

ORIGINAL ARTICLE

Intraperitoneal injection of the pancreatic peptide amylin potently reduces behavioral impairment and brain amyloid pathology in murine models of Alzheimer's disease

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Amylin, a pancreatic peptide, and amyloid-beta peptides (A β), a major component of Alzheimer's disease (AD) brain, share similar β -sheet secondary structures, but it is not known whether pancreatic amylin affects amyloid pathogenesis in the AD brain. Using AD mouse models, we investigated the effects of amylin and its clinical analog, pramlintide, on AD pathogenesis. Surprisingly, chronic intraperitoneal (i.p.) injection of AD animals with either amylin or pramlintide reduces the amyloid burden as well as lowers the concentrations of A β in the brain. These treatments significantly improve their learning and memory assessed by two behavioral tests, Y maze and Morris water maze. Both amylin and pramlintide treatments increase the concentrations of A β 1-42 in cerebral spinal fluid (CSF). A single i.p. injection of either peptide also induces a surge of A β in the serum, the magnitude of which is proportionate to the amount of A β in brain tissue. One intracerebroventricular injection of amylin induces a more significant surge in serum A β than one i.p. injection of the peptide. In 330 human plasma samples, a positive association between amylin and A β 1-42 as well as A β 1-40 is found only in patients with AD or amnesic mild cognitive impairment. As amylin readily crosses the blood–brain barrier, our study demonstrates that peripheral amylin's action on the central nervous system results in translocation of A β from the brain into the CSF and blood that could be an explanation for a positive relationship between amylin and A β in blood. As naturally occurring amylin may play a role in regulating A β in brain, amylin class peptides may provide a new avenue for both treatment and diagnosis of AD.

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INTRODUCTION

Amylin is a short peptide of 37 amino acids produced and secreted by the pancreas. It passes through the blood–brain barrier (BBB) easily^{1,2} and mediates brain functions, including inhibiting the appetite to improve glucose metabolism,³ relaxing cerebrovascular structure^{4,5} and probably enhancing neural regeneration.⁶ Despite the fact that amylin is a natural peptide in the body with physiological functions, it could aggregate in the pancreas in type 2 diabetes.⁷ Thus, an amylin analog pramlintide was developed, and it serves as an effective drug in clinical use for diabetes.^{8,9} Pramlintide contains 3 amino-acid differences from amylin, so does not aggregate like amylin, but it mediates all of amylin's functions in the brain.

Amylin and amyloid-beta peptide (A β), the major component of brain Alzheimer's disease (AD) pathology, share several features, including having similar β -sheet secondary structures,¹⁰ binding to the same amylin receptor¹¹ and being degraded by the same protease, insulin-degrading enzyme.^{12–14} Interestingly, several studies show that monomeric amylin and its analogs inhibit the formation of A β aggregation *in vitro*.^{15–19} A recent study found an accumulation of amylin amyloid in the cerebrovascular system in the AD brain,²⁰ whereas abundant A β in the AD brain may block

the ability of amylin to bind its receptor to hinder normal amylin functions that are essential for the brain.³ All these prompted us to hypothesize that exogenous amylin class peptides would influence amyloid pathology in the AD brain. Because of their effects on glucose metabolism, cerebral vasculature, and possible neuroregeneration, the influence of amylin class peptides could be positive, especially using unamyloidogenic pramlintide. Using AD murine models, we investigated the effects of amylin and its clinical analog on A β amyloid pathogenesis in AD.

METHODS

Mice and experimental treatments

5XFAD mice, which are APP/PS1 double transgenic mice with five familial AD mutations,²¹ were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Tg2576 (APPsw, K670N/M671L) transgenic mice²² were originally purchased from Taconic (Hudson, NY, USA) and were maintained on a B6SJL/F1/J background at the Boston VA animal facility before this study. Dutch amyloid precursor protein (APP) mice, which are APP transgenic mice carrying the Dutch mutation (E693Q),²³ were generated by Dr Hui Zheng's laboratory. Non-transgenic B6SJL/F1/J wild type were used as control mice. For this study, all mice were maintained in microisolator

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cages in the Animal Facilities of Boston University School of Medicine, Boston, MA, USA.

Amylin was purchased from AnaSpec (Fremont, CA, USA), and pramlintide was from Amylin Pharmaceutical (San Diego, CA, USA). Experimental groups of animals were matched for age, gender and weight. Female 5XFAD mice aged 3.5 months, female Tg2576 mice aged 9 months and Dutch APP mice aged 12 months were used. For the chronic treatment experiments, mice were treated with intraperitoneal (i.p.) injection of amylin ($200 \mu\text{g kg}^{-1}$) (5XFAD $n=20$; Tg2576 $n=8$) or pramlintide ($200 \mu\text{g kg}^{-1}$) (Tg2576 $n=8$) vs phosphate-buffered saline (PBS; 5XFAD $n=20$; Tg2576 $n=8$) once daily for 10 weeks. For the challenge experiments, the mice received a single i.p. injection of amylin ($200 \mu\text{g kg}^{-1}$) (5XFAD $n=8$; Tg2576 $n=20$; Dutch APP $n=6$) or pramlintide ($200 \mu\text{g kg}^{-1}$) (Tg2576, $n=8$) vs PBS (5XFAD, $n=8$; Tg2576, $n=20$; Dutch APP, $n=6$) or intracerebroventricular (i.c.v.) injection of amylin ($200 \mu\text{g kg}^{-1}$) (5XFAD, $n=6$). For i.c.v. injection, the animals were under anesthesia to receive the procedure described previously.²⁴ A 1-mm burr hole was drilled at a stereotactically defined ventricular location (1 mm caudal to the bregma, 1.3 mm lateral to the midline and at 2 mm depth to the pia surface), and a pulled glass pipette mounted onto a Nanoject II injector (Drummond Scientific Company, Broomall, PA, USA) was used to inject PBS or amylin. Blood draws were conducted before and after the injection to isolate serum. To collect cerebral spinal fluid (CSF), the cisterna magna was exposed under anesthesia in these mice and a pulled glass micro-pipette was used to do lumbar puncture to obtain CSF (<http://www.jove.com/video/960/a-technique-for-serial-collection-cerebrospinal-fluid-from-cisterna>). Generally, 10–20 μl of CSF were collected from each procedure. All animal procedures were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Boston University Animal Care and Use Committee.

Analysis of amyloid burden in brain

Brains of the mice were removed, post-fixed in 4% paraformaldehyde for 4 h, treated with 30% sucrose (in PBS) for 24 h and then embedded in Tis-sue-Tek optimal cutting temperature. Serial coronal cryosections (8–10 μm) were cut and stored at 20 °C. After air drying and washing with PBS, quenching of endogenous peroxidase activity was performed by incubating the sections for 30 min in 0.3% H_2O_2 in methanol followed by PBS washes. Slides were preincubated in blocking solution (5% (vol/vol) goat serum (Sigma, St Louis, MO, USA) and 0.3% (vol/vol) Triton-X100 in PBS) for 1 h at room temperature, followed by mouse-on-mouse blocking reagent (Vector Labs, Inc., Burlingame, CA, USA, MKB-2213) incubation for 1 h. Primary antibody for the amyloid beta (mouse mAb anti-6E10, SIG-39320, 1:300, Covance, Princeton, NJ, USA) was incubated overnight. Secondary antibodies used were biotinylated mouse (Vector Labs, Inc.; 1:500) for immunohistochemistry. Immunobinding of primary antibodies was detected by biotin-conjugated secondary antibodies and Vectastain ABC kit (Vector Labs, Inc.) using DAB (3,3'-diaminobenzidine; Vector Labs, Inc.) as a substrate for peroxidase and counter-staining with hematoxylin.

End products were visualized as eight-bit RBG images using the NIS Elements BR 3.2 software (<http://www.nikoninstruments.com/Products/Software/NIS-Elements-Br-Microscope-Imaging-Software>) at a total magnification of $\times 40$. For analysis of the amyloid burden, the ImageJ software (<http://rsbweb.nih.gov/ij/>) was used. After adjusting for threshold, ImageJ was used to count all plaques and to measure the area taken up by plaques, average size of the plaques and the percentage of total brain area occupied by plaques. The intensity \times size values of the plaques were measured by analyzing average raw gray levels of the plaques in the image, normalizing them to the background of the slide not taken up by brain and multiplying by the average size of the plaques in the image. The brain area (cortex or hippocampus or thalamus) was outlined using the edit plane function. The number of plaques in the outlined structure was also recorded. Data were pooled from three independent readers who were blind to the treatment for all three sections and averaged. The outcomes of amyloid burden in the brain between treatment and controls were compared by using the Student's *t*-test.

Characterizing APP processing and measurements of A β levels

Brain proteins were extracted by TBS-X or extracted and fractionated into three parts: (1) TBS-soluble fraction, (2) TBS-X fraction, and (3) formic acid-soluble fraction, as described.²⁵ The presence of APP processing was visualized by using western blots with human APP-specific antibody, 6E10, after fractionating brain proteins and using reduced SDS-PAGE (sodium

dodecyl sulfate-polyacrylamide gel electrophoresis). The amounts of full-length APP and C99 fragments from the TBS-X soluble fraction and of secreted APP (sAPP) from the TBS-soluble fraction were also quantitated and compared using *t*-tests.

Cerebral A β levels were assayed in the total protein by TBS-X extraction of hemi-brain sucrose homogenates. The A β levels in serum, plasma and CSF were assayed after the samples were collected and centrifuged. We used an enzyme-linked immunosorbent assay method^{26,27} in which A β was trapped with either monoclonal antibody to the C-terminal of A β 1–40 (2G3) or A β 1–42 (21F12) and then detected with biotin-conjugated to the N-terminus of A β . A dilution of 6E10 was optimized to detect A β in the range of 50–800 pg protein in the brain samples. The dilution of blood and CSF samples was between twofold and fivefold; the lowest detection level for each A β peptide in blood was 1.6 pg ml⁻¹ determined from a standard curve. Streptavidin-conjugated alkaline phosphatase (Promega, Madison, WI, USA) was added and incubated, and the signal was amplified by adding alkaline phosphatase fluorescent substrate (Promega), which was then measured.

Behavioral tests and data analysis

All behavioral experiments were done blind with respect to the treatment and the genotype of the mice. Behavioral data were analyzed using *t*-tests to determine the significance of differences.

Y maze test with spontaneous alternation performance²¹ ($n=10$ per group) was performed three times with different groups of 5XFAD, Tg2576 and control mice after the treatments. Each mouse was placed in the center of the symmetrical Y maze and was allowed to explore freely through the maze during a 5-min session. The sequence and total number of arms entered were recorded. Percentage of alternation was determined as follows: number of trials containing entries into all three arms/maximum possible alternations $\times 100$. The maximum possible alterations = total number of arms entered – 2.

Morris water maze test with spatial learning and memory performance²⁸ was used with 5XFAD mice ($n=10$ per group) after the completion of treatment. All mice underwent reference memory training with a hidden platform in one quadrant of the pool for 10 days with four trials per day. After the last trial of day 10, the platform was removed, and each mouse received one 60-s swim probe trial. Escape latency (in seconds) is reported in Figure 1. Other indices, including length of swim path, swim speed, percentage of time in the outer zone and percentage of time and path in each quadrant of the pool were also recorded using an HVS image video tracking system (Reston, VA, USA).

In vitro BACE1 activity assay

BACE1 activity was measured by incubating recombinant BACE1 with a 9mer substrate (DabcyI-SEVNLDAEF-Edans). In brief, 0.2 μg of recombinant BACE1 was pre-incubated for 5 min in the presence of PBS, a known BACE1 inhibitor,²⁹ or amylin or pramlintide in BACE1 activity assay buffer (50 mM NaOAc (pH 4.5), 1 mg ml⁻¹ BSA, 15 mM EDTA (pH 4.5), 0.8% CHAPS). Then the substrate at 5 μM was added to the reaction, and samples were incubated for 2 h at 37 °C. Cleavage of BACE1 substrate was measured in fluorescence intensities (excitation at 340 nm and emission at 492 nm) using EnVision Multilabel Reader (PerkinElmer, Boston, MA, USA). A specific BACE1 activity was calculated after subtracting the assay background. For measuring IC50 of amylin, different concentrations of amylin were incubated with recombinant BACE1, and BACE1 activity was measured as described above.

Human study

The plasma samples from the Nutrition, Aging and Memory in the Elderly (NAME) study²⁷ were used for this study. The protocol and consent form were approved by the Institutional Review Board of Tufts University New England Medical Center. The measurements of plasma A β 1–42 and A β 1–40 are described above. An enzyme-linked immunosorbent assay was used to measure plasma amylin according to the manufacturer's instructions (LINCO Research, St Charles, MO, USA).

Diagnosis of dementia. The diagnosis of dementia was based on the DSM-IV (Diagnostic and Statistical Manual of Mental Disorders, fourth edition) criteria. NINCDS-ADRDA guidelines³⁰ were used to determine whether criteria were met for a diagnosis of possible or probable AD.

Diagnosis of amnesic mild cognitive impairment (MCI). The diagnostic criteria for MCI were based on Petersen *et al.*³¹ 1999 guidelines, with some

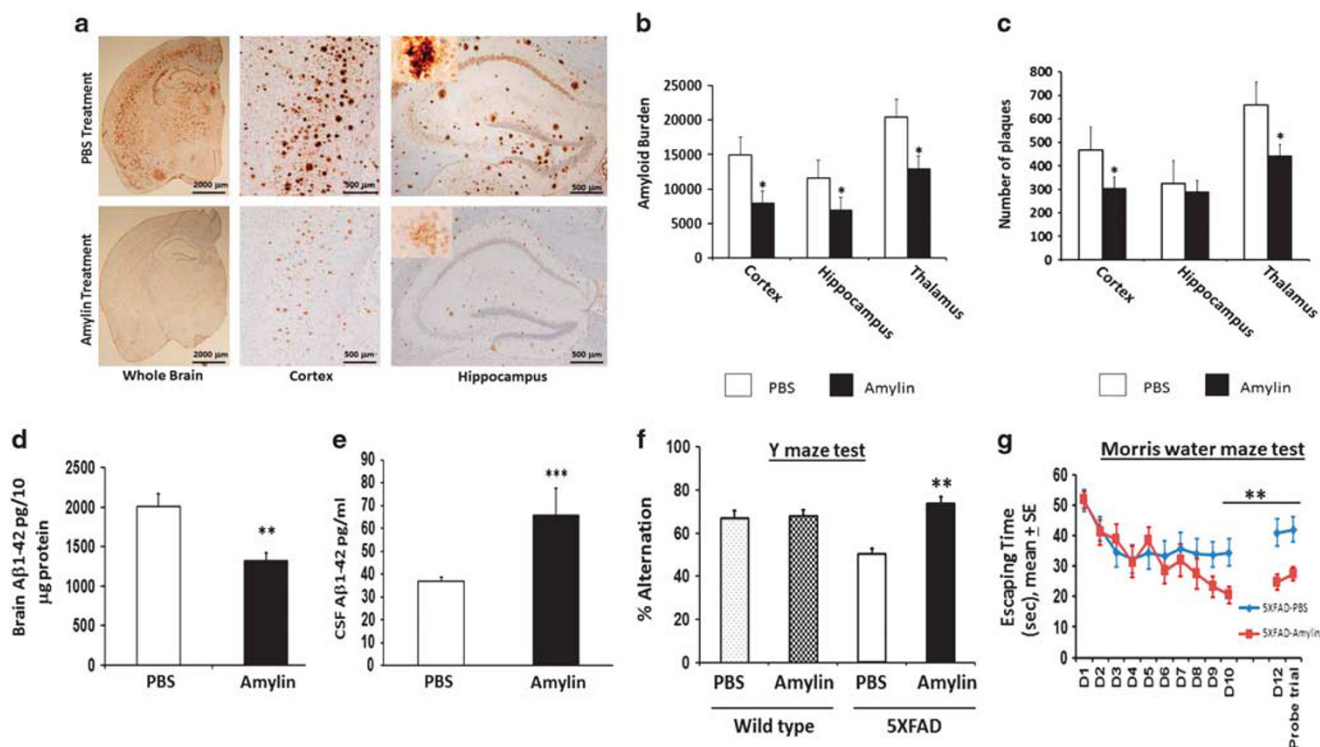


Figure 1. Amylin treatment of 5XFAD mice reduces the amyloid burden and improves their learning and memory. At 3.5 months of age, 5XFAD mice were treated by intraperitoneal injection of phosphate-buffered saline (PBS) or amylin ($200 \mu\text{g kg}^{-1}$) daily for 10 weeks ($n = 10$ per group) (Supplementary Table S1). **(a)** Dense-cored A β plaque burden is reduced in the whole brain, including the cerebral cortex and the hippocampus. **(b)** Amylin-treated mice had a significant reduction in A β amyloid burden in the cortex, hippocampus and thalamus quantitated by average amyloid intensity \times plaque size. **(c)** Compared with PBS treatment, the amylin-treated mice also had fewer plaques in the cortex and thalamus, while the hippocampus region only showed a tendency. Compared with the PBS treatment, the amylin-treated mice had lower concentration of A β 1-42 (pg per 10 μg brain protein) in the brain **(d)** ($P = 0.005$) but had higher concentrations of A β 1-42 in CSF (pg ml^{-1}) **(e)** ($P = 0.04$). The amylin-treated 5XFAD mice illustrated improved cognition by showing increased percentage of alternation in the Y maze test **(f)** ($P = 0.001$) and by showing shortened times in Morris water maze test **(g)** in finding the hidden platform at day 10 (D10) ($P = 0.005$), in memory at day 12 (D12) after the completion of training and skipping day 11 ($P = 0.002$) and in the probe trial ($P = 0.03$). Mean \pm s.e. was used with * $P < 0.0001$; ** $P < 0.01$; *** $P < 0.05$.

modifications to broaden the concept of MCI. Those with memory impairment only or cognitive impairment including memory and other domains were considered to have amnesic MCI; those without forgetfulness and with impairments in other cognitive domains, such as executive and visuospatial dysfunction, were considered to have non-amnesic MCI.

Definition of the controls. Subjects were considered cognitively intact if they were not demented and scored no more than 1 s.d. below the mean of age- and education-defined strata on MMSE (mini mental state exam) and no more than 1.5 s.d. below the mean of age- and education-defined strata on the neuropsychological tests.

Statistical analysis. Statistical analysis was performed using SAS (version 9.1, Middleton, MA, USA). Normally distributed variables such as age and MMSE scores were presented as mean \pm s.d. and were compared using analysis of variance. Amylin, A β 1-42 and A β 1-40 were transformed to Log10 for association analyses owing to their skewed distributions. Univariate analyses were performed to determine the correlation coefficients between amylin and A β 1-42 or A β 1-40. Multivariate linear regression was performed to evaluate the associations between amylin and A β 1-42 or A β 1-40 after adjusting for potential confounders.

RESULTS

Treatment with amylin or its analog reduces the AD pathology. We treated APP transgenic mice 5XFAD, which have abundant A β 1-42 but little or no A β 1-40 in the brain,²¹ by i.p. injection of amylin ($200 \mu\text{g kg}^{-1}$) once daily for 10 weeks ($n = 10$ in each

group) (Figure 1 and Supplementary Table S1). Compared with controls, amylin treatment significantly reduced the amyloid pathology in the cortex, hippocampus and thalamus (Figure 1a). Amylin-treated mice had the reduction in both the size and intensity of amyloid plaques in these brain regions ($P < 0.0001$; Figure 1b) and had decreased numbers of amyloid plaques wherever measured, except for such a tendency in the hippocampus (Figure 1c). Measurements of A β indicated that the amylin treatment also reduced the level of A β 1-42 in brain tissue ($P = 0.005$; Figure 1d) but increased the level of A β 1-42 in CSF ($P = 0.04$; Figure 1e).

To validate the amylin specificity of this effect, we used either amylin or pramlintide to treat another AD mouse line, Tg2576²² (Figure 2 and Supplementary Table S2). Unlike 5XFAD mice, Tg2576 mice generate both A β 1-42 and A β 1-40 in the brain. Amylin and pramlintide treatments had similar effects in reducing A β 1-42 and A β 1-40 in the brain (Figure 2a). Although both amylin and pramlintide treatment effectively increased A β 1-42 in CSF, neither affected A β 1-40 in CSF to a statistically significant level (Figure 2b).

Treatment with amylin or its analog improves learning and memory

To evaluate the effect of amylin treatment on learning and memory, we conducted Y maze and Morris water maze behavioral tests in these mice. We found that amylin treatment significantly

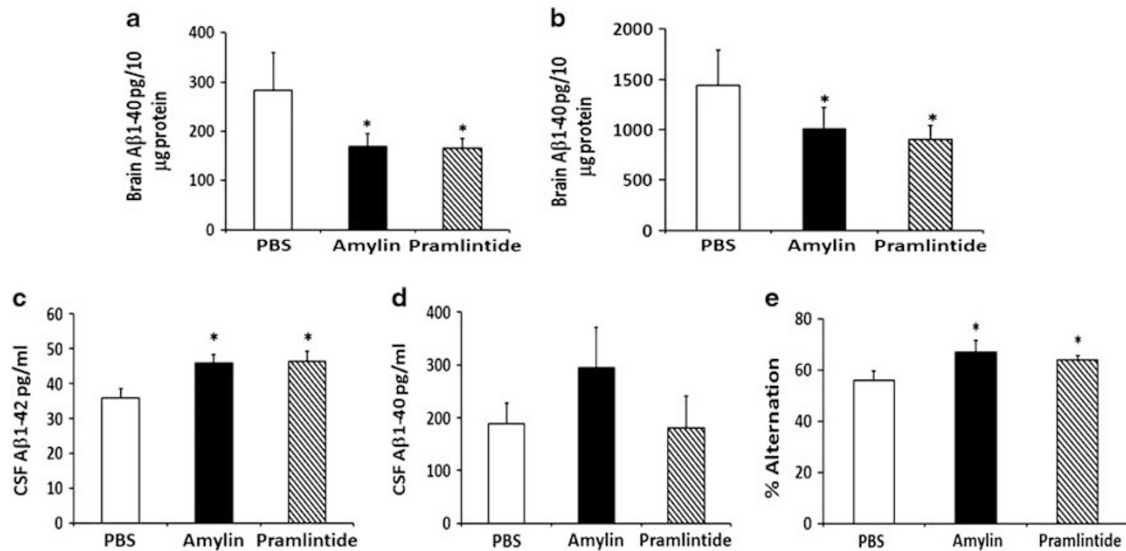


Figure 2. Treatment of Tg2576 mice with amylin or its analog pramlintide reduces the amyloid burden and improves their learning and memory. At 9 months of age, Tg2576 mice were treated with intraperitoneal injection of phosphate-buffered saline (PBS), amylin ($200 \mu\text{g kg}^{-1}$) or pramlintide ($200 \mu\text{g kg}^{-1}$) daily for 10 weeks ($n=8$ per group) (Supplementary Table S2). Both amylin- and pramlintide-treated mice had reduced concentrations of (a) Aβ1-42 (measured as pg per $10 \mu\text{g}$ brain protein) and (b) Aβ1-40 in the brain compared with PBS-treated controls. (c) Both amylin- and pramlintide-treated mice had increased concentrations of Aβ1-42 (pg ml^{-1}) in CSF, but (d) neither treatment significantly affected the concentration of Aβ1-40. (e) Compared with PBS treatment, both amylin- and pramlintide-treated mice exhibited improved cognition by showing increased percentage of alternation in the Y maze test. Mean \pm s.e. was used with * $P < 0.05$.

increased the average percentage of alternation in the Y maze test in 5XFAD mice ($P=0.0001$) but not in control mice (Figure 1f). In the Morris water maze test, amylin treatment reduced the time for acquisition during maze training ($P=0.01$) and shortened the time for memory retention ($P=0.007$) and for probe trial ($P=0.02$) 2 days after maze training (Figure 1g). And in Tg2576 mice, both amylin and pramlintide treatments equally improved the performance of mice in the Y maze test (Figure 2c). With two different behavioral tests in two different AD mouse models, these data demonstrate that both peripheral treatments with either amylin or pramlintide improved learning and memory in these mice.

Amylin and pramlintide enhance the removal of Aβ out of the brain

To elucidate the mechanism by which amylin class peptides reduce the amyloid pathology in the brain but increased the concentrations of Aβ1-42 in CSF (Figures 1 and 2), we first tested the possibility that amylin class peptides stimulate the removal of the toxic Aβ from the AD brain to another compartment of the body. We measured Aβ levels in serum before and after i.p. injection of a single dose of amylin or pramlintide into the AD mice. A single dose of amylin in Tg2576 mice, but not in control mice, produced a significant increase in serum Aβ1-40 and Aβ1-42 at different time points (Supplementary Figure 1) up to 24 h (Figure 3a). In contrast to amylin, injection of PBS did not induce the Aβ surge in blood in Tg2576 mice, indicating that the increase of serum Aβ was provoked by the injection of amylin (Figure 3a). Consistently, a single i.p. injection of pramlintide also provoked surges of Aβ1-40 and Aβ1-42 in serum similar to those induced by amylin (Figure 3b).

To further determine that the surge of Aβ in serum provoked by amylin challenge reflected the Aβ pathology in the brain, we repeated the experiment using 5XFAD mice, which have abundant Aβ1-42 but little or no Aβ1-40 in the brain. We found that a single i.p. injection of amylin into 5XFAD mice resulted in an increased

level of Aβ1-42 but not of Aβ1-40 in serum (Figure 3c). We then assessed the response in another APP mouse line, which carries the knock-in Dutch mutation of APP in the context of Swedish and London mutations and mainly produces Aβ1-40 but little Aβ1-42 in the brain. Amylin challenge in these mice provoked a surge of Aβ1-40 but not of Aβ1-42 in the blood (Figure 3d). Using 5XFAD mice of different ages, we found that peripheral amylin injection resulted in increases in serum Aβ levels that were proportionate to the levels of Aβ in the brain (Figure 4a).

Despite the fact that all of the APP transgenic mice studied here express substantial APP in the brain, they probably express little APP in peripheral tissues. To confirm that the source of the Aβ surge in serum is the brain, and is mediated by the effects of amylin on the brain, we assessed the surge of Aβ in serum following a single injection of amylin into the brain ventricles of these APP mice. I.c.v. injection of amylin ($200 \mu\text{g kg}^{-1}$) provoked a much greater surge of Aβ in serum than a peripheral i.p. injection of amylin ($200 \mu\text{g kg}^{-1}$) in the same APP mice (Figure 4b). These data suggest that because peripherally administered amylin or pramlintide must cross the BBB to influence the amyloid pathology in the brain, the effect of i.p. injection was not as pronounced as that produced by i.c.v. injection.

Amylin inhibits BACE1 activity

We next examined the effects of amylin on APP expression and processing in the brain of Tg2576 mice. There were no observable differences between the control and treatment groups in the expression levels of APP mRNA (Supplementary Figure S2) or in the levels of full-length APP protein or in the levels of one form of secreted APP (sAPPα) in brain extracts (Figure 5a). However, compared with controls, mice treated with amylin had lower levels of the APP processing fragments C99³² (Figures 5a and 5b). The β-site APP cleaving enzyme (BACE1) is the key protease initiating the generation of C99 by cleaving APP leading to further production of Aβ.^{33,34} In an *in vitro* assay using purified BACE1, we found

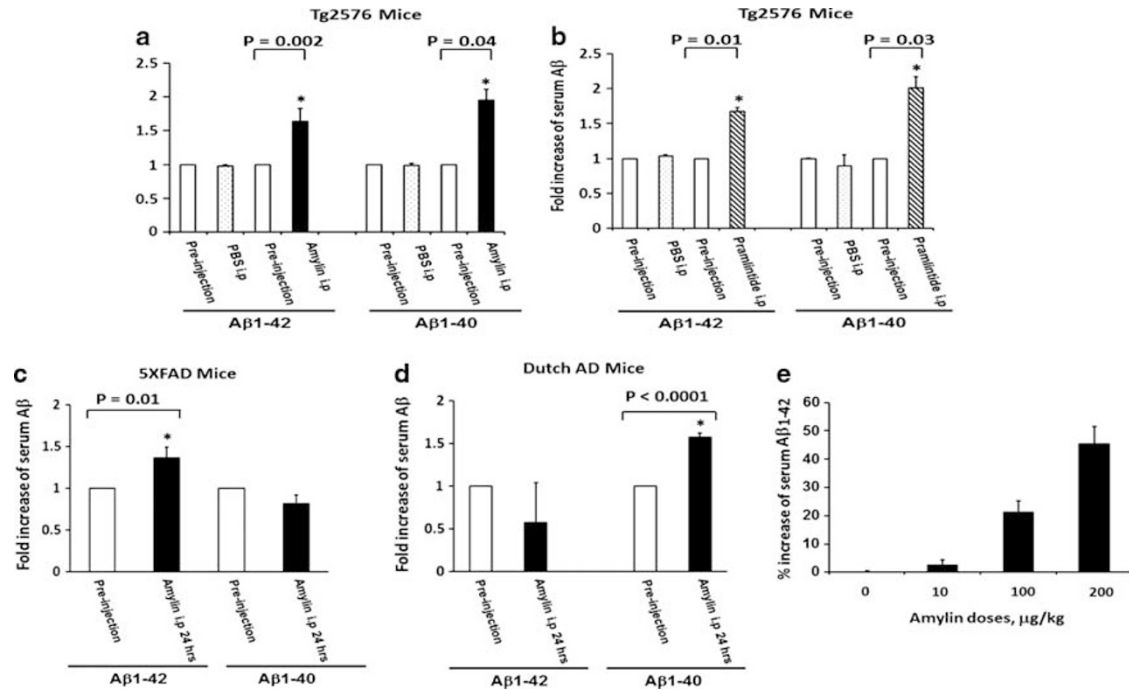


Figure 3. A single injection of amylin or pramlintide induces a change of serum A β in the amyloid precursor protein (APP) transgenic mice. After one intraperitoneal (i.p.) injection of phosphate-buffered saline vs amylin (a) or pramlintide (b) ($200 \mu\text{g kg}^{-1}$) in Tg2576 mice, changes in levels of serum A β 1-42 and A β 1-40 were observed. A single i.p. injection of amylin induced an increase in serum A β 1-42 but not A β 1-40 in 5XFAD mice (c); in contrast, i.p. injection of amylin induced an increase in serum A β 1-40 but not A β 1-42 in Dutch APP mice (d). In 5XFAD mice, the changes in serum A β 1-42 in response to a challenge with amylin were dose dependent (e). Significance of changes in A β levels before and after the injection was determined by Student's *t*-test. * $P < 0.05$.

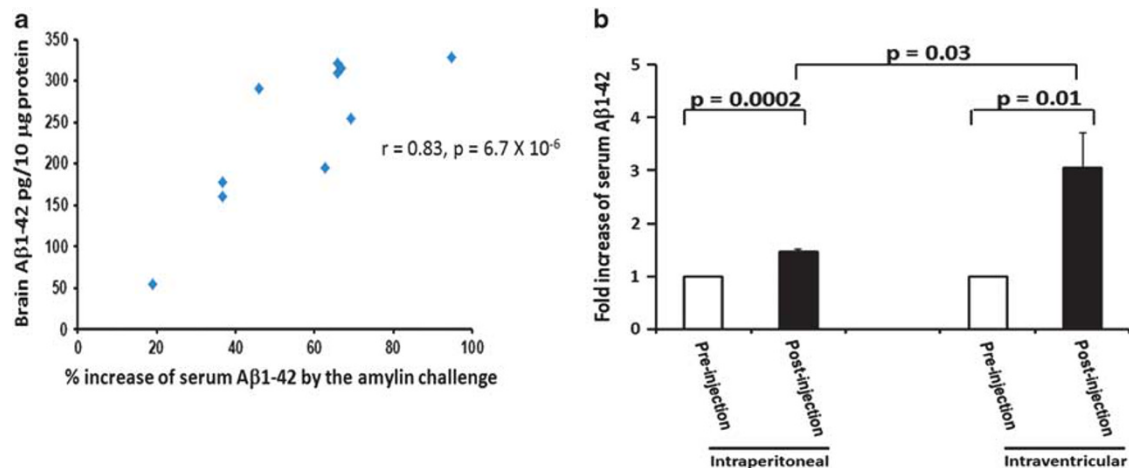


Figure 4. Change in serum A β levels induced by amylin challenge and A β pathology in the brain. Serum samples were isolated before and after 5XFAD mice, which were at different ages, were challenged by a single intraperitoneal (i.p.) injection of amylin. The mice were killed immediately after the amylin challenge procedure, and the brain protein was extracted. The percentage of changes in serum A β 1-42 induced by a single i.p. injection of amylin significantly correlated with the amounts of A β 1-42 in their brains ($r = 0.83$, $P = 6.7 \times 10^{-6}$) (a). Amylin-induced serum A β 1-42 changes induced by i.p. injection or intracerebroventricular (i.c.v.) injection in the 5XFAD mice were compared (b). Higher levels of increased serum A β 1-42 induced by i.c.v. injection of amylin than by i.p. injection of amylin were observed ($P = 0.03$) in these mice.

that amylin significantly inhibited BACE1 activity (70%) in a dose-dependent manner (Figures 5c and 5d). The IC₅₀ for BACE1 inhibition by amylin was $6.9 \mu\text{M}$. Surprisingly, pramlintide, which only has 3 amino-acid differences from amylin, showed little or no ability to inhibit BACE1 activity in this assay and did not reduce

C99 levels compared with saline-treated controls (data not shown). As pramlintide treatment reduced A β in the brain to the same extent as amylin treatment (Figure 2), these data suggest that inhibition of BACE1 is not the major mechanism by which amylin class peptides reduce the A β burden in the brain.

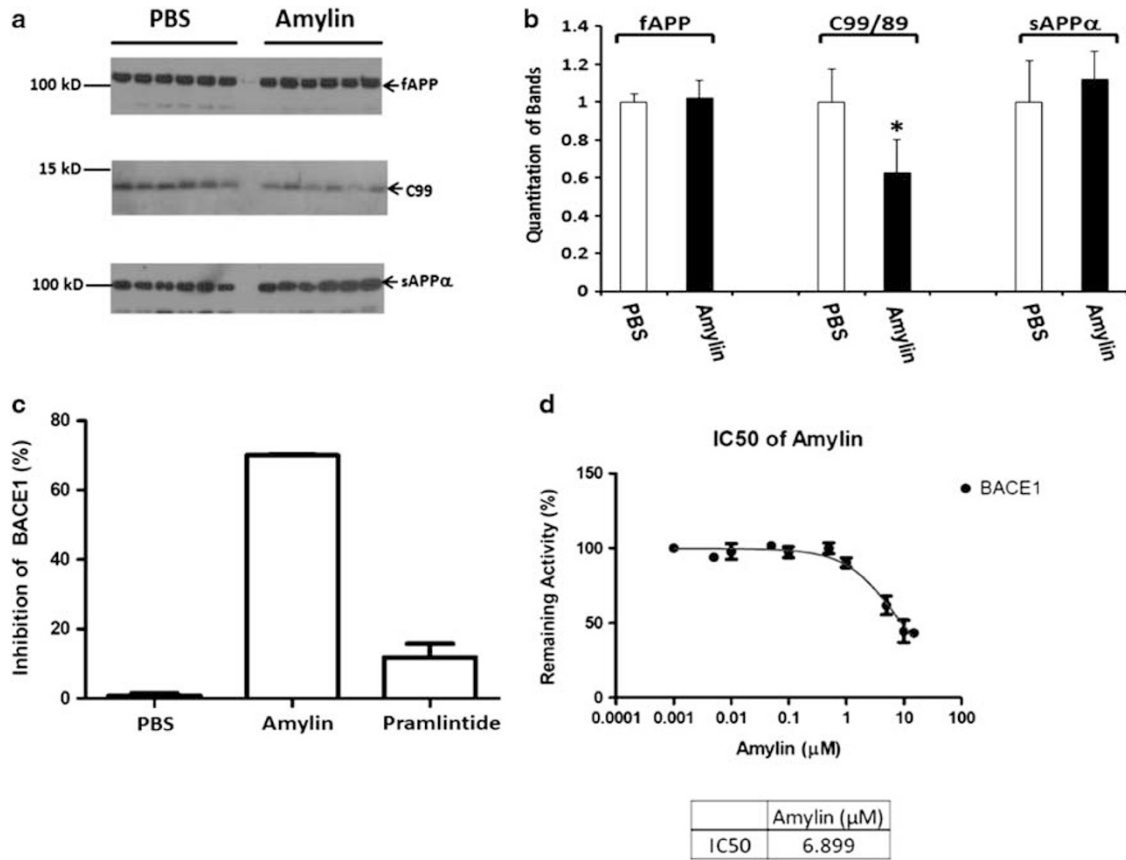


Figure 5. Characterization of the effect of amylin on amyloid precursor protein (APP) processing and BACE1. (a) Detection of full-length APP (fAPP) and APP processed products by 6E10 antibody in western blotting in brain homogenates from Tg2576 mice treated with phosphate-buffered saline (PBS) or amylin ($n=6$ per group), and (b) quantitation of the results (mean \pm s.e.) are shown. Although there were no differences in the levels fAPP and secreted APP cleaved by α secretase (sAPP α) between PBS control and the amylin treatment, there was an increase in C99 fragment from APP cleaved by BACE1 in the brains treated with amylin compared with PBS treatment (* $P=0.004$). (c) *In vitro* assay using purified BACE1 showed that amylin significantly inhibited BACE1 activity, but pramlintide had little inhibition for BACE1. The concentration of amylin necessary to inhibit 50% BACE1 activity (IC50) in this assay is 6.9 μ M (d).

Table 1. Correlations between A β and amylin in plasma in humans

Diagnoses	Controls, N = 145	Amnestic, MCI N = 16	Alzheimer's disease, N = 42
Age, year, mean \pm s.d.*	72.3 \pm 8.0	75.7 \pm 8.7	80.5 \pm 8.1
MMSE, mean \pm s.d.*	27.1 \pm 2.6	26.4 \pm 2.5	22.2 \pm 3.3
Log10 amylin with Log10 A β 1-42	$r=+0.06$, $P=0.46$	$r=+0.73$, $P=0.001$	$r=+0.52$, $P=0.0004$
Log10 amylin with Log10 A β 1-40	$r=+0.02$, $P=0.83$	$r=+0.58$, $P=0.02$	$r=+0.29$, $P=0.06$

Abbreviations: MCI, mild cognitive impairment; MMSE, Mini Mental State Exam. Using analysis of variance, age and average MMSE scores in the subgroups of the controls, amnestic MCI and Alzheimer's disease are compared. * $P < 0.0001$. Pearson's analyses were performed to determine the correlation coefficient between plasma A β 40 or A β 42 and amylin in different subgroups: the controls, amnestic MCI and Alzheimer's disease. P -values for statistical significance are shown.

The association between amylin and A β in human plasma
If these observations made in mice that exogenously and peripherally added amylin removed A β out of the brain into the blood causing increased levels of both amylin and A β in blood were implied to the naturally occurring amylin and applicable to human AD patients, we should be able to see a positive association between amylin and A β in blood in humans. We assessed amylin and A β levels in plasma samples from a human study with diagnoses³⁵ and did find that amylin was positively

associated with A β 1-42 as well as A β 1-40 in plasma after adjusting for the confounders (Supplementary Table S3). After dividing the subjects into subgroups based on the diagnoses and using univariate analysis, we found that plasma amylin was associated with A β 1-42 ($r=+0.52$, $P=0.0004$) and only tended to be associated with A β 1-40 ($r=+0.29$, $P=0.06$) among AD subjects ($n=42$) (Table 1). The correlations between plasma amylin and A β 1-42 ($r=+0.73$, $P=0.001$) or A β 1-40 ($r=+0.58$, $P=0.02$) were stronger among the elderly with amnestic MCI ($n=16$), a

prodromal stage of AD. In contrast, there was no correlation between plasma amylin and either form of A β among the elderly who had normal cognition ($n = 145$). There were no differences in the concentrations of amylin and A β among the three subgroups, and these relationships between amylin and A β were also not found among patients with other types of dementia and non-amnesic MCI (data not shown).

DISCUSSION

These data indicate that long-term peripheral amylin treatment improved learning and memory in the AD mice. Reducing neurotoxic A β species, especially monomers and small oligomers,³⁶ rather than decreasing plaque burden *per se*, is probably more critical for and relevant to cognitive improvement by the treatment of AD in our experiments. Indeed, one study shows that hyper-aggregation of A β is even protective for neurodegeneration.³⁷ In addition, these A β species in the AD brain may block the ability of amylin to bind to its receptor¹¹ and lead to the loss or reduction of amylin's activities, which are essential for the brain.³⁻⁶ Another example of the loss of function in AD is that some early onset AD cases may manifest in cognitive decline as a result of the loss of presenilin function.³⁸ The drug inhibiting presenilin function worsens cognition in AD patients.³⁹

Our study and others argue for a therapeutic application of amylin class peptides for AD through the following mechanism. First, amylin and pramlintide enhance the removal of A β from the brain and its transfer into the blood, probably through their effects on cerebral vasculature as amylin has been shown to improve cerebral vasculature.⁵ Dysfunction of the BBB, decreased cerebral blood flow and impaired vascular clearance of A β from the brain are all thought to contribute to AD pathogenesis.⁴⁰ Solanezumab, an immune drug that also removes A β from the AD brain into blood,⁴¹ has been shown to delay cognitive decline in those who have an early stage of AD.⁴² Induced removal of A β from the brain into the blood by the amylin class peptides also could be used in a challenge test to specifically reflect and diagnose AD pathology in the brain.

Second, despite the fact that BACE is considered to be a prime target for developing the drugs for AD, and that BACE1 knockout in the APP transgenic mice significantly reduce the amyloid burden in the brain⁴³ and are viable,⁴⁴ most peptide-based BACE1 inhibitors failed as drugs for AD owing to their inability to pass through the BBB.⁴⁵ Our study shed some light that amylin, but not pramlintide, is a BACE1 inhibitor crossing the BBB. However, unlike other published BACE1 inhibitors, which decrease A β in CSF and blood,^{46,47} treatment with amylin increased the concentrations of A β 1-42 in CSF and blood. Additionally, although pramlintide was unable to inhibit BACE1, pramlintide equally reduced amyloid pathology in the brain like amylin. These data suggest that enhancing A β removal from the brain and translocation of A β into the blood, rather than inhibition of BACE1, is the major mechanism by which amylin class peptides reduce the A β burden in the brain in our study.

Third, evidence from numerous studies suggests other benefits of amylin class peptides for AD. As brain imaging studies have demonstrated perturbed cerebral glucose metabolism in the AD brain,⁴⁸ glucose metabolism should be a target for drug development for AD. Because of their ability to cross the BBB, both amylin and pramlintide could potentially improve glucose metabolism in the brain.⁴⁹ Additionally, several studies show that amylin and its analogs have an effect to inhibit the formation of A β aggregation and reduces cell death caused by the A β aggregates,¹⁵⁻¹⁹ which is a key element in AD pathogenesis. As in type 2 diabetes large amounts of amylin can aggregate in the pancreas, amylin itself may not be suitable to treat AD in patients who also have diabetes. It is noteworthy that pramlintide does not form aggregates and has become an effective and safe drug for

diabetes.⁷ Our results certainly suggest a therapeutic potential of pramlintide for AD and warrant a clinical trial in AD with off-label use.

When exogenous and synthetic amylin was i.p injected into mice in our experiments, it increased the level of amylin in the blood as expected while also increasing the blood levels of A β through translocating A β out of the brain into the blood. If endogenous pancreatic amylin in the body has the same role in regulating A β in brain, amylin and A β in blood should be positively associated. Thus the finding of a positive association between amylin and A β , especially A β 1-42, in plasma in AD and amnesic MCI patients supports the relevance of our findings in mice. Other drugs targeting A β clearance and removing A β out of the brain were shown to be effective for prodromal or early stage of AD.⁵⁰ However, there is broad agreement that more therapeutic avenues need to be explored in addition to targeting A β for the treatment of AD. Amylin class drugs not only remove A β from the brain, as demonstrated by our study, but can also improve glucose metabolism⁵¹ and cerebrovasculature^{4,5} in the AD brain. Some amylin analogs may be BACE1 inhibitors in the brain. Based on their multiple effects, we propose that the amylin class peptides have potential to become a new avenue as a challenge test for diagnosis of amnesic MCI and AD and as a therapeutic for the disease.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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