrating the restrictive permeability of the BBB compared with peripheral tissues as analyzed previously in detail for peptides and polar solutes (34,35). We found that nilvadipine does not increase the permeability of the BBB to Evans blue, suggesting that nilvadipine is impacting the transport of A $\beta$  across the BBB. The molecular mechanisms responsible for the transport of A $\beta$  across the BBB have been studied extensively. The receptor for advanced glycation end products (RAGE) has been identified as the main receptor responsible for the influx of circulating A $\beta$  to the brain whereas the lipoprotein receptor (LRP-1) has been identified as the receptor mediating the efflux of A $\beta$  from the brain into the circulation (36,37). We have confirmed that LRP-1 and RAGE are the main receptors mediating the transport of A $\beta$  in our *in vitro* model of the BBB employing HBMEC (23). Our data showing that nilvadipine can selectively improve the transport of A $\beta$  from the brain side to the peripheral side of this *in vitro* BBB model suggest that nilvadipine is either inhibiting RAGE or is facilitating LRP-1-mediated A $\beta$  transport. Nilvadipine has been shown to inhibit nuclear factor- $\kappa$ B (NF $\kappa$ B)-dependent transcription (38) and, as expected for an NF $\kappa$ B inhibitor,

nilvadipine users have reduced plasma cytokine levels (39). Interestingly, both RAGE and BACE-1 expression are regulated by NF $\kappa$ B (40–43). In particular, inhibition of NF $\kappa$ B signaling decreases RAGE expression in endothelial cells (44) and reduces A $\beta$  production and BACE-1 expression levels (21,45,46). The inhibition of NF $\kappa$ B by nilvadipine therefore may represent a plausible mechanism responsible for both the inhibition of A $\beta$  production and for the increased clearance of A $\beta$  across the BBB observed following nilvadipine treatment.

We next investigated the effect of a chronic nilvadipine administration in transgenic mouse models of AD. Chronic oral dosage with nilvadipine resulted in an approximately 40% to 50% reduction in  $\beta$ -amyloid burden in Tg PS1/APPsw and Tg APPsw mice. In addition, improvement of cognitive behavior was observed after nilvadipine administration in Tg PS1/APPsw mice showing that nilvadipine can reduce memory deficits associated with the  $\beta$ -amyloid load in Tg PS1/APPsw mice.

We have shown previously that nilvadipine increases cerebral blood flow in a transgenic mouse model of AD (47). In addition, nilvadipine has been shown to improve spatial memory in a rat model of AD (48), consistent with our findings in transgenic mouse models of AD. Interestingly, nilvadipine, but not amlodipine, has been shown to improve cerebral blood flow and cognitive function, and to reduce the rate of conversion to AD in patients with mild cognitive impairment (10). Moreover, in an open-label safety and tolerability trial with nilvadipine in AD patients, we observed that cognition was stabilized in AD patients treated with nilvadipine, whereas untreated subjects showed cognitive decline (49).

The proteases that generate A $\beta$  directly are considered major targets for AD. However, direct inhibitors of these proteases with a clinically appropriate profile have been difficult to discover so far. Brain A $\beta$  accumulation theoretically is dependent on the balance between A $\beta$  production and A $\beta$  clearance. The routes



of A $\beta$  elimination involve both the catabolism of A $\beta$  (50) and its transport across the BBB (37,51,52). The clearance of A $\beta$  across the BBB plays a critical role in brain A $\beta$  accumulation (53,54) and has been suggested to represent the major pathway leading to brain A $\beta$  elimination (55). Recently, a study measuring both A $\beta$  production and clearance rates in AD and healthy controls has revealed that the production of A $\beta$  is similar in AD and controls, whereas the clearance rate of A $\beta$  is decreased by around 30% in AD compared with healthy patients, showing that A $\beta$  clearance mechanisms are defective in AD brains (56,57). Our study identifies and characterizes two clinically used antihypertensive DHPs with a proven safety record in elderly patients, nilvadipine and nitrendipine, as dual modulators of APP processing and of the clearance of A $\beta$  across the BBB. To our knowledge, these two DHPs represent the first small molecules identified so far that are able to stimulate the clearance of A $\beta$  across the BBB. These data may explain why nilvadipine and nitrendipine have shown beneficial effects in AD patients (7,8,10,49). Since the efficacy of nilvadipine and nitrendipine toward A $\beta$  accumulation is independent of their antihypertensive effect, as other antihypertensive DHPs are inefficient, we hypothesize that compounds derived from the chemical structure of A $\beta$ -lowering DHPs could be optimized to negate their antihypertensive activity, and at the same time, enhance their A $\beta$ -lowering properties, providing a new class of AD medications.

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#### DISCLOSURES

Several authors have financial interests in the commercialization of nilvadipine and related DHPs as clinical treatments for the prevention and mitigation of Alzheimer disease. These authors are D Paris, G Ait-Ghezala, F Crawford and MJ Mullan.

#### REFERENCES

1. Perl DP. (2010) Neuropathology of Alzheimer's disease. *Mt. Sinai J. Med.* 77:32–42.
2. Chow VW, Mattson MP, Wong PC, Gleichmann M. (2010) An overview of APP processing enzymes and products. *Neuromolecular Med.* 12:1–12.
3. Lublin AL, Gandy S. (2010) Amyloid-beta oligomers: possible roles as key neurotoxins in Alzheimer's Disease. *Mt. Sinai J. Med.* 77:43–9.
4. Gleichmann M, Mattson MP. (2010) Alzheimer's disease and neuronal network activity. *Neuromolecular Med.* 12:44–7.
5. Citron M. (2010) Alzheimer's disease: strategies for disease modification. *Nat. Rev. Drug. Discov.* 9:387–98.
6. Vassar R, et al. (1999) Beta-secretase cleavage of Alzheimer's amyloid precursor protein by the transmembrane aspartic protease BACE. *Science.* 286:735–41.
7. Forette F, et al. (1998) Prevention of dementia in randomised double-blind placebo-controlled Systolic Hypertension in Europe (Syst-Eur) trial. *Lancet.* 352:1347–51.
8. Forette F, et al. (2002) The prevention of dementia with antihypertensive treatment: new evidence from the Systolic Hypertension in Europe (Syst-Eur) study. *Arch. Intern. Med.* 162:2046–52.
9. Hanon O, Forette F. (2004) Prevention of dementia: lessons from SYST-EUR and PROGRESS. *J. Neurol. Sci.* 226:71–4.
10. Hanyu H, et al. (2007) Nilvadipine prevents cognitive decline of patients with mild cognitive impairment. *Int. J. Geriatr. Psychiatry.* 22:1264–6.
11. Matsuda H, et al. (2008) Effect of nilvadipine on regional cerebral blood flow in a patient with early Alzheimer disease. *Clin. Nucl. Med.* 33:34–5.
12. Sato T, et al. (2008) A patient with early Alzheimer's disease who showed improvement of cognitive function and cerebral perfusion by combined therapy of nilvadipine and PPAR gamma agonists. *Nippon Ronen Igakkai Zasshi.* 45:428–33.
13. Khachaturian AS, et al. (2006) Antihypertensive medication use and incident Alzheimer disease: the Cache County Study. *Arch. Neurol.* 63:686–92.
14. Yasar S, Corrada M, Brookmeyer R, Kawas C. (2005) Calcium channel blockers and risk of AD: the Baltimore Longitudinal Study of Aging. *Neurobiol. Aging.* 26:157–63.
15. Duron E, Hanon O. (2010) Antihypertensive treatments, cognitive decline, and dementia. *J. Alzheimers Dis.* 20:903–14.
16. Fournier A, et al. (2009) Prevention of dementia by antihypertensive drugs: how AT1-receptor-blockers and dihydropyridines better prevent dementia in hypertensive patients than thiazides and ACE-inhibitors. *Expert Rev. Neurother.* 9:1413–31.
17. Bellew KM, et al. (2004) Hypertension and the rate of cognitive decline in patients with dementia of the Alzheimer type. *Alzheimer Dis. Assoc. Disord.* 18:208–13.
18. SHEP Cooperative Research Group. (1991) Prevention of stroke by antihypertensive drug treatment in older persons with isolated systolic hypertension. Final results of the Systolic Hypertension in the Elderly Program (SHEP). *JAMA.* 265:3255–64.
19. Maxwell CJ, Hogan DB, Ebly EM. (1999) Calcium-channel blockers and cognitive function in elderly people: results from the Canadian Study of Health and Aging. *CMAJ.* 161:501–6.
20. Koo EH, Squazzo SL. (1994) Evidence that production and release of amyloid beta-protein involves the endocytic pathway. *J. Biol. Chem.* 269:17386–9.
21. Paris D, et al. (2010) Reduction of beta-amyloid pathology by celastrol in a transgenic mouse model of Alzheimer's disease. *J. Neuroinflammation.* 7:17.
22. Holcomb L, et al. (1998) Accelerated Alzheimer-type phenotype in transgenic mice carrying both mutant amyloid precursor protein and presenilin 1 transgenes. *Nat. Med.* 4:97–100.
23. Bachmeier C, Mullan M, Paris D. (2010) Characterization and use of human brain microvascular endothelial cells to examine beta-amyloid exchange in the blood-brain barrier. *Cytotechnology.* 2010;62:519–29.
24. Yuan W, Li G, Gil ES, Lowe TL, Fu BM. (2010) Effect of surface charge of immortalized mouse cerebral endothelial cell monolayer on transport of charged solutes. *Ann. Biomed. Eng.* 38:1463–72.
25. Methia N, et al. (2001) ApoE deficiency compromises the blood brain barrier especially after injury. *Mol. Med.* 7:810–5.
26. Epiphany S, et al. (2010) VEGF promotes malaria-associated acute lung injury in mice. *PLoS Pathog.* 6:e1000916.
27. Hsiao K, et al. (1996) Correlative memory deficits, Abeta elevation, and amyloid plaques in transgenic mice. *Science.* 274:99–102.
28. Morris R. (1984) Developments of a water-maze procedure for studying spatial learning in the rat. *J. Neurosci. Methods.* 11:47–60.
29. Furukawa T, et al. (1999) Selectivities of dihydropyridine derivatives in blocking Ca(2+) channel subtypes expressed in Xenopus oocytes. *J. Pharmacol. Exp. Ther.* 291:464–73.
30. Uchida S, Yamada S, Nagai K, Deguchi Y, Kimura R. (1997) Brain pharmacokinetics and in vivo receptor binding of 1,4-dihydropyridine calcium channel antagonists. *Life Sci.* 61:2083–90.
31. Nag S, Picard P, Stewart DJ. (2001) Expression of nitric oxide synthases and nitrotyrosine during blood-brain barrier breakdown and repair after cold injury. *Lab. Invest.* 81:41–9.

32. Janus C, *et al.* (2000) Spatial learning in transgenic mice expressing human presenilin 1 (PS1) transgenes. *Neurobiol. Aging*. 21:541–9.
33. Hanon O, *et al.* (2006) Relationship between anti-hypertensive drug therapy and cognitive function in elderly hypertensive patients with memory complaints. *J. Hypertens*. 24:2101–7.
34. Mann GE, Zlokovic BV, Yudilevich DL. (1985) Evidence for a lactate transport system in the sarcolemmal membrane of the perfused rabbit heart: kinetics of unidirectional influx, carrier specificity and effects of glucagon. *Biochim. Biophys. Acta*. 819:241–8.
35. Zlokovic BV, Segal MB, Begley DJ, Davson H, Rakic L. (1985) Permeability of the blood-cerebrospinal fluid and blood-brain barriers to thyrotropin-releasing hormone. *Brain Res*. 358:191–9.
36. Deane R, *et al.* (2003) RAGE mediates amyloid-beta peptide transport across the blood-brain barrier and accumulation in brain. *Nat. Med.* 9:907–13.
37. Shibata M, *et al.* (2000) Clearance of Alzheimer's amyloid-ss(1–40) peptide from brain by LDL receptor-related protein-1 at the blood-brain barrier. *J. Clin. Invest.* 106:1489–99.
38. Iwasaki Y, *et al.* (2004) Nilvadipine inhibits nuclear factor-kappaB-dependent transcription in hepatic cells. *Clin. Chim. Acta*. 350:151–7.
39. Kagawa H, Nomura S, Ozaki Y, Nagahama M, Fukuhara S. (1999) Effects of nilvadipine on cytokine-levels and soluble factors in collagen disease complicated with essential hypertension. *Clin. Exp. Hypertens*. 21:1177–88.
40. Tanaka N, *et al.* (2000) The receptor for advanced glycation end products is induced by the glycation products themselves and tumor necrosis factor-alpha through nuclear factor-kappa B, and by 17beta-estradiol through Sp-1 in human vascular endothelial cells. *J. Biol. Chem.* 275:25781–90.
41. Penzo M, *et al.* (2010) Inhibitor of NF-kappa B kinases alpha and beta are both essential for high mobility group box 1-mediated chemotaxis [corrected]. *J. Immunol.* 184:4497–509; erratum 184:7314.
42. Sambamurti K, Kinsey R, Maloney B, Ge YW, Lahiri DK. (2004) Gene structure and organization of the human beta-secretase (BACE) promoter. *Faseb J*. 18:1034–6.
43. Buggia-Prevot V, Sevalle J, Rossner S, Checler F. (2008) NFkappaB-dependent control of BACE1 promoter transactivation by Abeta42. *J. Biol. Chem.* 283:10037–47.
44. Marx N, *et al.* (2004) Thiazolidinediones reduce endothelial expression of receptors for advanced glycation end products. *Diabetes*. 53:2662–8.
45. Guglielmotto M, *et al.* (2010) AGEs/RAGE complex upregulates BACE1 via NF-kappaB pathway activation. *Neurobiol. Aging*. 2010, Jul 16 [Epub ahead of print].
46. Paris D, *et al.* (2007) Inhibition of Abeta production by NF-kappaB inhibitors. *Neurosci. Lett.* 415:11–6.
47. Paris D, *et al.* (2004) Nilvadipine antagonizes both Abeta vasoactivity in isolated arteries, and the reduced cerebral blood flow in APPsw transgenic mice. *Brain Res*. 999:53–61.
48. Iwasaki K, *et al.* (2007) Nilvadipine prevents the impairment of spatial memory induced by cerebral ischemia combined with beta-amyloid in rats. *Biol. Pharm. Bull.* 30:698–701.
49. Kennelly S, *et al.* APOE ε4 specific short-term cognitive benefits of nilvadipine intervention in Alzheimer's patients in an open-label trial. *Ther. Adv. Neurol. Disord.* Accepted July 2010.
50. Iwata N, *et al.* (2000) Identification of the major Abeta1–42-degrading catabolic pathway in brain parenchyma: suppression leads to biochemical and pathological deposition. *Nat. Med.* 6:143–50.
51. Monro OR, *et al.* (2002) Substitution at codon 22 reduces clearance of Alzheimer's amyloid-beta peptide from the cerebrospinal fluid and prevents its transport from the central nervous system into blood. *Neurobiol. Aging*. 23:405–12.
52. Deane R, *et al.* (2004) LRP / amyloid beta-peptide interaction mediates differential brain efflux of Abeta isoforms. *Neuron*. 43:333–44.
53. Bell RD, Zlokovic BV. (2009) Neurovascular mechanisms and blood-brain barrier disorder in Alzheimer's disease. *Acta Neuropathol.* 118:103–13.
54. Deane R, Bell RD, Sagare A, Zlokovic BV. (2009) Clearance of amyloid-beta peptide across the blood-brain barrier: implication for therapies in Alzheimer's disease. *CNS Neurol. Disord. Drug Targets*. 8:16–30.
55. Zlokovic BV, Yamada S, Holtzman D, Ghiso J, Frangione B. (2000) Clearance of amyloid beta-peptide from brain: transport or metabolism? *Nat. Med.* 6:718–9.
56. Mawuenyega KG, *et al.* (2010) Decreased clearance of CNS beta-amyloid in Alzheimer's disease. *Science*. 330:1774.
57. Bateman RJ, *et al.* (2006) Human amyloid-beta synthesis and clearance rates as measured in cerebrospinal fluid in vivo. *Nat. Med.* 12:856–61.