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Days-to-criterion as an indicator of toxicity associated with human Alzheimer amyloid- β oligomers

Sam Gandy, M.D., Ph.D.^{1,2,¶}, Adam J. Simon, Ph.D.^{3,*}, John W. Steele, B.A.^{1,*}, Alex L. Lublin, Ph.D.^{1,*}, James J. Lah, M.D., Ph.D.⁴, Lary C. Walker, Ph.D.^{4,5}, Allan I. Levey, M.D., Ph.D.⁴, Grant A. Krafft, Ph.D.⁶, Efrat Levy, Ph.D.⁷, Frédéric Checler, Ph.D.⁸, Charles Glabe, Ph.D.⁹, Warren Bilker, Ph.D.¹⁰, Ted Abel, Ph.D.¹¹, James Schmeidler, Ph.D.¹², and Michelle E. Ehrlich, M.D.¹³

Sam Gandy: samuel.gandy@mssm.edu; Adam J. Simon: adam.jay.simon@gmail.com; John W. Steele: john.steele@mssm.edu; Alex L. Lublin: alex.lublin@mssm.edu; James J. Lah: james.lah@emory.edu; Lary C. Walker: lary.walker@emory.edu; Allan I. Levey: allan.levey@emory.edu; Grant A. Krafft: gkrafft@acumenpharm.com; Efrat Levy: evely@nki.rfmh.org; Frédéric Checler: checler@ipmc.cnrs.fr; Charles Glabe: cglabe@uci.edu; Warren Bilker: warren@mail.med.upenn.edu; Ted Abel: abele@sas.upenn.edu; James Schmeidler: james.schmeidler@mssm.edu; Michelle E. Ehrlich: michelle.ehrlich@mssm.edu

¹ Departments of Neurology and Psychiatry and Alzheimer's Disease Research Center, Mount Sinai School of Medicine, New York, NY 10029

² James J. Peters VA Medical Center, Bronx, NY 10461

³ Neuronostics, Yardley, PA, 19067

⁴ Department of Neurology, Center for Neurodegenerative Disease, Emory University, Atlanta, GA 30322

⁵ Yerkes Primate Research Facility, Emory University, Atlanta, GA 30322

⁶ Acumen Pharmaceuticals, Inc., Livermore, CA 94551

⁷ Center for Dementia Research, New York University and the Nathan S. Kline Institute for Psychiatric Research, Orangeburg, NY 10962

⁸ Institute de Pharmacologie Moleculaire et Cellulaire, Valbonne, France 06560

⁹ Department of Neurology, University of California Irvine School of Medicine, CA 92697

[¶]To whom correspondence should be addressed to: Sam Gandy, M.D., Ph.D., Departments of Neurology and Psychiatry, Mount Sinai School of Medicine, One Gustave L. Levy Place, Box 1137, New York, NY 10029 USA, samuel.gandy@mssm.edu.

*These authors contributed equally

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S.G., J.W.S., and A.L. prepared the manuscript. J.W.S., W.B., A.L.L. and J.S. performed statistical analysis of all data. L.C.W. and F.C. contributed to the histological analyses. A.J.S. and G.K. designed and performed ADDL ELISAs. C.G., T.A., E.L., G.K., L.C.W., A.I.L., and M.E.E., provided critical reading of the manuscript and were instrumental in the design and execution of histology, microscopy, and behavioral experiments. M.E.E. and S.G. procured funding for the project.

¹⁰ Department of Psychiatry, University of Pennsylvania, Philadelphia PA 19104

¹¹ Department of Biology, University of Pennsylvania, Philadelphia PA 19104

¹² Department of Psychiatry, Mount Sinai School of Medicine, New York, NY 10029

¹³ Departments of Neurology and Pediatrics and Alzheimer's Disease Research Center, Mount Sinai School of Medicine, New York, NY 10029

Abstract

Objectives—Recent evidence suggests that high molecular weight soluble oligomeric A β (oA β) assemblies (also known as A β -derived diffusible ligands, or ADDLs) may represent a primary neurotoxic basis for cognitive failure in AD. To date, *in vivo* studies of oA β /ADDLs have involved injection of assemblies purified from the cerebrospinal fluid (CSF) of human subjects with Alzheimer's disease or from the conditioned media of A β -secreting cells into experimental animals. We sought to study the bioactivities of endogenously formed oA β /ADDLs generated *in situ* from the physiological processing of human APP transgenes.

Methods—We produced and histologically characterized single transgenic mice overexpressing APP^{E693Q} or APP^{E693Q} X PS1 Δ E9 bigenic mice. APP^{E693Q} mice were studied in the Morris water maze (MWM) task at 6 and 12 months of age. Following the second MWM evaluation, mice were sacrificed, and brains were assayed for A β total, A β 40, A β 42, and oA β /ADDL by ELISA and were also histologically examined. Based on results from the oA β /ADDL ELISA, we assigned individual APP^{E693Q} mice to either an “undetectable oA β /ADDLs group” or a “readily detectable oA β /ADDLs group”. A days-to-criterion (DTC) analysis was used to determine delays in acquisition of the MWM task.

Results—Both single transgenic and bigenic mice developed intraneuronal accumulation of APP/A β , though only Dutch APP^{E693Q} X PS1 Δ E9 bigenic mice developed amyloid plaques. The APP^{E693Q} mice did not develop amyloid plaques at any age studied, up to 30 months. APP^{E693Q} mice were tested for spatial learning and memory, and only 12-month old APP^{E693Q} mice with readily detectable oA β /ADDLs displayed a significant delay in acquisition of the MWM task when compared to NTg littermates.

Interpretation—These data suggest that cerebral oA β /ADDL assemblies generated in brain *in situ* from human APP transgenes may be associated with cognitive impairment. We propose that a DTC analysis may be a sensitive method for assessing the cognitive impact in mice of endogenously generated oligomeric human A β assemblies.

SEARCH TERMS

(1) Amyloid; (2) Alzheimer's Disease; (3) Spatial Recognition; (4) Days-to-Criterion; (5) Amyloid Precursor Protein

INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disorder and is the most common cause of senile dementia. Rare familial forms of AD are caused by genes that modulate metabolism of the amyloid- β peptide (A β) (for review, see ref. 1), and progression of all forms of AD involves the accumulation in brain of insoluble spherical deposits of aggregated A β known as amyloid plaques². A reformulation of the amyloid cascade hypothesis has shifted focus from the hallmark amyloid plaques to high molecular weight soluble assemblies of oligomeric A β (oA β , also known as A β -derived diffusible ligands; ADDLs)^{3–7} as the proximate neurotoxins underlying AD.

Recent evidence has implicated $\alpha\text{A}\beta/\text{ADDLs}$ β in cognitive decline^{8,9}. Electrophysiological studies have shown that addition of $\alpha\text{A}\beta/\text{ADDLs}$ to hippocampal slices results in an inhibition of long-term potentiation (LTP), a cellular model of learning and memory⁶. These results were corroborated *in vivo* via demonstration of deficits in learning and memory performance following injection of $\alpha\text{A}\beta/\text{ADDLs}$ directly into the hippocampi of living rats^{4,10}.

In this report, we utilized an *in vivo* model of AD that produce soluble $\alpha\text{A}\beta/\text{ADDLs}$ β either with (APP^{E693Q} X PS1 Δ E9) or without (APP^{E693Q}) β -amyloid plaques in the brain¹¹. We show that the levels of $\alpha\text{A}\beta/\text{ADDLs}$ are associated with impaired acquisition of the Morris water maze (MWM) task by APP^{E693Q} mice. We propose that days-to-criterion (DTC) analyses might be especially sensitive for assessing deficits associated with $\alpha\text{A}\beta/\text{ADDLs}$ generated from the physiological processing of transgenic human APP.

METHODS

Experimental animals

Generation of C57BL/6J-TgN(Thy1-APP^{E693Q}, APP751 numbering) transgenic mice was performed as described by Gandy *et al* (2007)¹². Briefly, pTSC21, the mouse Thy1.2 expression cassette¹³ was digested and blunt-ended at the unique *Xho*I site, and the APP751^(E693Q) cDNA (provided by Dr. Efrat Levy, New York University)¹⁴ was inserted into the Thy1.2 cassette. The 5' end of the cDNA was modified to introduce a Kozak sequence, with primers

5'GCCCCGCGCAGGGGCGCCATGCTGC

CCGGTTTG-3' and 5'CGGGGCGCGTCC

CCGCGGTACGACGGGCCAAAC-3', using the Quik Change Site-Directed Mutagenesis kit (Stratagene). The DNA for injection was released with *Pvu*II, purified from an agarose gel, dialyzed and injected following routine protocol. Generation of transgenic PS1 Δ E9 mice was previously described¹². Experimentally naïve male and female non-transgenic littermates (NTg n=8), APP^{E693Q} single transgenic mice (n=17), or APP^{E693Q} X PS1 Δ E9 bigenic mice (n=12) were maintained and bred under standard conditions consistent with National Institutes of Health guidelines for animal care and approved by the Institutional Animal Care and Use Committee of the James J. Peters Veterans Affairs Medical Center. Mice were handled for 2 minutes per day for 3 days prior to pre-training on MWM.

Western blot analysis of APP expression

Brains were homogenized on ice and extracts were denatured in SDS loading buffer. Samples were separated by standard SDS-polyacrylamide gel electrophoresis and electrophoretically transferred to polyvinylidene fluoride membrane (Millipore, Bedford, MA). The following primary antibodies were used: pan-species anti-APP C-terminus specific pAb369 (previously described¹⁵) or anti-human $\text{A}\beta_{1-16}$ human APP/ $\text{A}\beta$ specific mAb6E10 (Covance).

Immunohistochemistry and Electron Microscopy

Mice were anesthetized by CO₂ exposure and transcardially perfused with cold saline, followed by fixation in 4% phosphate-buffered paraformaldehyde. Coronal sections (40 μ m) were cut with a Leica vibratome 2000 (Nussloch, Germany), cryoprotected, and stored at -20°C. Cresyl violet, hematoxylin and eosin (H&E) staining are done according to standard protocols. For light microscopy, tissue blocks were frozen on dry ice and sectioned at 40 μ m on a freezing microtome. For electron microscopy, blocks of forebrain, motor cortex,

hippocampus, and cerebellum were sectioned at 40 μ m on a vibratome (Technical Products International, St. Louis, MO).

Immunohistochemical processing was performed with free-floating sections and immunoperoxidase using previously described methods¹⁶. The following antibodies were used: mAb 4G8 and mAb 6E10 (Covance), polyclonal antibodies to the A β carboxy terminus [polyclonal antibody FCA3340 and FCA3542 specific for A β 40 or A β 42, respectively, generous gift from F. Checler] and anti-GLUT4 (Chemicon). Biotinylated goat anti-rat IgG (1:200 dilution; Vector Labs, Burlingame, CA) and avidin-biotinylated horseradish peroxidase complex (Vectastain Elite, Vector Labs) were used to localize the primary antibody. Immunoreactivity was visualized with 0.05% 3,3-diaminobenzidine tetrahydrochloride (DAB) and 0.01% H₂O₂ in 50 mM Tris, pH 7.6. Sections for light microscopy were slide-mounted, air-dried, dehydrated through a graded alcohol series and xylenes, and, finally, coverslipped for microscopic examination. Sections for electron microscopic immunohistochemistry were post-fixed in 1% osmium tetroxide, stained with 2% uranyl acetate, dehydrated through graded alcohol and propylene oxide, and embedded in Eponate 12 resin. Ultrathin sections were cut on a Reichert Ultracut S ultramicrotome (Leica, Deerfield, IL) and collected on mesh copper grids for examination on a JEOL JEM-100C transmission electron microscope. Control sections were processed in parallel, in which the primary antibody was either omitted or pre-absorbed with the corresponding antigen. Sections for electron microscopic immunohistochemistry were post-fixed in 1% osmium tetroxide, stained with 2% uranyl acetate, dehydrated through graded alcohol and propylene oxide, and embedded in Eponate 12 resin.

Thioflavin-S staining

Floating sections were washed in PBS and mounted on Superfrost Plus slides coated with Vectabond (Vector Laboratories, Burlingame, CA), before being processed for Thioflavin-S (Thio-S). Briefly, sections were post-fixed in 10% formalin for 10 minutes, then washed in PBS. After incubation for 10 minutes in 0.25% potassium permanganate, sections were washed in PBS and incubated in 2% potassium metabisulfite and 1% oxalic acid until they appeared white. Sections were then washed in water and stained for 10 minutes with a solution of 0.015% Thio-S in 50% ethanol. Finally, sections were washed in 50% ethanol and in water, then dried, and dipped into Histo-Clear before being coverslipped with Permount. All chemicals were from Sigma (St. Louis, MO). Finally, the sections were coverslipped with Vectashield (Vector Laboratories), and sealed with nail polish.

Analysis of cerebral microhemorrhage

Cerebral hemorrhage, when present, is typically accompanied by a delayed appearance of hemosiderin-positive microglia¹⁷. Perls's Berlin blue-stained clusters of hemosiderin staining were qualitatively evaluated (presence/absence) from sections throughout the neocortex, hippocampus, and thalamus. An additional set of every 10th section was stained for H&E and screened for acute intraparenchymal hemorrhage.

A β ELISAs (A β 40, A β 42, oligomeric A β /ADDLs)

A β was detected by incubating horseradish peroxidase-conjugated JRF/Atot/17 (human A β) or JRF/rA1-15/2 (murine A β) as detection antibody. For ELISA determination of oA β /ADDLs, the identical monoclonal antibody (6E10) was used for both capture and detection¹⁸. Therefore, only species with at least two mAb6E10 epitopes were detected. ELISA plates were developed using a color reaction (TMB Microwell Peroxidase Substrate System, Kirkegaard & Perry, Gaithersburg, MD), and the A450 was read and quantified by comparison to covalently cross-linked A β dimer standards.

Morris water maze behavioral analysis

Experimentally naïve mice were trained on the MWM task at 6 months of age, extinguished, then trained and tested again at 12 months of age, all according to a standard protocol¹⁹. The water maze was a circular pool (120 cm in diameter). White nontoxic tempera paint was mixed with water to make the water opaque. Hidden 0.5 cm beneath the surface of the water was a circular platform (11.2 cm in diameter). The path of the mouse was recorded with a video tracking system (HVS Image, Buckingham, UK). The water maze was located in a 5.2 m × 2.1-m room. There were different cues on each wall of the room: along one wall is a 90 cm × 60-cm poster; along another wall is a coat rack and a 30 cm × 30-cm black triangle; along the third wall is a deflated multicolored inner tube, measuring 45 cm in diameter; and hung in the center of the curtain is a 30-cm diameter inflated yellow ball. A video camera was mounted above the center of the pool. During pre-training, mice were trained to sit on the platform. This training occurred in a 5-gallon (19-L) bucket in a room that was different from the experimental room. Pre-training consisted of three trials. In the first trial, mice were placed into the bucket and allowed to swim to a visible platform located in the center of the bucket, where they sat for 30 s. The second trial was identical to the first, except that the platform is submerged 0.5 cm beneath the surface of the water. The third trial was identical to the second, except that mice sat on the platform for 60s.

During the training/acquisition phase, on 12 consecutive days, mice received four trials per day during which the platform was hidden 0.5 cm beneath the surface of the water in a constant location (one of two locations were used, balanced across subjects). A different starting location was used on each trial, which consists of a swim followed by a 20-s platform sit. Any mouse that does not find the platform within 60s was guided to it by the experimenter. The inter-trial interval (ITI) is 4–6 min (this ITI is used in all subsequent experiments). During the ITI, mice sat in their home cages, which are kept near the computer and out of sight of the water maze throughout each session. The 4-6-min ITI was long enough for the mice to dry themselves before the next trial.

The MWM task was extinguished by standard extinction protocol at 6 months. Briefly, four 60-s trials in which they swam in the pool in the absence of the platform, but shower curtains were hung around the pool to block the distal cues in the room from view. Care is taken to ensure that mice are removed from different locations on each trial. Preference during extinction and probe trials was assessed by analyzing time spent searching in the target quadrant compared with time spent searching in the other three quadrants. Mice were sacrificed and analyzed at 13 months of age, following MWM testing at 12 months and two probe trials. ANOVAs at each time point were used to determine whether there exists a link between MWM and levels of A β species in each brain region. Mice were group housed with ad libitum access to food and water and maintained on a 12:12 light:dark cycle with lights on at 7AM. All experiments were conducted during the light period between the hours of 9AM and 5PM.

Statistical analysis of MWM behavior

An overhead video camera was used to capture swim pattern and time in each quadrant for all mice during training/acquisition and probe trials. Average escape latency was calculated for each animal on each day of the training/acquisition period. For probe trials, time spent in each quadrant was calculated. A days-to-criterion (DTC) analysis has been previously used to analyze acquisition of the MWM task²⁰. Mice met criterion if they had escape latencies of less than 25s on two consecutive trials, indicating reliable performance of the task. The 25s criterion was based on the third quartile escape latencies for day 11 of training, indicating that at least 75% of animals tested found the escape platform within approximately 25s

(quartile ranks not shown). Each animal received a DTC score of up to 12 reflecting the day on which they met criterion for acquisition of the task.

Repeated measures analysis of variance (ANOVA) was utilized to analyze escape latency for the 12 days of training/acquisition. Multivariate ANOVA (MANOVA) was used to compare escape latency from each day of training/acquisition with animals grouped by genotype or ADDL level. For DTC analysis, a fourth root ($x^{0.25}$) transformation of DTC score was used to account for long right tail distribution, allowing for assumption of normal distribution in subsequent parametric tests. One-way ANOVAs were used to compare mean group differences on probe trials and also for DTC analysis. Levene's statistic was computed to determine homogeneity of variance between groups. For all ANOVAs, a Bonferroni's (homogeneity of variance assumed) or a Dunnett's T3 (homogeneity of variance not assumed) multiple comparisons was used to determine between-groups differences for ADDL level and independent samples *t*-tests were used to compare APP^{E693Q} versus NTg mice for DTC and at probe trials. A Kaplan-Meier survival analysis and Mantel-Cox log rank multiple comparisons were employed to further analyze between-groups differences for DTC by ADDL level (see also Supplementary Table 2*b–d*). These tests, which used untransformed data, were primarily utilized to validate the fourth-root-transformed mean DTC data for use in parametric tests. Significance is reported for all tests with a $p \leq 0.05$ using two-tailed $\alpha = 0.05$; *p*-values for all tests are reported in Supplementary Table 2.

RESULTS

Biochemical characterization of APP^{E693Q} mice

Several missense mutations within the A β domain of the amyloid precursor protein (APP) have been associated with an increase in the propensity of the peptide to form oA β /ADDL assemblies²¹. All these mutations are located near the middle of the A β domain, where they have been proposed to disrupt salt bridges that, when present, stabilize parallel β -sheets and promote fibrillogenesis. The model suggests that, because the salt bridges cannot form, fibrillogenesis is destabilized, and the formation of oA β /ADDL assemblies is favored. Based on these observations, we sought to determine whether the APP^{E693Q} mutation generates A β with a high propensity to form soluble oligomers, without plaque pathology.

Brains of six individual lines of APP^{E693Q} transgenic mice (all F1 generation) were analyzed for levels of huAPP expression by western blot analysis (Figure 1*a*). Using rabbit anti-pan-APP cytoplasmic tail pAb369 and human-APP (A β _{1–16})-specific mouse mAb6E10, we were able to confirm transgene protein expression in the brains of transgenic animals. Since APP-CTFs are the immediate precursors for metabolism and generation of the A β peptide, their detectability is an important measure in order to account for all the catabolic fragments of APP along the pathway to A β generation. In comparison to several other transgenic AD models, which utilize the Swedish APP^{K670N, M671L} mutation (i.e. Tg2576 and TgCRND8) to increase total production of A β via increasing BACE cleavage of APP²², the APP^{E693Q} mutation is not preferentially cleaved by BACE. Moreover, APP^{E693Q} C99 (β -CTF) levels are not obviously increased as in the Tg2576 or TgCRND8 lines (Figure 1*b*).

Immunohistological analysis of APP^{E693Q} and APP^{E693Q} X PS1 Δ E9 mice

The hereditary APP^{E693Q} mutation has been described as an autosomal dominant form of cerebral amyloid angiopathy (CAA) with cerebral hemorrhage¹¹. APP^{E693Q} single transgenic mice were analyzed for vascular pathology using immunohistochemistry, revealing initial appearance of amyloid-laden cerebral vessels in APP^{E693Q} mice at 12 months or older, as compared to their non-transgenic littermates (Figure 2*a*). Moreover, Perls' blue stain of

APP^{E693Q} brain tissue revealed occasional vessels outlined by hemosiderin, representing extravasation of blood, which is likely due to a combination of aging and CAA (Figure 2b). There was no evidence of gross hemorrhage.

Human APP-overexpressing mouse models of AD have been reported to display learning deficits prior to plaque deposition, though all of these murine models do eventually develop plaques^{23,24}. Our APP^{E693Q} mice, however, never develop senile plaques, up to at least 30 months of age, in comparison to the APP^{E693Q} mouse model developed by the Jucker laboratory, which develop some diffuse plaques¹¹. To accelerate the progression of plaque-like Alzheimer's related pathology and, importantly, to validate the integrity of the APP^{E693Q} transgene, we crossed APP^{E693Q} mice with a mice overexpressing the familial AD-associated exon 9-deleted PS1 mutant (PS1ΔE9). Both APP^{E693Q} single transgenic and APP^{E693Q} X PS1ΔE9 were further studied using immunohistochemistry with mAb6E10 and a rabbit pAb anti-Aβ₄₂-C-terminus-specific antibody. In both lines of mice, mAb6E10 and pAb anti-Aβ₄₂-immunopositive staining of intraneuronal vesicles was detectable as early as 2 months of age, and mAb6E10 showed typical amyloid plaques in the brains of APP^{E693Q} X PS1ΔE9 bigenic mice as early as 11 months of age (Figure 2c), but again not in APP^{E693Q} single transgenic littermates (see Supplementary Figure 1). We compared intraneuronal mAb6E10-positive and mAb4G8-positive (pan-species anti-Aβ₁₇₋₂₁) vesicular staining patterns in APP^{E693Q} single transgenic mice versus the well-characterized Tg2576 mouse model of AD (Figure 2d)²⁴. The intensity and granularity of staining in APP^{E693Q} mice appears to be more robust compared to that observed in the Tg2576 mouse. Moreover, immunoelectron microscopy revealed APP/Aβ-immunopositivity associated with the multivesicular bodies (MVBs/lipofuscin) of the late endosomal/lysosomal system (Figure 3) of both APP^{E693Q} single transgenic and APP^{E693Q} X PS1ΔE9 bigenic mice. In comparison to APP^{E693Q} alone, APP^{E693Q} X PS1ΔE9 mice have an increased Aβ₄₂/Aβ₄₀ ratio and develop plaques (Figure 2c and Supplementary Figure 1).

APP^{E693Q} mice exhibit an oAβ/ADDL-dependent deficit in acquisition of the Morris water maze

To investigate the role of soluble oAβ/ADDLs in AD-related deficits in learning and memory, we employed the Morris water maze (MWM) to analyze deficits in spatial learning and memory at 6 and 12 months of age. Ultimately, APP^{E693Q} X PS1ΔE9 mice were excluded from oAβ/ADDL-related MWM behavioral statistical analysis since only 3 of 12 total mice formed detectable levels of oAβ/ADDLs.

The MWM is a widely used measure of both short- and long-term spatial memory, in which the animal uses spatial cues within the test room to find a hidden escape platform. Visuospatial function has been correlated with functional status in AD patients²⁵ and hippocampal dysfunction associated with AD typically results in poor performance on visuospatial and spatial orientation-related tasks^{26,27}. APP^{E693Q} single transgenic mice and their non-transgenic littermates (NTg) were trained and tested on the MWM at 6 months of age, extinguished, and then trained and tested again at 12 months of age. For 11 consecutive days, mice were trained to swim to an escape platform within 60s, and escape latency was recorded at each trial. At 12 and 21 days post-training, mice were placed in the tank without an escape platform, and time spent swimming in each quadrant during this "probe" trial was recorded. No significant differences were observed between NTg and APP^{E693Q} mice at 6 months of age during training or probe trials, and swim speed did not vary by genotype (data not shown). Further, at 12 months of age no difference was observed between NTg and APP^{E693Q} mice at either probe trial. However, a large amount of intra-genotype variability was observed for APP^{E693Q} mice during training, notably in the later days of training (Figure 4).

Based on the hypothesis that oA β /ADDL levels might explain behavioral differences between individual APP^{E693Q} mice, we used a duplicate-epitope sandwich ELISA¹⁸ to measure oA β /ADDL levels in all tested mice at 13 months of age. A duplicate epitope sandwich ELISA utilizes the same antibody for both capture and detection, resulting in detection of a substrate with two or more of the identical antigen site i.e. dimers or larger. However, we cannot exclude the possibility that APP fragments other than A β might also aggregate and contribute to the ELISA signal. This limitation is inherent in the method.

Based on oA β /ADDL levels (Supplementary Table 1), NTg or APP^{E693Q} were grouped as follows: NTg mice (NTg mice are unable to form oligomers in the absence of the human APP transgene, n=8); undetectable (ud)A β /ADDL mice (mice with oA β /ADDL levels below the lower limit of reliable quantitation; LLRQ; 39pg/g, n=12); or readily detectable (d)oA β /ADDL mice (mice with oA β /ADDL levels above the LLRQ, n=5). No difference was observed at either probe trial (Figure 5a) at 12 months of age when mice were grouped by oA β /ADDL level. Analysis of escape latency during the training period revealed significant between-subjects differences for (d)oA β /ADDL mice (p=0.027), but not (ud)oA β /ADDL mice (p=0.227), in comparison to NTg mice (Figure 5). A Bonferroni's post-hoc analysis revealed significant differences for escape latency only between (d)oA β /ADDL and NTg mice on day 6 (p=0.021) and also on day 5 and day 9 between (d)oA β /ADDL and (ud)oA β /ADDL (p=0.010, p=0.002, respectively), (d)oA β /ADDL and NTg (p=0.010, p<0.001, respectively), but not (ud)oA β /ADDL versus NTg (p=0.579). Notably, no significant differences in escape latency were observed between NTg, (ud)oA β /ADDL, or (d)oA β /ADDL groups on the final day of training/acquisition (Figure 5b), indicating that APP^{E693Q} mice did eventually learn the MWM task.

Throughout the 12-day acquisition phase, typically mean escape latency decreases from day-to-day for NTg mice (Figure 4b). In order to more efficiently analyze the relationship between oA β /ADDL level and acquisition of the MWM task, we used a days-to-criterion (DTC) analysis of escape latency²⁰. Briefly, we established the criterion for reliable performance of the acquired MWM task as two consecutive trials with escape latencies of 25 seconds or less, where DTC represents the day on which criterion was met. There was a significant increase in DTC for (d)oA β /ADDL (M=10.6) compared to NTg mice (M=5.5; p=0.01), but no significant difference was observed between (ud)oA β /ADDL and NTg mice (Figure 5). Taken together, these results indicate an oA β /ADDL level-dependent delay in acquisition of the MWM task in APP^{E693Q} transgenic mice at 12 months of age.

DISCUSSION

We provide evidence that APP^{E693Q} single transgenic mice develop a significant oA β /ADDL-dependent delay in acquisition of the MWM task at 12 months of age that is not dependent on the development of AD-like plaque pathology or macrohemorrhage. APP^{E693Q} single transgenic mice, as old as 30 months, did not develop senile plaques in contrast to APP^{E693Q} X PS1 Δ E9 bigenic mice, which developed plaques by 12 months of age.

Both APP^{E693Q} single transgenic and APP^{E693Q} X PS1 Δ E9 exhibited robust accumulation of intraneuronal APP/A β -like immunoreactivity within MVBs/lipofuscin. Recent evidence supports a toxic role of intraneuronal accumulation of APP/A β ²⁸, and activity-induced reduction of intraneuronal A β has been shown to protect against A β -related synaptic alterations²⁹. Based on the previous findings that oA β /ADDL formation may be initiated intracellularly^{30–33} and the work reported here, we suggest that the intraneuronal accumulation of APP/A β observed in APP^{E693Q} mice may represent one site for the initiation of oA β /ADDL formation (Figures 2 and 3). By studying the effects of oA β /

ADDLs generated in brain *in situ*, the current study is highly novel, since all studies to date have involved external application or intracerebral injection of partially purified oA β /ADDLs preparations^{4–10}.

Impairment of spatial navigation on the hidden goal task (a human analogue of the MWM) was recently associated with hippocampal dysfunction, wherein patients with hippocampal-related mild cognitive impairment (MCI) and AD patients displayed nearly identical delays in acquisition compared to both controls and non-hippocampal-related MCI patients²⁶. Moreover, 21–22-week old (early plaque pathology) TgCRND8 mice also showed a delayed acquisition of the MWM task without long-term deficits at probe trials, whereas 38–42-week old (late plaque pathology) TgCRND8 mice displayed a delayed acquisition of the MWM with long-term deficits at the Day 12 probe trial in comparison to NTg littermates²³. Jacobsen and colleagues (2006)³⁴ also described early, pre-plaque deficits in acquisition of spatial orientation of 3 month-old Tg2576 mice on the contextual fear conditioning task. However, none of these studies investigated the association of oA β /ADDLs with the observed deficits in spatial learning and memory. Here, we report that 12-month old APP^{E693Q} mice displayed an oA β /ADDL-dependent delay in acquisition of the MWM task compared to NTg littermates, suggesting that more discrete deficits of spatial orientation may be an early marker of AD-like cognitive decline. Importantly, we provide evidence that, in a mouse model in which oA β /ADDLs are generated *in situ* from physiological processing of transgenic human APP, these deficits in spatial orientation are oA β /ADDL-dependent. A recent publication implicated a correlation of A-11-positive oA β levels with deficits related to acquisition of spatial memory on the MWM task of 2-year old APP23/Abca1 mice³⁵. Taken together with these findings, our results suggest that 12-month old APP^{E693Q} mice may represent a “pre-clinical” model of AD, although further work is required to determine whether APP^{E693Q} mice acquire even more severe long-term spatial deficits at a later age (i.e., 18 and 24 months). Without development of plaque pathology or long-term spatial navigation deficits such as those described in 16-month old APP^{K670L, M671N} X PS1 Δ E9 bigenic mice²⁷, 12-month old APP^{E693Q} mice provide a model for studying specific oA β /ADDL-related deficits in spatial learning and memory. We propose that DTC analysis may represent a particularly sensitive measure of pre-pathological oA β /ADDL-related clinical deficits in the acquisition of tasks requiring spatial orientation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Immunoblot characterization of APP^{E693Q} single transgenic mice

(A) Both blots are from identical samples. Lane 1 is from a non-transgenic mouse brain. Lanes 2–7 are from the brains of 6 F1 generation mice from 6 unique APP^{E693Q} single transgenic mice. Levels of holoAPP expression were measured from each mouse based on levels of mature and immature APP as measured by pan-species anti-APP cytoplasmic tail pAb369 or human APP/A β -specific mAb6E10. Lane 5 represents an F1 mouse from the line of the APP^{E693Q} breeder mice used in this experiment, indicating a 5–8-fold overexpression of holoAPP in comparison to non-transgenic mice (Lane 1). Immunoreactivity to mAb6E10 (bottom) represents expression of the human APP transgene. (B) Immunoblot characterization of APP-CTFs in the brains of transgenic and non-transgenic mice is shown. In comparison to mice harboring the Swedish (APP^{K670N, M671L}) mutation (our laboratory's unpublished Swedish mouse) or TgCRND8 (APP^{K670N, M671L, V717F}), courtesy of Dr. David Westaway, University of Toronto), the APP^{E693Q} mutation does not appear to alter cleavage by either α - or β -secretases, noted by the lack of C99-CTF (β -CTF) in comparison to both TgCRND8 and Tg2576 mice (Swedish APP, courtesy of Dr. Karen Hsiao) shown here.

Figure 2. Pathological analysis of CAA and amyloid deposition in APP^{E693Q} and APP^{E693Q} X PS1ΔE9 mice

(A) Cerebral amyloid angiopathy is represented by vascular pathology in APP^{E693Q} mice. A representative image of staining of amyloid-laden cerebral vessels in an APP^{E693Q} mouse (right) compared to a NTg (left) is shown here. (B) Representative Perls' Berlin Blue stain of the brain of a 20-month old APP^{E693Q} mouse shows hemosiderin indicative of the local extravasion of blood (arrows). This degree of Perls' positivity is likely due to a combination of both aging and CAA. (C) Immunostaining of the hippocampus of APP^{E693Q} X PS1ΔE9 bigenic mouse using mAb6E10 antibody, revealing the development of plaque pathology, which is not present in APP^{E693Q} single transgenic animals. (D) Intraneuronal APP/Aβ accumulation in APP^{E693Q} mice compared to Tg2576 mice represented by immunostaining with mAb6E10 and mAb4G8. Vesicular staining in the two murine lines was found to differ qualitatively and quantitatively, with APP^{E693Q} showing more discrete and more intense immunoreactivity compared to Tg2576 mice.

Figure 3. Immunoelectron microscopy reveals APP accumulation in intraneuronal organelles
 Representative immunoelectron microscopy images of pAb369-immunopositive staining of small-, medium-, and especially large-sized cytoplasmic vesicular structures, including the internal and external lamellae of multivesicular bodies (MVBs) and external lamellae of dense lysosomes and lipofuscin. APP accumulation in MVBs and dense lysosomes has been previously associated with increased pathogenic processing of APP^{31,32}. These results suggest that APP^{E693Q} transgenic mice exhibit a large amount of intraneuronal accumulation of APP.

Figure 4. Behavioral characterization of APP^{E693Q} on the MWM task

Non-transgenic (n=8) and APP^{E693Q} single transgenic (n=17) mice were trained for 12 days and then tested on probe trials at 12 and 21 days-post training at 6 months of age, extinguished, then trained and tested again at 12 months of age. No significant differences were observed between NTg and APP^{E693Q} mice during training or probe test (either 12 or 21 days) at 6 months of age. (A) No significant differences were observed for percent of time in the target quadrant between 12-month old NTg and APP^{E693Q} mice at either 12 (gray; $p=0.06$) or 21 (purple; $p=0.754$) day probe trials. (B) During the 12-day training/acquisition period, NTg mice reached the escape platform with shorter escape latencies from day-to-day and with a low amount of variability among NTg mice, indicating acquisition of the task. (C) No difference was observed between NTg and APP^{E693Q} during the 12-day training period. In comparison to NTg littermates, APP^{E693Q} single transgenic mice displayed a large amount of intragenotype variability, especially in the later days of training. Levene's test for equality of variances for NTg versus APP^{E693Q} mice during training revealed significantly non-homogeneous variances on day 8 [$F(2,22)=5.208$, $p=0.014$], day 10 [$F(2,22)=3.634$, $p=0.043$], day 11 [$F(2,22)=3.428$, $p=0.05$], and day 12 [$F(2,22)=4.108$, $p=0.03$]. Error bars: $\pm$ S.E.M.

Figure 5. Aged APP^{E693Q} mice exhibited an oAβ/ADDL-dependent delay in acquisition of the MWM task

We tested the a priori hypothesis that cerebral oAβ/ADDL level accounts for cognitive deficits on the MWM task in non-plaque forming, pre-pathological APP^{E693Q} single transgenic mice. Post-mortem biochemical analysis of brain oAβ/ADDL concentration was used to group experimental mice as NTg (unable to make oAβ/ADDLs, n=8), or APP^{E693Q} with undetectable (below LLRQ; (ud)oAβ/ADDL, n=12) or detectable (above LLRQ; (d)oAβ/ADDL, n=5) ADDLs. (A) No significant difference was observed for percent of time in target quadrant between 12-month old NTg, (ud)oAβ/ADDL, or (d)oAβ/ADDL groups at the day 12 post-training probe trial, suggesting that APP^{E693Q} single transgenic mice do not have oAβ/ADDL-dependent long-term memory deficits. (B) Further analysis showed no significant difference between 12-month old NTg and (ud)oAβ/ADDL or (d)oAβ/ADDL mice on day 12 of training, indicating that all mice performed equally, on average, on the final day of training. (C) A repeated measures ANOVA revealed significant between-groups differences for escape latency [$F(2,22)=6.005$, $p=0.008$] and Dunnett's T3 multiple comparisons analysis (homogeneity of variance not assumed) revealed a significant between-groups difference only between NTg and (d)oAβ/ADDL mice ($p=0.027$). A MANOVA was further utilized to determine the days on which between-subjects differences occurred, indicating a significant between-subjects effects on day 5 [$F(2,22)=6.551$, $p=0.006$], day 6 [$F(2,22)=4.641$, $p=0.021$], and day 9 [$F(2,22)=11.730$, $p<0.001$]. Bonferroni's multiple comparisons analysis (homogeneity of variance assumed) indicated a significantly higher escape latency only for (d)oAβ/ADDL mice compared to NTg mice on day 6 ($p=0.021$), and also on day 5 and day 9 of training between (d)oAβ/ADDL mice and both NTg ($p=0.010$, $p<0.001$, respectively) and (ud)oAβ/ADDL ($p=0.010$, $p=0.002$, respectively) mice. (D) A days-to-criterion (DTC) analysis was utilized to more specifically assess the relationship between oAβ/ADDL level and acquisition of the MWM task. A criterion score for reliable acquisition of the MWM task was set to two consecutive trials with escape latencies of 25 seconds or less and each mouse received a score reflecting the day on which criterion was met. A oneway ANOVA revealed significant between-groups differences for DTC [$F(2,22)=5.526$, $p=0.011$] and Bonferroni's multiple comparisons analysis (homogeneity of variance assumed) revealed a significant increase in DTC only for (d)oAβ/ADDL in comparison to NTg mice ($p=0.01$). Taken together, these results suggest that APP^{E693Q} mice do eventually acquire and retain the MWM task, however these mice

exhibit a clear $\alpha\beta$ /ADDL-dependent delay in acquisition of the task in comparison to NTg mice. Error bars: $\pm$ S.E.M; * $p < 0.05$; ** $p < 0.01$ with a two-tailed $\alpha = 0.05$.