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An anti-pyroglutamate-3 A β vaccine reduces plaques and improves cognition in APP^{swe}/PS1 E9 mice

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Abstract

Pyroglutamate-3 amyloid-beta (pGlu-3 A β) is an N-terminally truncated A β isoform likely playing a decisive role in Alzheimer's disease (AD) pathogenesis. Here, we describe a prophylactic passive

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Dr. Jen-Ulrich Rahfeld is a former employee of Probiodrug AG and holds stock options in the company.

Dr. Martin Kleinschmidt is a former employee of Probiodrug AG and holds stock options in the company.

Dr. Inge Lues is a Chief Development Officer and a stockholder of Probiodrug AG.

Dr. Stephan Schilling is a current employee of Probiodrug AG and holds stock options in the company.

Dr. Hans-Ulrich Demuth is the former Chief Scientific Officer of Probiodrug AG, and currently is a chief scientific advisor for Probiodrug AG and a stockholder of the company.

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immunization study in APPswe/PS1 E9 mice using a novel pGlu-3 A β IgG1 monoclonal antibody (mAb), 07/1 (150 and 500 μ g i.p. weekly), and compare its efficacy with a general A β IgG1 mAb, 3A1 (200 μ g i.p. weekly) as a positive control. After 28 weeks of treatment, plaque burden was reduced and cognitive performance of 07/1-immunized Tg mice, especially at the higher dose, was normalized to wild-type (Wt) levels in two hippocampal-dependent tests and partially spared compared to PBS-treated Tg mice. Mice that received 3A1 had reduced plaque burden but showed no cognitive benefit. In contrast to 3A1, treatment with 07/1 did not increase the concentration of A β in plasma, suggesting different modes of A β plaque clearance. In conclusion, early selective targeting of pGlu-3 A β by immunotherapy may be effective in lowering cerebral A β plaque burden and preventing cognitive decline in the clinical setting. Targeting this pathologically-modified form of A β thereby is unlikely to interfere with potential physiologic function(s) of A β that have been proposed.

Keywords

immunotherapy; pyroglutamate-3 amyloid-beta; APPswe/PS1 E9; microhemorrhage; vascular amyloid

1. Introduction

Alzheimer's disease (AD) represents the most common form of dementia and to date, there are no disease-modifying treatments effective in halting or slowing disease progression. The neuropathological hallmarks of AD include cerebral accumulation of amyloid-beta (A β) protein as extracellular deposits called plaques and intracellular inclusions of hyperphosphorylated tau termed neurofibrillary tangles (NFT). Formulated from both genetic and neuropathological analyses, the central principle of AD pathogenesis has focused on the accumulation of cerebral A β , which is summarized in the amyloid cascade hypothesis (Hardy and Selkoe, 2002). A β is generated in the amyloidogenic pathway from sequential cleavages of the amyloid precursor protein by two endopeptidases, β - and γ -secretase (Esler and Wolfe, 2001). In addition to C-terminal heterogeneity of A β species, e.g. A β (1-40) and A β (1-42), N-terminally truncated and posttranslationally generated variants are also observed in the forms of pyroglutamate-3 and pyroglutamate -11 (pGlu-3 and pGlu-11) A β (Sullivan et al., 2011).

Seminal work conducted by Saido and colleagues demonstrated that pGlu-3 A β was deposited in equal or greater abundance than full-length A β species starting at Asp1 in AD and Down syndrome (DS) brain tissues, which suggests that pGlu-3 A β 's aberrant accumulation in the brain may play an early and decisive role in AD etiology (Saido et al., 1995). Soon thereafter, others confirmed that pGlu-3 A β is present in diffuse and compacted plaques as well as cerebral amyloid angiopathy in both AD and DS brain, thus confirming this particular isoform as a disease-relevant A β species (Frost et al., 2013, Lemere et al., 1996, Miravalle et al., 2005, Piccini et al., 2005, Tekirian et al., 1998). Several groups have characterized the cerebral accumulation of pGlu-3 A β in a few mammalian models of sporadic cerebral amyloidosis, including aged beagle canines, non-human primates and polar bears (Frost et al., 2013, Hartig et al., 2010, Tekirian et al., 1998). Moreover,

examination of numerous AD-like transgenic mouse models revealed varying amounts of pGlu-3 A β peptides compared to general A β species, which differed greatly for age of onset, cortical brain region and overall abundance; however, general A β preceded pGlu-3 A β deposition in mouse models and the latter was observed in only a subset of diffuse, focal and vascular deposits (Frost et al., 2013, Guntert et al., 2006, Hartlage-Rubsamen et al., 2011, Jawhar et al., 2012, Mandler et al., 2012, Schieb et al., 2011, Wirths et al., 2010a).

The formation of pGlu-3 A β occurs by enzymatic truncation of the first two amino acids of full-length A β by unknown aminopeptidases, followed by cyclization of the free glutamic acid residue at position 3 by glutaminyl cyclase (QC or isoQC) to generate pGlu at the A β N-terminus (Cynis et al., 2008, Schilling et al., 2004, Schilling et al., 2008). This posttranslational modification has been shown to increase A β cytotoxicity in neuron and astrocyte cultures compared to non-pyroglutamatized A β species (Nussbaum et al., 2012, Russo et al., 2002). In agreement with this *in vitro* evidence, overexpression of pGlu-3 A β in transgenic murine models has been shown to induce selective neurodegeneration and behavioral deficits that correlate with the onset of cerebral pGlu-3 A β deposition (Alexandru et al., 2011, Becker et al., 2013, Wirths et al., 2009). The toxicity has been linked to increased hydrophobicity and aggregation propensity compared with full-length A β (Harigaya et al., 1995, He and Barrow, 1999, Schlenzig et al., 2009). Also, it has been demonstrated that pGlu-3 A β may act as a nidus for template-induced protein misfolding and oligomerization, both with itself and with free A β 1-42 to generate cytotoxic low-molecular weight oligomers (Nussbaum et al., 2012). Recently, QC expression and pGlu-3 A β accumulation in human AD brain has been shown to correlate with cognitive decline (Morawski et al., 2014, Pivtoraiko et al., 2014) and tau pathology (Mandler et al., 2014). Thus, cumulating evidence from human post-mortem tissue and mouse models suggest pGlu-modified A β as a species causally involved in AD progression and cognitive decline (Rijal Upadhaya et al., 2014, Saido et al., 1995).

Our pilot study data in APPswe/PS1 E9 mice suggested lowering of total A β (including pGlu-3 and non-pGlu-3 A β) and reduced microgliosis in the absence of microhemorrhage using a novel pGlu-3 A β IgG1 mAb, 07/1, in both prevention and therapeutic paradigms (Frost et al., 2012). However, given the small number of mice per group in our pilot study, the effect of pGlu-3 A β immunotherapy on cognition was not examined. In the current study, male APPswe/PS1 E9 Tg mice received weekly intraperitoneal injections of either 150 μ g or 500 μ g of 07/1 mAb for 28 weeks starting at six months of age. Two control groups were either vaccinated weekly with 200 μ g 3A1, a general A β IgG1 mAb that recognizes a non-pGlu epitope within the N-terminus, or treated with PBS. Effects on cognition were assessed using two hippocampal-dependent behavioral paradigms, the Water T Maze (WTM) and Contextual Fear Conditioning (CFC), along with characterization of locomotor activity in the Open Field test, and included age- and gender-matched wild-type (Wt) controls. Finally, detailed histopathological stainings and quantitative image analyses were performed on fixed brain sections to assess region-specific changes of A β plaque burden, associated gliosis and vascular integrity. Biochemical assessments on brain homogenates and terminal plasma samples were used to characterize A β levels in both the CNS and periphery of various A β species as well as exogenous antibody levels.

2. Materials and Methods

2.1 Animals

The current passive immunization study was conducted in male APP^{swE}/PS1^{E9} transgenic mice (henceforth, referred to as Tg mice) on a C57BL/6J background starting at 6 months of age. APP^{swE}/PS1^{E9} Tg mice express two human genes of familial AD, the APP^{K594N/M595L} Swedish and Presenilin 1 delta E9 (PS1^{E9}) (deletion of exon 9) under a mouse prion protein promoter (Jankowsky et al., 2004). Original Tg breeders were obtained from The Jackson Laboratory (Bar Harbor, ME) and were maintained in our colony by crossing male APP^{swE}/PS1^{E9} Tg mice with female C57BL/6J mice. All animal use was approved by the Harvard Standing Committee for Animal Use and was in compliance with all state and federal regulations. Initial accumulation of cerebral A β plaque burden has been reported to occur at 4-6 months of age in the cortex and hippocampus in APP^{swE}/PS1^{E9} mice (Garcia-Alloza et al., 2006, Jankowsky et al., 2004) whereas cerebral amyloid angiopathy (CAA) in the leptomeningeal vasculature starts at ~6 months (Garcia-Alloza et al., 2006). Manifestations of cognitive deficits have been observed in APP^{swE}/PS1^{E9} starting at 6-months of age (Park et al., 2006), which are exacerbated with age (Gimbel et al., 2010, Jankowsky et al., 2005).

2.2 Treatment

A total of 62 male mice were utilized in this study. Prior to the start of immunization, four APP^{swE}/PS1^{E9} Tg (avg. 5.6 mo $\pm$ 0.45) mice were sacrificed as baseline controls to assess cerebral A β plaque burden at the commencement of treatment. The remaining mice were divided into four groups and received one of the following treatments: 250 μ l sterile PBS (n=12; avg. 5.89 mo $\pm$ 0.13), 200 μ g 3A1, a general A β IgG1 mAb (n=11; avg 5.88 mo $\pm$ 0.15), 150 μ g 07/1, a pGlu-3 A β IgG1 mAb (n=12; avg. 5.74 $\pm$ 0.14) or 500 μ g of 07/1 (n=11; avg. 5.78 mo $\pm$ 0.14). The 3A1 mAb was generated and provided by Dr. Brian O'Nuallain (ARCND); the 07/1 mAb was generated and provided by Probiobdrug AG. A group of age- and gender-matched Wt littermates were injected with 250 μ l PBS (n=12; avg. 5.80 mo $\pm$ 0.12) and served as behavioral controls. Mice were treated with a total volume of 250 μ l (antibody or PBS) via intraperitoneal injection for 28 weeks.

2.3 Euthanasia and tissue preparation

Mice were euthanized, perfused and plasma harvested at 6 months (baseline) or 13 months of age as previously described (Frost et al., 2013). The brain was extracted and divided sagittally. The hippocampus, cortex and cerebellum were dissected from one hemisphere and snap frozen for biochemical analyses. The other hemisphere was drop-fixed in 4% paraformaldehyde (Electron Microscopy Sciences) for 24 h at 4°C, cryoprotected in graded sucrose solutions at 4°C and embedded in OCT compound (Tissue Tek).

2.4 Histology and quantitative image analysis

Ten-micron thick brain cryosections were cut with a Leica CM1850 cryostat and mounted on Colorfrost Plus slides (Fisher Scientific). Neuropathological analysis of A β plaque burden and associated gliosis were carried out with the following antibodies: R1282

(1:1000), a general A β rabbit polyclonal antibody (pAb) (gift D. Selkoe, Boston, MA); 82E1 (1:100), a mouse monoclonal antibody (mAb) recognizing A β 1-x (IBL); 07/2 (1:1000), a novel IgG2b mAb against pGlu-3 A β (Probiobdrug AG); Iba1 (1:500), a rabbit pAb that detects resting and activated microglia (Wako Chemicals); and CD68 (1:200), a rat mAb that detects activated microglia and macrophages with a phagocytic phenotype (AbD Serotec). Sections were air-dried for 30 min and washed with TBS for 2 $\times$ 5min. The remainder of the immunohistochemistry was carried out using the Elite ABC Kit (Vector Laboratories) as previously described (Frost et al., 2013). Staining for fibrillar amyloid with 1% Thioflavin S (Sigma-Aldrich) and microhemorrhage with hemosiderin (2% potassium ferrocyanide) was conducted as previously described (Liu et al., 2013).

Quantitative image analysis of the percent area of immunoreactivity (IR) and Thioflavin S staining using Bioquant image analysis software was conducted on six sections per mouse at three equidistant planes for hippocampus, frontal cortex and cerebellum for each of the following markers: R1282, 82E1, 07/2 and Thioflavin S. The percent area of immunoreactivity for Iba1 and CD68 IR was quantified in the hippocampus for three sections at three equidistant planes. The threshold of detection was held constant for each marker and the imager was blinded to treatment group.

2.5 Dot Blot

Earlier characterization of the 07/1 mAb included surface plasmon resonance and Western blot analyses (Frost et al., 2012). Here, we examined the specificity of the 07/1 and a control IgG1 mAb 3A1 by dot blot analysis as previously described (Frost et al., 2013). Briefly, synthetic peptides of varying lengths were immobilized on a 0.2 μ m nitrocellulose membrane at 1000, 100, 10 and 0.1 ng, respectively. Synthetic A β peptides 1-7, 3-9, 3-13, 1-15, 1-40 were synthesized at the Biopolymer Laboratory at University of California, Los Angeles. A β (3-40), pGlu-3 A β (9) and pGlu-3 A β (40) peptides were generated at Probiobdrug AG (Halle, Germany). After blocking with Odyssey Blocking Buffer (Li-Cor Biosciences) for 2 h at RT, membranes were incubated overnight with shaking at 4°C with either 07/1 at 0.54 μ g/ml or 3A1 0.2 μ g/ml, washed the following day with PBS and incubated with secondary antibody diluted in blocking buffer (1:10,000, Li-Cor Biosciences) for 1 h at RT. Membranes were scanned with a LiCor scanner.

2.6 Brain homogenization and A β ELISA in plasma and brain samples

Brain tissue without cerebellum was homogenized in TBS (20 mM Tris, 137 mM NaCl, pH 7.6; 2 volumes of buffer per brain weight, Dounce homogenizer) containing protease inhibitor cocktail (Complete Mini, Roche) and 0.1 mM AEBSF, sonicated and centrifuged at 75,500 $\times$ g for 1 hour at 4°C. The supernatant was stored at -80°C and A β peptides were sequentially extracted with 2.5 ml 2% SDS in distilled water (SDS fraction), and 0.5 ml 70% formic acid (formic acid fraction). The formic acid extract was neutralized by addition of 3.5 M Tris solution and diluted to a final volume of 10 ml using ELISA blocking buffer (Pierce, Cat.No. 37571). The snap frozen cerebellum was weighed and homogenized in ten volumes of T-PER buffer (Pierce) containing phosphatase (Sigma-Aldrich) and protease (Roche) inhibitors using a dounce homogenizer. Samples were then centrifuged at 175,000g for 1 h at 4°C and the T-PER fraction (soluble) was aspirated off. The remaining pellet was

resuspended in 5M guanidine-HCl buffer at pH 8.0 and oscillated at RT for 4 h. The solution (insoluble) was aliquoted and stored at -80°C until use. $\text{A}\beta(\text{x-42})$ (MesoScale Discovery) and pGlu-3 $\text{A}\beta(42)$ (IBL) were measured by ELISA following the manufacturer's instructions.

2.7 Antibody concentration measurements in plasma and brain tissues

To quantify 3A1 and 07/1 exogenous antibody concentrations in brain homogenates and terminal plasma samples, 96-well plates were washed three times with a wash solution containing: 25 mM Tris, 150 mM NaCl, 0.1% BSA and 0.05% Tween 20 at pH 7.2 and subsequently coated with 30 ng of biotinylated $\text{A}\beta(1-18)$ peptide or 20 ng biotinylated pGlu3-12 $\text{A}\beta$ (Probiobdrug AG), respectively, at RT for 2 h. The plates were then blocked with PierceTM Protein-Free blocking buffer for 1 h at RT. After washing, antibody standards and brain or plasma samples were added to each respective plate and incubated at 4°C for 2h. The plates were washed three times and then incubated with anti-mouse IgG-HRP 100ng/ml at 4°C for 1h. For detection, SureBlueTM TMB substrate solution (KPL) was added to each well for 30 min at RT and then the reaction stopped by adding 100 μl of 1.2 N H_2SO_4 . A Benchmark Microplate Reader (Bio-Rad) was used to measure optical density values at 450nm.

2.8 Behavioral Tests

Behavioral testing of all mice was performed starting at ~12 months of age.

2.8.1 Open Field—The Open Field test is used to assess spontaneous locomotor activity by measuring the total distance traveled as well anxiety level based upon the amount of time spent in the center. Mice are placed in the center of a Plexiglas[®] chamber (27-L $\times$ 27-W $\times$ 20-H cm; Med Associates) and are allowed to explore freely for 1 h. During that time, mice are monitored using a computer-assisted infrared tracking system, which computes the total distance traveled measured in 10-min bins.

2.8.2 Contextual fear conditioning—Based on Pavlovian classical conditioning, contextual fear conditioning is a test to evaluate memory and based on the tendency of a mouse to elicit a fear response (freezing) when re-introduced to a context where an aversive stimulus (foot shock) was inflicted. During the training phase, mice are placed in the conditioning chamber (31-L $\times$ 25-W $\times$ 25-H cm, Med Associates) with Plexiglas[®] sidewalls, stainless steel end walls and a floor comprised of steel bars. For 2 min, mice are allowed to explore the chamber after which time they receive a 2 sec foot shock (0.5mA) followed by another foot shock after 2 min. One min following the second foot shock, the mouse is removed from the chamber. Twenty-four h later, the mouse is returned to the chamber for 5 min without a foot shock. Freezing behavior is tracked and scored by Topscan software (Clever Sys).

2.8.3 Water T Maze—This behavioral paradigm, encompassing similar principles to the Morris Water Maze, is used to assess spatial learning and memory in which a mouse uses spatial cues to navigate to find an escape platform submerged just below the water's surface. In addition, the test also measures cognitive flexibility through a reversal trial requiring the

mice to learn a new platform location. The testing apparatus is a plus maze (each arm 14.1-L and 4.6-W cm) made of clear Plexiglas® with each arm designated as North (N), South (S), East (E) or West (W). For the mice to either choose only the E or W arm for escape, dividers are placed to block off the N or S arms. Mice are placed in the N or S arms, in a semi-random order, at the start of each trial. The maze is filled with 25-26 °C water and made opaque by adding non-toxic white paint so the mouse cannot see the submerged escape platform. Initially for the acquisition trial, the hidden escape platform is placed in the E arm. Mice are carried to the starting point and the experimenter scores a correct or incorrect response for each trial based on whether the arm with the escape platform is chosen. Regardless of the response, mice are allowed to remain on the platform for 10 sec at the end. Mice are given 10 trials/day and the percent correct responses are calculated by averaging correct responses across the 10 trials for each day. Acquisition criterion is achieved when a mouse scores 80% or more correct responses over two consecutive days. After all mice reach acquisition criterion, the reversal trial begins: the platform is moved to the opposite side (W) and the same procedure is repeated until all mice learn the platform's new location.

2.9 Statistics

With the exception of the behavioral data, statistical analyses were conducted with Prism 5 (GraphPad) using One-way ANOVA and Tukey's post-hoc test. For behavioral data, Sigma Plot (Systat Software Inc) was used along with Fisher's PLSD. A p value <0.05 was considered significant. All data are expressed as the mean $\pm$ SEM, unless otherwise noted.

3. Results

3.1 Antibody characterization

07/1 mAb was previously characterized by both Western blot and surface plasmon resonance (Frost et al., 2012). The 07/1 mAb was shown to detect only pGlu-3 A β peptide and not A β (1-40) or A β (3-x) peptides by Western blot. In addition, using surface plasmon resonance, 07/1 mAb exhibited a high affinity for pGlu-3 A β (40/42) but did not bind to other pyroglutamated peptides or hormones. To characterize the specificity of 3A1 IgG1 mAb, a dot blot assay was conducted using synthetic A β peptides of varying lengths including A β 1-7, 3-9, pE3-9, 3-13, 1-15, 3-40, pE3-40, and 1-40. In parallel, another dot blot was loaded with the same peptides listed above and probed with 07/1 mAb to confirm its specificity. The 3A1 mAb that detects a non-pGlu-3 A β epitope within the A β N-terminus, bound A β 1-15 and 1-40 peptides at 1000 and 100 ng, but did not bind any of the other A β peptides including pGlu-3 A β (Figure 1A). In contrast, 07/1 mAb detected pGlu-3 A β (9) (pE3-9) at 1000, 100, 10 ng and pGlu-3 A β (40) (pE3-40) at 1000ng, but not their N-terminally truncated precursors A β (3-9) or A β (3-40), respectively (Figure 1B).

3.2 A β plaque burden at the start of dosing

Four male APPswe/PS1 E9 Tg mice were sacrificed as baseline control to assess cerebral A β plaque burden at the start of the study. As shown in Supplemental Figure 1, immunolabeling of fixed brain sections with R1282, a general A β pAb, showed IR in the cortex (CTX; S1E) and hippocampus (HC; S1A) but not in the cerebellum (CB; S1I). A β -IR plaques containing pGlu- A β were detected with 07/2 mAb in CTX (S1F), but not in HC

(S1B) or CB (S1J). Sections labeled with 3A1 mAb confirmed R1282 staining in all three brain regions (S1C,G,K). Thioflavin S-positive fibrillar amyloid plaques were observed in the CTX (S1H, asterisk) but not in HC (S1D) or CB (S1L). Vascular amyloid was not detected. These data confirm that vaccination was initiated at the early stages of general A β deposition and the very early stages of cerebral accumulation of pGlu-3 A β and fibrillar amyloid.

3.3 07/1 mAb partially protects against age-related cognitive deficits

3.3.1 Open Field—Starting at 12-13 months of age, behavioral testing was initiated to measure the effects of passive immunization on hippocampal-dependent learning and memory as well as locomotor activity and anxiety. Vaccinated and PBS-treated Tg mice were compared to PBS-treated age and gender-matched Wt control mice. All APP^{swe}/PS1 E9 Tg mice showed increased locomotor activity illustrated by a significantly greater ($p<0.05$) distance traveled in the testing chamber during the first ten minutes of the Open Field test compared to Wt mice; however, from 10-60 min, there were no significant differences in the total distance traveled between groups (Fig 2A). APP^{swe}/PS1 E9 Tg mice showed a similar amount of time spent in the center of the Open Field compared Wt-PBS mice (data not shown), suggesting there were no genotype or no effects of antibody treatment on anxiety-like behavior.

3.3.2 Contextual Fear Conditioning—Contextual Fear Conditioning was performed after testing in the Open Field and Water T Maze. During the Contextual Fear Conditioning (CFC) training phase (i.e. learning), all APP^{swe}/PS1 E9 Tg mice showed significantly less freezing behavior ($p<0.05$) after the first and second foot shocks compared to PBS-treated Wt controls, however no antibody treatment effects were observed (Fig 2B). During the CFC context test (i.e. fear memory), PBS-treated Tg mice froze significantly less ($p<0.05$) than PBS-treated Wt mice, indicating that 13 month-old male APP^{swe}/PS1 E9 display deficits in contextual memory compared to age- and gender-matched Wt littermates. 3A1-immunized mice also froze less than PBS-treated Tg mice ($p<0.05$; Fig 2C). Weekly immunization of Tg mice with 07/1 mAb partially protected against these deficits as both the 150 μ g and 500 μ g 07/1 immunization groups were not statistically different than Wt-PBS; however, both groups were also not statistically different than PBS-immunized Tg mice either, even though a trend for better performance was seen in 150 μ g 07/1-immunized Tg mice (Fig 2C).

3.3.3 Water T maze—Similar to the Morris Water Maze (MWM), the Water T Maze (WTM) requires mice to utilize visual and spatial cues to locate a submerged escape platform and is used to assess spatial learning and memory (acquisition trials) and cognitive flexibility (reversal trials). Deficits in spatial learning and memory in the MWM have been reported for 12 months and older APP^{swe}/PS1 E9 Tg mice (Liu et al., 2013, Puolivali et al., 2002). During the 7-day acquisition period in our study, 12-13 month-old PBS-treated APP^{swe}/PS1 E9 Tg mice made significantly fewer correct responses on days 3-7 ($p<0.05$; Fig 2D) and took significantly longer to reach the acquisition criterion than ageand gender-matched PBS-treated Wt mice ($p<0.05$; Fig 2E), demonstrating deficits in spatial learning and memory at this age. In contrast, APP^{swe}/PS1 E9 Tg mice receiving weekly doses of

500µg 07/1 mAb had the most correct responses during the course of the 7-day acquisition trial ($p=0.059$ overall posthoc; Fig 2D). Moreover, 500µg 07/1-immunized Tg mice reached the acquisition criterion significantly faster ($p<0.05$; Fig 2E) than PBS-treated Tg mice and were not statistically different than PBS-treated Wt mice (Fig 2E), suggesting that 07/1 mAb partially-protected against spatial learning deficits in APPswe/PS1 E9 Tg mice.

Consistent with the acquisition trial results, in the reversal phase of the WTM, which tests memory and cognitive flexibility, PBS-treated Tg mice showed fewer correct responses ($p<0.05$; Fig 2F) and required significantly more days to achieve reversal criterion ($p<0.05$; Fig 2G) than the PBS-treated Wt mice, confirming cognitive deficits in 12 month-old APPswe/PS1 E9 Tg mice. No significant differences were observed between 07/1-immunized Tg mice and PBS-immunized Tg mice in the percent of correct responses over five days (Fig 2F) or the number of days required to reach reversal criterion (Fig 2G), indicating no robust antibody treatment effects on cognitive flexibility. Of note, 500µg 07/1-immunized Tg mice, unlike other treatment groups, performed similar to PBS-treated Wt mice, suggesting a trend for protection against cognitive impairment (Fig 2G). No significant cognitive benefits were observed in the 3A1-immunized Tg mice. [VUM Complete]

3.4 07/1 mAb vaccination reduced total A β deposition

One week following the final injection, mice were sacrificed for neuropathological and biochemical analyses to examine changes in the CNS and periphery after immunization. APPswe/PS1 E9 begin accumulating general A β around 4-6 months of age in the cortex and hippocampus (Garcia-Alloza et al., 2006, Jankowsky et al., 2004). Although we have detected pGlu-3 A β in a very small subset of general A β -positive plaques in the hippocampus in APPswe/PS1 E9 mice at 6-months of age (Frost et al., 2013), typically cerebral pGlu-3 A β accumulation occurs after 8-mo of age in this mouse model.

In hippocampus, R1282 IR was reduced by 35% in 500µg 07/1-immunized Tg mice ($p<0.05$), 45% in 150µg 07/1-immunized Tg mice ($p<0.01$) and 58% in 3A1-immunized Tg mice ($p<0.001$) compared to PBS-treated Tg mice (Fig 3A-D, Q). Similar treatment-specific reductions were observed for A β (1-x) deposition in the hippocampus (Fig 3E-H). APPswe/PS1 E9 mice immunized with either 150µg or 500µg 07/1 mAb exhibited reductions in 82E1 IR of 35% ($p<0.001$) and 32% ($p<0.001$), respectively, compared to PBS-treated Tg mice, while 3A1-immunized Tg mice showed a 42% reduction ($p<0.001$) of A β 1-x deposition compared to PBS-treated Tg mice (Fig 3Q). Dramatic reductions in pGlu-3 A β depositions were observed in all vaccinated mice in the hippocampus (Fig 3J-L). Image analysis revealed that 500µg 07/1-immunized Tg mice had a 50% reduction ($p<0.001$), 150µg 07/1-immunized Tg mice had a 58% reduction ($p<0.001$) and 3A1-immunized Tg mice had a 48% reduction in pGlu-3 A β deposition compared to PBS-treated Tg mice (Fig 3I,Q). Changes in Thioflavin S-positive plaques were less robust, as only 150µg 07/1-immunized and 3A1-immunized Tg mice had significant reductions of 22% ($p<0.05$) and 29%, respectively ($p<0.01$), relative to PBS-treated Tg mice (Fig 3Q).

In parallel to significant plaque reduction in hippocampus, similar results were also observed in both the frontal cortex and cerebellum. In frontal cortex, general A β IR was reduced 20%

($p < 0.05$) in 150 μ g 07/1-immunized and 25% ($p < 0.01$) in 500 μ g 07/1-immunized Tg mice and 44% ($p < 0.001$) in 3A1-immunized Tg mice compared to PBS-treated Tg controls (Fig 3R). Complementary to general A β reductions, A β (1-x) deposition was reduced by 23% (n.s.) in 500 μ g 07/1-immunized Tg and 22% (n.s.) in 150 μ g 07/1-immunized Tg mice, while 3A1-immunized Tg mice displayed a 46% reduction ($p < 0.05$) in A β 1-x relative to PBS-treated Tg controls (Fig 3R). Pyroglutamate-3 A β deposition was reduced by a 30% ($p < 0.05$) in 500 μ g 07/1-immunized Tg mice and 37% ($p < 0.01$) in 150 μ g 07/1-immunized Tg mice, while 3A1-immunized Tg mice had a 40% reduction in pGlu-3 A β ($p < 0.01$) compared to PBS-treated Tg mice. As in the hippocampus, Thioflavin S-positive plaques were less effectively cleared in the frontal cortex, and only 3A1-immunized Tg mice exhibited a significant 27% reduction of 07/2 IR ($p < 0.05$; Fig 3R).

In the cerebellum, the greatest reductions were observed in general A β and A β 1-x IR in which 150 μ g 07/1-immunized and 500 μ g 07/1-immunized Tg mice had reductions of 27% ($p < 0.05$) and 43% ($p < 0.001$) in general A β deposition and 52% ($p < 0.01$) and 36% (n.s.) of A β (1-x) IR, respectively (Fig 3S). 3A1-immunized Tg mice also showed a 47% reduction in general A β ($p < 0.001$) and a 45% reduction in A β (1-x) deposition ($p < 0.05$) compared to PBS-treated Tg mice (Fig 3S). No significant differences in pGlu-3 A β deposition in cerebellum were noted in any immunization group and only 3A1-immunized Tg mice exhibited a significant 29% reduction in Thioflavin S-positive plaques ($p < 0.05$; Fig 3S).

To examine for changes in vascular amyloid, quantitative image analysis was performed on Thioflavin S-positive blood vessels in the cerebellum. Parenchymal and leptomenigeal blood vessels were quantified. Although there was a trend for reduction in all immunizations groups, compared to PBS-treated Tg controls, no significant differences were observed (data not shown). Furthermore, hemosiderin staining was performed for the detection of microhemorrhages. Very rare microhemorrhages (1-2 per mouse) were noted in a small subset of mice per group ($n = 2-4$), particularly in the leptomenigeal blood vessels in cortex and cerebellum, however, no significant differences were observed between groups (data not shown), indicating that vaccination with 07/1 and 3A1 did not increase the incidence of microhemorrhage.

3.5 Biochemical changes in A β levels in brain homogenates

Biochemical analyses from hippocampal, cortical and cerebellar soluble and insoluble brain homogenates did not confirm reductions in cerebral A β levels observed by neuropathological analyses. These data are summarized in Table 1. Fresh frozen hippocampi and cortices were homogenized to collect three separate fractions, TBS, SDS and formic acid-soluble. A β levels in TBS-soluble fractions were below the detection limit for both A β (x-42) and pGlu-3 A β (42) ELISAs and subsequently omitted from Table 1. There were no significant changes in A β (x-42) or pGlu-3 A β (42) levels in either the SDS (soluble) or formic acid (insoluble) fractions from hippocampus or cortex in any immunization group compared PBS-treated Tg mice (Table 1). In an effort to liberate more soluble A β , the cerebellum was homogenized in T-PER buffer (soluble fraction) initially, followed by guanidine-hydrochloric acid (insoluble fraction). Interestingly, there was a significant increase in soluble A β (x-42) levels after immunization in 500 μ g ($p < 0.001$) and 150 μ g

($p < 0.05$) that was also observed in 3A1-immunized mice ($p < 0.01$). As in the TBS fractions from hippocampus and cortex, pGlu-3 A β was undetectable in the T-PER soluble fractions from all groups and was found in very low abundance in the insoluble fraction in cerebellum, but remained unchanged with immunization (Table 1).

3.6 Plasma A β levels

Previous studies, including our own, have demonstrated a disruption in A β homeostasis after A β immunotherapy resulting in an efflux of A β from the brain into the periphery, known as the peripheral sink hypothesis (DeMattos et al., 2001, Lemere et al., 2003). To investigate whether there were changes in peripheral A β levels, plasma samples were collected from all mice and A β was quantified by ELISA. Interestingly, we detected no significant differences in plasma A β (1-x), A β (x-38), A β (x-40), and A β (x-42) between baseline (6 month-old) and PBS-treated APPswe/PS1 E9 control (13 month-old) mice, demonstrating that plasma A β levels remained constant with aging, even with increasing cerebral A β accumulation (Figure 4A-D). In 3A1-immunized Tg mice, very dramatic increases in plasma A β (1-x) ($p < 0.001$), A β (x-38) ($p < 0.001$), A β (x-40) ($p < 0.001$), A β (x-42) ($p < 0.001$), were observed relative to PBS-treated Tg controls, however no changes in any plasma A β species measured was observed in 07/1-immunized Tg mice (Figure 4A-D). These data suggest different mechanisms of action between 07/1 and 3A1 mAbs. In agreement with our previous pilot study, pGlu-3 A β (42) was not detected in plasma with or without immunization (data not shown). To date, pGlu-3 A β has not been detected outside of the CNS or in CSF in humans (Bibl et al., 2012, Wu et al., 2014).

3.7 Exogenous antibody concentrations in CNS and periphery

In order to assess the amounts of 07/1 and 3A1 IgG1 exogenous antibodies present in the CNS and the periphery at sacrifice, antibodies levels were assessed by neuropathological staining and measured via ELISA in plasma and T-PER-soluble cerebellar brain homogenates. Initially, brain sections were immunolabeled with a biotinylated anti-mouse IgG1 (S2E-H) antibody and stained with R1282 (S2A-D) and 07/2 (S2I-L), respectively on adjacent serial sections. IgG1 IR was detected in 500 μ g (S2H) and 150 μ g 07/1 immunized mice (S2G) which colocalized with a subset of R1282 (S2C,D) and 07/2-immunoreactive plaques (S2K,L). This was observed to a lesser extent in PBS-injected controls (S2A,E,I). Intense IgG1 IR was observed in 3A1-immunized mice (S2F) that colocalized with near all general A β deposition (S2B) and a subset of pGlu-3 A β plaques (S2J).

Moreover, antibody concentrations were assessed in plasma and cerebellar brain homogenates. To determine the antibodies directed against general A β and pGlu3-A β , 96-well plates were coated with A β 1-18 and pGlu3-12 A β peptides, respectively and incubated with the samples from treated and untreated mice. We observed a dose-dependent increase in plasma pGlu-3 A β antibody levels in 07/1-500 μ g Tg (~85,000 ng/ml) and 07/1 150 μ g-injected Tg mice (~20,000 ng/ml) (S2P). No antibodies recognizing pGlu3-12 A β were detected in plasma from 3A1 or PBS-injected mice, further supporting 07/1 and 3A1 mAb's specificities (S2P). In contrast, circulating antibodies that recognized A β 1-18 were present in 3A1-immunized mouse plasma (~60,000 ng/ml) (S2O) but not in either of the 07/1 immunization groups or PBS-injected Tg controls (S2O). In parallel, dose-dependent

increases in exogenous antibody concentrations corresponding to the antibody doses injected were observed when performing the aforementioned assays utilizing the Tper soluble fractions from post-perfusion cerebellar homogenates. This demonstrates that a fraction of the exogenous antibodies administered in the periphery were able to penetrate the CNS. In the cerebellum, ~14,000 pg/ml antibodies that recognized pGlu3-12 A β from 500 μ g 07/1-immunized Tg mice and ~3,000 pg/ml from 150 μ g 07/1-immunized Tg mice were detected (S2N). These amounts represent 0.016% and 0.015%, respectively the circulating antibody concentrations measured in plasma. No pGlu3-A β specific antibodies were detected in soluble cerebellar homogenates from 3A1-immunized or PBS-injected mice (S2N). However, a smaller fraction compared to the amount of A β 1-18-recognizing antibodies in plasma representing 0.003% was detected in soluble cerebellar homogenates from 3A1-immunized (2,000 pg/ml) mice but no appreciable amounts of A β 1-18-recognizing antibodies from 07/1 or PBS-injected Tg mice were detected (S2M).

3.8 Glial changes with 3A1 but not 07/1 vaccination

Changes in microgliosis were assessed in hippocampus on sections immunolabeled with Iba1 and CD68. As demonstrated in previously published reports (Frautschy et al., 1998, Gordon et al., 2002), we found Iba1 and CD68-positive microglia clustered around cerebral A β plaques in all mice (Fig 5A-L). Quantitative image analysis on three sections at three equidistant planes revealed similar Iba1 IR in hippocampus between all groups (Fig 5M). Furthermore, we observed a significant reduction in CD68-IR microglia in 3A1-immunized Tg mice compared to PBS-treated Tg mice (Fig 5F, N), but not in 150 μ g 07/1-immunized or 500 μ g 07/1-immunized Tg mice (Fig 5I,L,N). Also, we considered that the reduction in microglia may be due to a concomitant decrease in plaque burden. This was addressed by comparing the ratios of general A β , A β (1-x) and pGlu-3 A β deposition to Iba1 and CD68 IR in the hippocampus. We observed no significant changes in the ratios of general A β to Iba1 (S3A) or CD68 (S3B), A β 1-x to Iba1 (S3C) or CD68 (S3D) or pGlu-3 A β to CD68 (S3F) with any immunization group compared to PBS-injected Tg controls. However, we did observe significant reductions in the ratios of pGlu-3 A β deposition to Iba1 IR in 3A1 ($p<0.05$) 150 μ g-07/1 ($p<0.01$) and 500 μ g 07/1-immunized Tg mice ($p<0.001$) (S3E). Double immunofluorescence and confocal microscopy with sections immunolabeled for A β 1-x and CD68 or pGlu-3 A β and CD68 did not show any difference in the number of A β - and CD68-double positive microglia in hippocampus at the very end of immunization (data not shown).

4. Discussion

The main objective of the present study was to assess the effect of passive immunization with a pGlu-3 A β specific antibody, 07/1 at two different doses, on cognitive status, especially spatial learning and memory. The 3A1 IgG1 mAb, detecting full-length A β , was used as a positive control as numerous studies have shown passive immunotherapy utilizing antibodies against various A β epitopes to be safe and efficacious in lowering plaque burden and having positive effects on cognition as reviewed in (Lemere and Masliah, 2010). Our previous pilot study demonstrated prevention of cerebral accumulation of general and pGlu-3 A β species in APPswe/PS1 E9 mice after immunization with 07/1, when peripherally administered at onset of plaque deposition at 6 months of age (Frost et al.,

2012). Consequences of the treatment for cognition, however, have not been assessed and are generally sparsely investigated in preclinical immunization trials. Here, we tested the 07/1 mAb at two different doses, 150 and 500 μ g, to investigate whether there would be a dose-dependent clearance of cerebral A β and/or cognitive benefits. Importantly, we confirmed our previous results and observed a significant lowering of total A β plaque deposition in the absence of microhemorrhage and, for the first time, demonstrate rescue of cognitive deficits with anti-pGlu-3 A β passive immunotherapy in a preclinical AD-like mouse model.

Due to its known aberrant accumulation early in AD pathogenesis and ability to precipitate aggregation of other A β isoforms in a prion-like manner, pGlu-3 emerged as a target for AD treatment, including the development of pGlu-3 A β vaccines. Our study is in agreement with the first published report of a passive vaccine selectively targeting pGlu-3 A β oligomers from the T. Bayer Lab who found that immunizing four 4.5-month old 5xFAD mice for 6-weeks lowered cerebral A β accumulation and plaque burden and normalized anxiety-like behavior (Wirhith et al., 2010b). Subsequently, Eli Lilly developed a pGlu-3 A β -specific antibody mE8, that when used for passive immunization in PDAPP mice, significantly reduced cerebral A β levels (as measured by ELISA) in a therapeutic but not prevention trial (Demattos et al., 2012). Two different Ig isotypes, IgG1 and IgG2a, were compared to examine the role of microglial effector functions on pGlu-3 A β -specific immunotherapy. While both antibodies elicited significant changes in A β levels, the IgG2a antibody was shown to clear more A β *in vivo* and *ex vivo* (in a phagocytosis assay), consistent with earlier work on the effects of A β mAb Ig isotypes on plaque clearance (Bard et al., 2003). Our pilot (Frost et al., 2012) and current pGlu-3 A β passive vaccine studies are in agreement with T. Bayer's study in terms of plaque-lowering effects; however, our biochemical results differ from theirs in that we have not observed biochemical confirmation of cerebral A β -lowering, possibly due to issues with either tissue homogenization or a differential clearance of deposits of different sizes. Differing clearance of plaques has been observed previously also with gantenerumab (Bohrmann et al., 2012), and similar effects might account for the apparent discrepancy between quantitative image analysis of plaque size and the A β concentration in the brain.

Our study also differs from that of DeMattos and colleagues (Demattos et al., 2012) in that we observed significant plaque-lowering in a prevention study but they did not. Possible explanations for this difference may include a lack of target engagement by mE8 in the young PDAPP mice (Demattos et al., 2012) in which immunization started at 9 months of age and lasted 3 months. PDAPP express low levels of pGlu-3 A β even at 12 months, so it is conceivable that there was not enough cerebral deposition in the 12 month-old mice, at the end of the study, to see a change. This would indicate that the plaque-lowering effects on other A β species occurs after pGlu-3 A β aggregation and plaque deposition, possibly as a "seed" is underway. On the other hand, we have shown previously that APPswe/PS1 E9 Tg mice start accumulating pGlu-3 A β as early as 6 months of age (Frost et al., 2013), although the baseline mice (6 month-old) in this study were mostly devoid of pGlu-3 A β deposits. However, based on our previous work, it is reasonable to assume that pGlu-3 formation and deposition would have begun shortly after 6 months of age in these animals and was well

established in 13 month-old PBS-treated Tg mice. Thus, our current results provide support for a “seeding” effect of pGlu-3 A β in brain.

Notably, 07/1 mAb is the first anti-pyroglutamate-3 A β antibody to demonstrate cognitive improvement when administered prophylactically in a preclinical murine model of AD, as described below. APPswe/PS1 E9 Tg mice have been shown to exhibit deficits in spatial learning and memory in the Morris Water Maze starting at 12 months of age and older compared to Wt littermates (Liu et al., 2013, Puolivali et al., 2002). In parallel, in the WTM, a test of hippocampal-dependent spatial learning and memory, we found that PBS-treated APPswe/PS1 E9 Tg mice made significantly more errors than PBS-treated Wt littermates in both the acquisition and reversal trials, and required a significantly longer time to achieve the acquisition and reversal criterion, thereby confirming that male APPswe/PS1 E9 Tg mice exhibit deficits in spatial learning and memory at 13 months of age. Furthermore, when collapsing all individual day data, the 500 μ g 07/1-immunized APPswe/PS1 E9 Tg mice overall had a trend for more percent correct responses, reached the acquisition criterion significantly faster than PBS-treated Tg controls and were not statistically different than PBS-treated Wt littermates for either measure; however, no antibody treatment rescued cognitive deficits in the reversal task, suggesting that 07/1 mAb immunization was more protective for acquisition but not reversal learning and memory in APPswe/PS1 E9 Tg mice. VUM

In contrast to the WTM data, a significant effect of 07/1 treatment in Open Field or CFC was not observed. These assays were performed because previous reports showed increased hyperactivity and exploratory behavior (Filali et al., 2011, Lalonde et al., 2005) and decreased levels of contextual fear conditioning (e.g., Cramer et al., 2012) in APPswe/PS1 E9 Tg mice.

Detailed neuropathological analyses and accompanying quantitative image analysis, demonstrated significant reductions in the deposition of general A β , A β (1-x) and pGlu-3 A β species in several brain regions, particularly the hippocampus. Overall, there was not a dose-dependent clearance when comparing 150 μ g 07/1-immunized and 500 μ g 07/1-immunized Tg mice to PBS-treated Tg controls as both doses prevented cerebral A β deposition similarly. This might be caused by the low abundance of pGlu-3 A β in the brain. Because the accessible pGlu-epitope is already occupied at the low dose of 07/1, glia-mediated reduction of A β is elicited at similar extent at both doses. Future studies will test treatment with even lower doses. Moreover, the 07/1 mAb appeared not as effective as the 3A1 mAb in preventing deposition of fibrillar amyloid, general A β and A β (1-x). However, 07/1-immunized Tg mice displayed a robust reduction in pGlu-3 A β in the hippocampus, which complements the improvements noted in the acquisition phase of the WTM and the CFC context test. The insignificant differences between 3A1 and 07/1 in reduction of general A β and pGlu3-A β might be explained by two mechanisms: I., pGlu-A β formation is downstream of A β generation by β - and γ -secretase. Thus, removing the precursor of pGlu-A β , i.e. A β (1-40/42) by 3A1 is expected to also reduce pGlu3-A β and II., antibody decoration of mixed aggregates consisting of full-length A β and pGlu3-A β is expected to result in phagocytosis of both species. Conversely, treatment with 07/1 reduces general A β by the same mechanisms, which was observed as well.

No statistical differences were observed in the WTM between 3A1-immunized Tg and PBS Tg controls, even though plaque-lowering was observed and the antibody levels in plasma was in-between the concentration of both 07/1 doses (S2O,P).

The synergistic effect of the 07/1 antibody on behavior and histopathology is possibly due to direct targeting of a modified species with extremely toxic potential. Indeed, there are several reports suggesting a physiological role of “full-length” A β peptides (A β 1-x). Among them, antimicrobial activity (Soscia et al., 2010) and prevention of aggregation of highly amyloidogenic species (McGowan et al., 2005), as well as a regulatory role of A β for synaptic activity has been postulated (Puzzo and Arancio, 2013, Puzzo et al., 2011, Puzzo et al., 2008). Possibly, the lack of 07/1 mAb's ability to bind these forms of the peptide does not interfere with the regulatory function of distinct A β isoforms. Thereby, the neuropathological data in our study confirm that selectively targeting pGlu-3 A β by passive immunotherapy was effective in lowering not only pGlu-3 A β but also non-pGlu-3 A β species, which are co-aggregated with pGlu-A β . Although the exact mechanism of cerebral A β clearance has yet to be elucidated, several clearance mechanisms induced by A β immunotherapy have been proposed. They include: (1) Fc receptor (FcR) dependent (Bard et al., 2003, Bard et al., 2000, Ferrer et al., 2004, Nicoll et al., 2003) or -independent (Bacskai et al., 2002, Das et al., 2003) mediated-phagocytosis by microglia, (2) antibody-mediated disaggregation and neutralization A β toxicity (Bacskai et al., 2001, Frenkel et al., 2000, Solomon et al., 1997), (3) a disruption in A β homeostasis where there is an efflux in cerebral A β to the periphery, known as the “peripheral sink hypothesis” (DeMattos et al., 2001, Lemere et al., 2003) or intracerebral sequestration of monomeric A β (Yamada et al., 2009).

It is possible that the mechanisms of action of 07/1 and 3A1 mAbs are distinct and multifactorial. Our immunohistochemical data demonstrate target engagement of both 07/1 and 3A1 antibodies in the brain (Supplementary Figure 2). In both the 500 μ g and 150 μ g 07/1-immunized Tg mice, we found that over 0.01% of the antibodies that were detected in the plasma were present in the brain, which is in agreement with previous studies (Levites et al., 2006, Mably et al., 2015). The lower percentage of general A β -specific antibodies detected in the cerebellum from 3A1-immunized mice may be because fewer unbound antibodies were present, which is clearly evident from the neuropathology and is probably due to greater deposition of general A β compared to pGlu-3 A β in APPswe/PS1 E9 mice at 13 months of age.

Considering the proven target engagement in the brain, the dramatic increases in A β 1-x, x-38, x-40 and x-42 species in terminal plasma samples from 3A1-immunized Tg mice provide a striking difference and might be suggestive of peripheral clearance by the 3A1 mAb. In addition, this observed effect may be due, in part, to the 3A1 mAb's ability to bind to peripheral A β , which in itself has been shown to increase A β 's half-life (Golde et al., 2009, Seubert et al., 2008). Importantly, we did not detect pGlu-3 A β in plasma from any mice in this study, confirming an earlier report that pGlu-3 A β is located exclusive in the brain and not detected in the periphery (Bibl et al., 2012, Wu et al., 2014). Thus, it is unlikely that the 07/1 antibody was saturated in the periphery, suggesting a more centrally-mediated effect on plaque-lowering and cognitive protection.

It has been reported that microglia undergo transient changes in activation states throughout the course of A β immunotherapy (Wilcock et al., 2004a). Although we did not detect changes in Iba1 or CD68 IR in hippocampus at the end of the study in 07/1-immunized mice at either dose, this does not exclude the possibility that FcR-mediated clearance of A β aggregates by microglia occurred early and may have begun to subside by the end of vaccination; however, we did observe significant decreases in the ratios of pGlu-3 A β deposition to Iba1 IR in the hippocampus in all immunization groups, illustrating that pGlu-3 A β clearance did not parallel the reduction in microgliosis because the microglia could still be aiding in A β clearance at the time of sacrifice. Alternatively, the 07/1 mAb may have prevented aggregation of pGlu-3 A β , resulting in increased levels of soluble A β (as observed in cerebellum). Future advances using *in vivo* imaging techniques may aid in the understanding of microglia activation over the course of immunotherapy in both preclinical models and AD patients.

In addition to lowering cerebral A β plaque burden and protecting against cognitive deficits, A β passive immunotherapy has been shown to induce microhemorrhages in preclinical murine models (Pfeifer et al., 2002, Racke et al., 2005, Wilcock et al., 2004b). This finding was observed in human clinical trials, especially in patients with one or two Apolipoprotein E ϵ 4 alleles (Sperling et al., 2012). Importantly, as in our pilot study, we observed no increase in the incidence of microhemorrhage using pGlu-3 A β antibody (07/1 mAb) for immunotherapy. However, the mice in the present study had no evidence of vascular amyloid at the start of dosing and no significant changes in vascular amyloid following immunization. Future studies will be conducted in older Tg mice with more cerebral A β and vascular amyloid at the start of immunization to further examine the ability of 07/1 to safely prevent A β aggregation and/or clear plaques without inducing microhemorrhages.

5. Conclusion

We have demonstrated the ability of two different doses of the pGlu-3 A β antibody 07/1 to clear plaques and partially protect cognition, in absence of any additional microhemorrhages. The advantages of passive immunotherapy selectively targeting pGlu-3 A β may be to capture and detoxify a highly pathogenic A β isoform that plays a critical role in A β aggregation. Since we have yet to elucidate the physiological role of A β , it is best to target a modified, toxic A β species to avoid deleterious downstream effects that may result from clearing full-length A β . Furthermore, since pGlu-3 A β is either undetectable in the periphery (Bibl et al., 2012) or only found in individuals with genetic overproduction of A β (e.g. Down syndrome) (Mehta et al., Pyroglutamate-3 Amyloid Beta Protein Plasma Levels in Down Syndrome (DS), Alzheimer's Association International Conference 2014, Presentation Number: P4-050), the injected exogenous antibodies are unlikely to become saturated in the periphery, leaving unbound antibodies free to bind to pGlu-3 A β aggregates and/or deposits in the brain. Therefore, we conclude that passive immunotherapy targeting pGlu-3 A β may have strong potential for preventing or protecting against AD in its early stages.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- Alexandru A, Jagla W, Graubner S, Becker A, Bauscher C, Kohlmann S, et al. Selective hippocampal neurodegeneration in transgenic mice expressing small amounts of truncated Abeta is induced by pyroglutamate-Abeta formation. *J Neurosci*. 2011; 31:12790–801. [PubMed: 21900558]
- Bacsikai BJ, Kajdasz ST, Christie RH, Carter C, Games D, Seubert P, et al. Imaging of amyloid-beta deposits in brains of living mice permits direct observation of clearance of plaques with immunotherapy. *Nature medicine*. 2001; 7:369–72.
- Bacsikai BJ, Kajdasz ST, McLellan ME, Games D, Seubert P, Schenk D, et al. Non-Fc-mediated mechanisms are involved in clearance of amyloid-beta in vivo by immunotherapy. *J Neurosci*. 2002; 22:7873–8. [PubMed: 12223540]
- Bard F, Barbour R, Cannon C, Carretto R, Fox M, Games D, et al. Epitope and isotype specificities of antibodies to beta -amyloid peptide for protection against Alzheimer's disease-like neuropathology. *Proc Natl Acad Sci U S A*. 2003; 100:2023–8. [PubMed: 12566568]
- Bard F, Cannon C, Barbour R, Burke RL, Games D, Grajeda H, et al. Peripherally administered antibodies against amyloid beta-peptide enter the central nervous system and reduce pathology in a mouse model of Alzheimer disease. *Nature medicine*. 2000; 6:916–9.
- Becker A, Kohlmann S, Alexandru A, Jagla W, Canneva F, Bauscher C, et al. Glutaminyl cyclase-mediated toxicity of pyroglutamate-beta amyloid induces striatal neurodegeneration. *BMC Neurosci*. 2013; 14:108. [PubMed: 24083638]
- Bibl M, Gallus M, Welge V, Lehmann S, Sparbier K, Esselmann H, et al. Characterization of cerebrospinal fluid aminoterminaly truncated and oxidized amyloid-beta peptides. *Proteomics Clin Appl*. 2012; 6:163–9. [PubMed: 22532452]
- Bohrmann B, Baumann K, Benz J, Gerber F, Huber W, Knoflach F, et al. Gantenerumab: a novel human anti-Abeta antibody demonstrates sustained cerebral amyloid-beta binding and elicits cell-mediated removal of human amyloid-beta. *J Alzheimers Dis*. 2012; 28:49–69. [PubMed: 21955818]
- Cramer PE, Cirrito JR, Wesson DW, Lee CY, Karlo JC, Zinn AE, Casali BT, Restivo JL, Goebel WD, James MJ, Brunden KR, Wilson DA, Landreth GE. ApoE-directed therapeutics rapidly clear β -amyloid and reverse deficits in AD mouse models. *Science*. Mar 23. 2012; 335(6075):1503–6. [PubMed: 22323736]
- Cynis H, Scheel E, Saido TC, Schilling S, Demuth HU. Amyloidogenic processing of amyloid precursor protein: evidence of a pivotal role of glutaminyl cyclase in generation of pyroglutamate-modified amyloid-beta. *Biochemistry*. 2008; 47:7405–13. [PubMed: 18570439]
- Das P, Howard V, Loosbrock N, Dickson D, Murphy MP, Golde TE. Amyloid-beta immunization effectively reduces amyloid deposition in FcRgamma $^{-/-}$ knock-out mice. *J Neurosci*. 2003; 23:8532–8. [PubMed: 13679422]
- DeMattos RB, Bales KR, Cummins DJ, Dodart JC, Paul SM, Holtzman DM. Peripheral anti-A beta antibody alters CNS and plasma A beta clearance and decreases brain A beta burden in a mouse model of Alzheimer's disease. *Proc Natl Acad Sci U S A*. 2001; 98:8850–5. [PubMed: 11438712]
- Demattos RB, Lu J, Tang Y, Racke MM, DeLong CA, Tzaferis JA, et al. A plaque-specific antibody clears existing beta-amyloid plaques in Alzheimer's disease mice. *Neuron*. 2012; 76:908–20. [PubMed: 23217740]
- Esler WP, Wolfe MS. A portrait of Alzheimer secretases--new features and familiar faces. *Science*. 2001; 293:1449–54. [PubMed: 11520976]

- Ferrer I, Boada Rovira M, Sanchez Guerra ML, Rey MJ, Costa-Jussa F. Neuropathology and pathogenesis of encephalitis following amyloid-beta immunization in Alzheimer's disease. *Brain Pathol.* 2004; 14:11–20. [PubMed: 14997933]
- Filali M, Lalonde R, Rivest S. Anomalies in social behaviors and exploratory activities in an APPsw/PS1 mouse model of Alzheimer's disease. *Physiol Behav.* 2011; 104:880–5. [PubMed: 21640739]
- Frautschy SA, Yang F, Irizarry M, Hyman B, Saido TC, Hsiao K, et al. Microglial response to amyloid plaques in APPsw transgenic mice. *Am J Pathol.* 1998; 152:307–17. [PubMed: 9422548]
- Frenkel D, Katz O, Solomon B. Immunization against Alzheimer's beta -amyloid plaques via EFRH phage administration. *Proc Natl Acad Sci U S A.* 2000; 97:11455–9. [PubMed: 11027345]
- Frost JL, Le KX, Cynis H, Ekpo E, Kleinschmidt M, Palmour RM, et al. Pyroglutamate-3 amyloid-beta deposition in the brains of humans, non-human primates, canines, and Alzheimer disease-like transgenic mouse models. *Am J Pathol.* 2013; 183:369–81. [PubMed: 23747948]
- Frost JL, Liu B, Kleinschmidt M, Schilling S, Demuth HU, Lemere CA. Passive immunization against pyroglutamate-3 amyloid-beta reduces plaque burden in Alzheimer-like transgenic mice: a pilot study. *Neurodegen Dis.* 2012; 10:265–70.
- Garcia-Alloza M, Robbins EM, Zhang-Nunes SX, Purcell SM, Betensky RA, Raju S, et al. Characterization of amyloid deposition in the APPsw/PS1dE9 mouse model of Alzheimer disease. *Neurobiol Dis.* 2006; 24:516–24. [PubMed: 17029828]
- Gimbel DA, Nygaard HB, Coffey EE, Gunther EC, Lauren J, Gimbel ZA, et al. Memory impairment in transgenic Alzheimer mice requires cellular prion protein. *J Neurosci.* 2010; 30:6367–74. [PubMed: 20445063]
- Golde TE, Das P, Levites Y. Quantitative and mechanistic studies of Abeta immunotherapy. *CNS Neurol Disord Drug Targets.* 2009; 8:31–49. [PubMed: 19275635]
- Gordon MN, Holcomb LA, Jantzen PT, DiCarlo G, Wilcock D, Boyett KW, et al. Time course of the development of Alzheimer-like pathology in the doubly transgenic PS1+APP mouse. *Exp Neurol.* 2002; 173:183–95. [PubMed: 11822882]
- Guntert A, Dobeli H, Bohrmann B. High sensitivity analysis of amyloid-beta peptide composition in amyloid deposits from human and PS2APP mouse brain. *Neuroscience.* 2006; 143:461–75. [PubMed: 17008022]
- Hardy J, Selkoe DJ. The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. *Science.* 2002; 297:353–6. [PubMed: 12130773]
- Harigaya Y, Shoji M, Kawarabayashi T, Kanai M, Nakamura T, Iizuka T, et al. Modified amyloid beta protein ending at 42 or 40 with different solubility accumulates in the brain of Alzheimer's disease. *Biochem Biophys Res Commun.* 1995; 211:1015–22. [PubMed: 7598687]
- Hartig W, Goldhammer S, Bauer U, Wegner F, Wirths O, Bayer TA, et al. Concomitant detection of beta-amyloid peptides with N-terminal truncation and different C-terminal endings in cortical plaques from cases with Alzheimer's disease, senile monkeys and triple transgenic mice. *J Chem Neuroanat.* 2010; 40:82–92. [PubMed: 20347032]
- Hartlage-Rubsamen M, Morawski M, Waniek A, Jager C, Zeitschel U, Koch B, et al. Glutaminy cyclase contributes to the formation of focal and diffuse pyroglutamate (pGlu)-Abeta deposits in hippocampus via distinct cellular mechanisms. *Acta Neuropathol.* 2011; 121:705–19. [PubMed: 21301857]
- He W, Barrow CJ. The A beta 3-pyroglutamyl and 11-pyroglutamyl peptides found in senile plaque have greater beta-sheet forming and aggregation propensities in vitro than full-length A beta. *Biochemistry.* 1999; 38:10871–7. [PubMed: 10451383]
- Jankowsky JL, Fadale DJ, Anderson J, Xu GM, Gonzales V, Jenkins NA, et al. Mutant presenilins specifically elevate the levels of the 42 residue beta-amyloid peptide in vivo: evidence for augmentation of a 42-specific gamma secretase. *Hum Mol Genet.* 2004; 13:159–70. [PubMed: 14645205]
- Jankowsky JL, Melnikova T, Fadale DJ, Xu GM, Slunt HH, Gonzales V, et al. Environmental enrichment mitigates cognitive deficits in a mouse model of Alzheimer's disease. *J Neurosci.* 2005; 25:5217–24. [PubMed: 15917461]

- Jawhar S, Trawicka A, Jenneckens C, Bayer TA, Wirths O. Motor deficits, neuron loss, and reduced anxiety coinciding with axonal degeneration and intraneuronal Abeta aggregation in the 5XFAD mouse model of Alzheimer's disease. *Neurobiol Aging*. 2012; 33:196, e29–40. [PubMed: 20619937]
- Lalonde R, Kim HD, Maxwell JA, Fukuchi K. Exploratory activity and spatial learning in 12-month-old APP(695)SWE/co+PS1/DeltaE9 mice with amyloid plaques. *Neurosci Lett*. 2005; 390:87–92. [PubMed: 16169151]
- Lemere CA, Blusztajn JK, Yamaguchi H, Wisniewski T, Saido TC, Selkoe DJ. Sequence of deposition of heterogeneous amyloid beta-peptides and APO E in Down syndrome: implications for initial events in amyloid plaque formation. *Neurobiol Dis*. 1996; 3:16–32. [PubMed: 9173910]
- Lemere CA, Masliah E. Can Alzheimer disease be prevented by amyloid-beta immunotherapy? *Nat Rev Neurol*. 2010; 6:108–19. [PubMed: 20140000]
- Lemere CA, Spooner ET, LaFrancois J, Malester B, Mori C, Leverone JF, et al. Evidence for peripheral clearance of cerebral Abeta protein following chronic, active Abeta immunization in PSAPP mice. *Neurobiol Dis*. 2003; 14:10–8. [PubMed: 13678662]
- Levites Y, Das P, Price RW, Rochette MJ, Kostura LA, McGowan EM, et al. Anti-Abeta42- and anti-Abeta40-specific mAbs attenuate amyloid deposition in an Alzheimer disease mouse model. *J Clin Invest*. 2006; 116:193–201. [PubMed: 16341263]
- Liu B, Frost JL, Sun J, Fu H, Grimes S, Blackburn P, et al. MER5101, a novel Abeta1-15:DT conjugate vaccine, generates a robust anti-Abeta antibody response and attenuates Abeta pathology and cognitive deficits in APPswe/PS1DeltaE9 transgenic mice. *J Neurosci*. 2013; 33:7027–37. [PubMed: 23595760]
- Mably AJ, Kanmert D, Mc Donald JM, Liu W, Caldarone BJ, Lemere CA, et al. Tau immunization: a cautionary tale? *Neurobiol Aging*. 2015; 36:1316–32. [PubMed: 25619661]
- Mandler M, Rockenstein E, Ubhi K, Hansen L, Adame A, Michael S, et al. Detection of peri-synaptic amyloid-beta pyroglutamate aggregates in early stages of Alzheimer's disease and in AbetaPP transgenic mice using a novel monoclonal antibody. *J Alzheimers Dis*. 2012; 28:783–94. [PubMed: 22064070]
- Mandler M, Walker L, Santic R, Hanson P, Upadhaya AR, Colloby SJ, et al. Pyroglutamylated amyloid-beta is associated with hyperphosphorylated tau and severity of Alzheimer's disease. *Acta Neuropathol*. 2014; 128:67–79. [PubMed: 24861310]
- McGowan E, Pickford F, Kim J, Onstead L, Eriksen J, Yu C, et al. Abeta42 is essential for parenchymal and vascular amyloid deposition in mice. *Neuron*. 2005; 47:191–9. [PubMed: 16039562]
- Miravalle L, Calero M, Takao M, Roher AE, Ghetti B, Vidal R. Amino-terminally truncated Abeta peptide species are the main component of cotton wool plaques. *Biochemistry*. 2005; 44:10810–21. [PubMed: 16086583]
- Morawski M, Schilling S, Kreuzberger M, Waniek A, Jager C, Koch B, et al. Glutaminy cyclase in human cortex: correlation with (pGlu)-amyloid-beta load and cognitive decline in Alzheimer's disease. *J Alzheimers Dis*. 2014; 39:385–400. [PubMed: 24164736]
- Nicoll JA, Wilkinson D, Holmes C, Steart P, Markham H, Weller RO. Neuropathology of human Alzheimer disease after immunization with amyloid-beta peptide: a case report. *Nature medicine*. 2003; 9:448–52.
- Nussbaum JM, Schilling S, Cynis H, Silva A, Swanson E, Wangsanut T, et al. Prion-like behaviour and tau-dependent cytotoxicity of pyroglutamylated amyloid-beta. *Nature*. 2012; 485:651–5. [PubMed: 22660329]
- Park JH, Gimbel DA, GrandPre T, Lee JK, Kim JE, Li W, et al. Alzheimer precursor protein interaction with the Nogo-66 receptor reduces amyloid-beta plaque deposition. *J Neurosci*. 2006; 26:1386–95. [PubMed: 16452662]
- Pfeifer M, Boncristiano S, Bondolfi L, Stalder A, Deller T, Staufenbiel M, et al. Cerebral hemorrhage after passive anti-Abeta immunotherapy. *Science*. 2002; 298:1379. [PubMed: 12434053]
- Piccini A, Russo C, Gliozzi A, Relini A, Vitali A, Borghi R, et al. beta-amyloid is different in normal aging and in Alzheimer disease. *J Biol Chem*. 2005; 280:34186–92. [PubMed: 16103127]

- Pivtoraiko VN, Abrahamson EE, Leurgans SE, DeKosky ST, Mufson EJ, Ikonomic MD. Cortical pyroglutamate amyloid-beta levels and cognitive decline in Alzheimer's disease. *Neurobiol Aging*. 2014
- Puolivali J, Wang J, Heikkinen T, Heikkilä M, Tapiola T, van Groen T, et al. Hippocampal A beta 42 levels correlate with spatial memory deficit in APP and PS1 double transgenic mice. *Neurobiol Dis*. 2002; 9:339–47. [PubMed: 11950278]
- Puzzo D, Arancio O. Amyloid-beta peptide: Dr. Jekyll or Mr. Hyde? *J Alzheimers Dis*. 2013; 33(Suppl 1):S111–20. [PubMed: 22735675]
- Puzzo D, Privitera L, Fa M, Staniszewski A, Hashimoto G, Aziz F, et al. Endogenous amyloid-beta is necessary for hippocampal synaptic plasticity and memory. *Ann Neurol*. 2011; 69:819–30. [PubMed: 21472769]
- Puzzo D, Privitera L, Leznik E, Fa M, Staniszewski A, Palmeri A, et al. Picomolar amyloid-beta positively modulates synaptic plasticity and memory in hippocampus. *J Neurosci*. 2008; 28:14537–45. [PubMed: 19118188]
- Racke MM, Boone LI, Hepburn DL, Parsadainian M, Bryan MT, Ness DK, et al. Exacerbation of cerebral amyloid angiopathy-associated microhemorrhage in amyloid precursor protein transgenic mice by immunotherapy is dependent on antibody recognition of deposited forms of amyloid beta. *J Neurosci*. 2005; 25:629–36. [PubMed: 15659599]
- Rijal Upadhaya A, Kosterin I, Kumar S, von Arnim CA, Yamaguchi H, Fandrich M, et al. Biochemical stages of amyloid-beta peptide aggregation and accumulation in the human brain and their association with symptomatic and pathologically preclinical Alzheimer's disease. *Brain*. 2014; 137:887–903. [PubMed: 24519982]
- Russo C, Violani E, Salis S, Venezia V, Dolcini V, Damonte G, et al. Pyroglutamate-modified amyloid beta-peptides--AbetaN3(pE)--strongly affect cultured neuron and astrocyte survival. *J Neurochem*. 2002; 82:1480–9. [PubMed: 12354296]
- Saido TC, Iwatsubo T, Mann DM, Shimada H, Ihara Y, Kawashima S. Dominant and differential deposition of distinct beta-amyloid peptide species, A beta N3(pE), in senile plaques. *Neuron*. 1995; 14:457–66. [PubMed: 7857653]
- Schieb H, Kratzin H, Jahn O, Mobius W, Rabe S, Staufienbiel M, et al. Beta-amyloid peptide variants in brains and cerebrospinal fluid from amyloid precursor protein (APP) transgenic mice: comparison with human Alzheimer amyloid. *J Biol Chem*. 2011; 286:33747–58. [PubMed: 21795681]
- Schilling S, Hoffmann T, Manhart S, Hoffmann M, Demuth HU. Glutaminyl cyclases unfold glutamyl cyclase activity under mild acid conditions. *FEBS Lett*. 2004; 563:191–6. [PubMed: 15063747]
- Schilling S, Zeitschel U, Hoffmann T, Heiser U, Francke M, Kehlen A, et al. Glutaminyl cyclase inhibition attenuates pyroglutamate Abeta and Alzheimer's disease-like pathology. *Nature medicine*. 2008; 14:1106–11.
- Schlenzig D, Manhart S, Cinar Y, Kleinschmidt M, Hause G, Willbold D, et al. Pyroglutamate formation influences solubility and amyloidogenicity of amyloid peptides. *Biochemistry*. 2009; 48:7072–8. [PubMed: 19518051]
- Seubert P, Barbour R, Khan K, Motter R, Tang P, Kholodenko D, et al. Antibody capture of soluble Abeta does not reduce cortical Abeta amyloidosis in the PDAPP mouse. *Neuro-degenerative diseases*. 2008; 5:65–71. [PubMed: 18182780]
- Solomon B, Koppel R, Frankel D, Hanan-Aharon E. Disaggregation of Alzheimer beta-amyloid by site-directed mAb. *Proc Natl Acad Sci U S A*. 1997; 94:4109–12. [PubMed: 9108113]
- Soscia SJ, Kirby JE, Washicosky KJ, Tucker SM, Ingelsson M, Hyman B, et al. The Alzheimer's disease-associated amyloid beta-protein is an antimicrobial peptide. *PLoS One*. 2010; 5:e9505. [PubMed: 20209079]
- Sperling R, Salloway S, Brooks DJ, Tampieri D, Barakos J, Fox NC, et al. Amyloid-related imaging abnormalities in patients with Alzheimer's disease treated with bapineuzumab: a retrospective analysis. *Lancet Neurol*. 2012; 11:241–9. [PubMed: 22305802]
- Sullivan CP, Berg EA, Elliott-Bryant R, Fishman JB, McKee AC, Morin PJ, et al. Pyroglutamate-Abeta 3 and 11 colocalize in amyloid plaques in Alzheimer's disease cerebral cortex with

- pyroglutamate-Aβeta 11 forming the central core. *Neurosci Lett*. 2011; 505:109–12. [PubMed: 22001577]
- Tekirian TL, Saido TC, Markesbery WR, Russell MJ, Wekstein DR, Patel E, et al. N-terminal heterogeneity of parenchymal and cerebrovascular Aβeta deposits. *J Neuropathol Exp Neurol*. 1998; 57:76–94. [PubMed: 9600199]
- Wilcock DM, Rojiani A, Rosenthal A, Levkowitz G, Subbarao S, Alamed J, et al. Passive amyloid immunotherapy clears amyloid and transiently activates microglia in a transgenic mouse model of amyloid deposition. *J Neurosci*. 2004a; 24:6144–51. [PubMed: 15240806]
- Wilcock DM, Rojiani A, Rosenthal A, Subbarao S, Freeman MJ, Gordon MN, et al. Passive immunotherapy against Aβeta in aged APP-transgenic mice reverses cognitive deficits and depletes parenchymal amyloid deposits in spite of increased vascular amyloid and microhemorrhage. *J Neuroinflammation*. 2004b; 1:24. [PubMed: 15588287]
- Wirths O, Bethge T, Marcello A, Harmeier A, Jawhar S, Lucassen PJ, et al. Pyroglutamate Aβeta pathology in APP/PS1KI mice, sporadic and familial Alzheimer's disease cases. *J Neural Transm*. 2010a; 117:85–96. [PubMed: 19823761]
- Wirths O, Breyhan H, Cynis H, Schilling S, Demuth HU, Bayer TA. Intraneuronal pyroglutamate-Aβeta 3-42 triggers neurodegeneration and lethal neurological deficits in a transgenic mouse model. *Acta Neuropathol*. 2009; 118:487–96. [PubMed: 19547991]
- Wirths O, Erck C, Martens H, Harmeier A, Geumann C, Jawhar S, et al. Identification of low molecular weight pyroglutamate A{βeta} oligomers in Alzheimer disease: a novel tool for therapy and diagnosis. *J Biol Chem*. 2010b; 285:41517–24.
- Wu G, Miller RA, Connolly B, Marcus J, Renger J, Savage MJ. Pyroglutamate-Modified Amyloid-beta Protein Demonstrates Similar Properties in an Alzheimer's Disease Familial Mutant Knock-In Mouse and Alzheimer's Disease Brain. *Neurodegener Dis*. 2014; 14:53–66. [PubMed: 24158021]
- Yamada K, Yabuki C, Seubert P, Schenk D, Hori Y, Ohtsuki S, et al. Aβeta immunotherapy: intracerebral sequestration of Aβeta by an anti-Aβeta monoclonal antibody 266 with high affinity to soluble Aβeta. *J Neurosci*. 2009; 29:11393–8. [PubMed: 19741145]

Highlights

- pyroglutamate-3 A β is emerging as a therapeutic target in Alzheimer's Disease
- a pyroglutamate-3 A β antibody reduces brain A β plaques in APPswe/PS1 E9 without microbleeds
- rescue of cognitive deficits in two hippocampal-dependent learning tests
- pan-A β antibody showed similar A β reduction but less cognitive benefit
- our novel vaccine may be a safe and effective treatment for Alzheimer's disease

Fig 1.

07/1 IgG1 monoclonal antibody (mAb) specifically recognizes Aβ starting at pyroGlu-3 while 3A1, a general IgG1 mAb, recognizes a non-pGlu epitope within the first 15 amino acids of Aβ. Synthetic Aβ peptides of varying lengths were immobilized on a 0.2 μm nitrocellulose membrane at 1000, 100, 10 and 0.1 ng concentrations, respectively. Membranes were probed with either 07/1 mAb at 0.54 μg/ml (A) or 3A1 mAb at 0.2 μg/ml (B). 07/1 mAb was shown to only detect AβpE3-9 and Aβp3-40 synthetic peptides (A) while 3A1 mAb recognized Aβ1-15 and Aβ1-40 but not pGlu-3 Aβ isoforms (B).

Fig 2.

Preventive passive immunization with a pGlu-3 mAb partially protects against cognitive deficits in APPswe/PS1 E9. At 12-13 months of age, APPswe/PS1 E9 Tg mice and age- and gender-matched wild-type (Wt) controls were tested in three behavioral paradigms, Open Field (A), Contextual Fear Conditioning (CFC) (B-C) and the Water T Maze (WTM) (D-G). In Open Field, all APPswe/PS1 E9 mice showed a significantly greater distance travel in the testing arena during the first 10 min ($p < 0.05$), however there were no differences in distance traveled between APPswe/PS1 E9 mice and Wt controls from 10-60

min (A). In CFC, during the training phase on day 1 (d1), all APPswe/PS1 E9 Tg mice displayed a significantly lower percent freezing after foot shocks 1 and 2 than Wt controls ($p<0.05$), however there were no antibody treatment effects (B). During the CFC context test, APPswe/PS1 E9 PBS-injected mice showed a significantly lower ($p<0.05$) percent freezing than Wt controls, which was also observed in APPswe/PS1 E9 3A1-immunized mice ($p<0.05$). APPswe/PS1 E9 mice 150 μ g 07/1-immunized mice showed a small trend ($p<0.099$) for improvement in contextual fear conditioning compared to Wt mice but were not statistically different from APPswe/PS1 E9 PBS-injected mice (C). In the acquisition phase of the WTM, APPswe/PS1 E9 PBS-injected mice had significantly fewer ($p<0.05$) correct responses than Wt controls on days 3-7 (D) and required significantly more ($p<0.05$) days to reach acquisition criterion than Wt littermates (E). Conversely, APPswe/PS1 E9 500 μ g 07/1-immunized mice achieved acquisition criterion faster ($p<0.05$) than APPswe/PS1 E9 PBS-injected mice and were not significantly different than Wt controls (E). During the reversal trial of the WTM, APPswe/PS1 E9 PBS-injected mice showed significantly fewer correct responses ($p<0.05$) (F) and took significantly longer ($p<0.05$) (G) to reach reversal criterion than Wt controls, however no antibody treatment improved reversal learning and memory (F,G). * indicates $p<0.05$. 1. One mouse from the 500 μ g 07/1-immunized group was excluded from statistical analyses for Open Field because it was 2.8 SD from the mean.

Fig 3.

Prophylactic passive immunization with 07/1 mAb at 150 and 500 μ g doses significantly lowered pGlu-3 A β deposition as well as general A β and A β 1-x species but not Thioflavin S-positive plaques (ThioS) in the brains of APPswe/PS1 E9 Tg mice. Representative photomicrographs of hippocampus from each treatment groups of serial sections immunolabeled with a general A β polyclonal antibody, R1282 (A-D), 82E1 mAb, which specifically recognizes A β starting at Asp1 (E-H), 07/2, a novel pGlu-3 A β IgG2b monoclonal antibody (I-L) and stained with ThioS for fibrillar amyloid (M-P). Quantitative

image analysis was performed on six stained sections at three equidistant planes for each using the aforementioned markers. In hippocampus, significant reductions in general A β , A β 1-x and pGlu-3 deposition we appreciated in 150 and 500 μ g 07/1-immunized mice as well as 200 μ g 3A1-immunized Tg mice compared to PBS-treated Tg controls (Q). In addition, significant reductions in general and pGlu-3 A β deposition were found in the frontal cortex in 150 μ g and 500 μ g 07/1-immunized mice and also in 3A1-immunized mice compared to PBS-treated Tg controls (R). In frontal cortex, no significant differences in A β 1-x deposition or ThioS-positive plaques at either dose of the 07/1 antibody was observed, however, there were significant reductions in A β 1-x and Thio S plaque burden in the frontal cortex of 3A1-immunized mice (R). In cerebellum, a significant reduction in general A β staining was observed in both 150 μ g and 500 μ g 07/1-immunized Tg mice and 3A1-immunized Tg mice compared to PBS-controls (S). When quantifying A β 1-x deposition, significant reductions were noted in 150 μ g 07/1-immunized and 3A1-immunized Tg mice compared to PBS-treated Tg controls. No significant changes in pGlu-3 A β deposition were found in the hippocampus with any treatment and only 3A1-immunized mice showed reductions in Thio S staining in cerebellum compared PBS controls (S). All data are expressed as the mean $\pm$ SEM. * indicates $p < 0.05$, ** indicates $p < 0.01$ and *** indicates $p < 0.001$.

Fig 4.

Vaccination with 3A1 mAb elicited a dramatic increase in plasma Aβ levels, while neither dose of 07/1 mAb caused changes in plasma Aβ at sacrifice. Aβ1-x, x-38, x-40 and x-42 levels were measured by sandwich ELISA in terminal plasma samples from baseline (6 mo) and injected mice (13 mo). Interestingly, plasma Aβ levels did not change from early stages of cerebral Aβ plaque deposition in baseline mice, PBS-injected transgenic mice or after immunization with 150 or 500μg 07/1 mAb (A-D); however, there was a very significant increase in plasma Aβ1-x ($p < 0.001$), x-38 ($p < 0.001$), x-40 ($p < 0.001$) and x-42 ($p < 0.001$) in 3A1-immunized APPswe/PS1 E9 mice compared to PBS-injected APPswe/PS1 E9 controls (A-D). All data are represented as the mean $\pm$ SEM. *** indicates $p < 0.001$.

Fig 5.

Immunohistochemical analysis of microgliosis in hippocampus. Adjacent serial sections were immunolabeled with a general A β polyclonal antibody, R1282 (A,D,G,J), Iba1 for resting and activated microglia (B,E,H,K) and CD68, a marker for activated microglia of a phagocytic phenotype (C,F,I,L). Representative photomicrographs from one mouse per group show that both Iba1 and CD68-positive microglia colocalized with general A β in all groups, however, overall there were no changes in Iba1 IR microglia between immunized (E,H,K) and PBS-treated Tg mice (B). It was evident that there was a reduction in plaque-associated CD68-positive microglia in 3A1-immunized Tg mice (F) compared to PBS-treated Tg controls (C) but not with either dose of the 07/1 mAb (I,L). Quantitative image analysis of six immunolabeled section, at three equidistant planes, of Iba1 IR in the entire hippocampus confirmed there were no changes in Iba1-positive microglia between groups (M). Quantification of CD68 immunoreactivity in hippocampus revealed a significant reduction ($p<0.05$) in CD68-positive microglia in 3A1-immunized Tg mice compared to PBS-treated Tg controls, while no changes were noted in 07/1-immunized Tg mice at either dose (N). All data are reported as the mean $\pm$ SEM. * indicates $p<0.05$.

Cerebral Aβ Biochemical Analysis

TABLE 1

Group	Fraction	Hippocampus ^a		Cortex ^a		Cerebellum ^b	
		Aβx-42	AβpE3-42	Aβx-42	AβpE3-42	Aβx-42	AβpE3-42
PBS	detergent-soluble	6.3 ± 0.8	33 ± 4	13.2 ± 4.6	64 ± 18	33 ± 0.3	undetectable
3A1 200μg	detergent-soluble	6.4 ± 0.9	36 ± 5	13.4 ± 4.3	65 ± 16	65 ± 41 ^{**}	undetectable
07/1 150μg	detergent-soluble	6.5 ± 0.4	36 ± 4	14.8 ± 3.7	64 ± 19	47 ± 38 [*]	undetectable
07/1 500μg	detergent-soluble	6.6 ± 0.7	37 ± 5	12.8 ± 5.7	61 ± 16	165 ± 133 ^{***}	undetectable
PBS	insoluble	4.8 ± 0.9	33 ± 1	7.5 ± 2.2	33 ± 6	4 ± 1	0.1 ± 0.01
3A1 200μg	insoluble	5.9 ± 0.8	36 ± 2	6.2 ± 3.0	28 ± 8	4 ± 0.2	0.1 ± 0.01
07/1 150μg	insoluble	5.3 ± 0.5	36 ± 1	5.9 ± 2.6	26 ± 9	4 ± 0.3	0.1 ± 0.02
07/1 500μg	insoluble	6.2 ± 0.6	33 ± 2	6.8 ± 2.8	28 ± 6	5 ± 0.8	0.02 ± 0.01

^a Detergent-soluble fraction is represented as the sodium dodecyl sulfate extract and the insoluble as the formic acid extract. All Aβx42 levels are presented in μg/g brain weight as the mean ± SEM and AβpE3-42 levels are presented in ng/g brain weight as the mean ± SEM.

^b Detergent-soluble fraction is represented as the T-PER extract and the insoluble as the guanidine-hydrochloride extract. Both Aβx42 and AβpE3-42 data are represented in ng/ml as the mean ± SEM.

* indicates p<0.05

** indicates p<0.01

*** indicates p<0.001.