Early accumulation of intracellular fibrillar oligomers and late congophilic amyloid angiopathy in mice expressing the Osaka intra-Aβ APP mutation

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Pathogenic amyloid-β peptide precursor (APP) mutations clustered around position 693 of APP—position 22 of the Aβ sequence—are commonly associated with congophilic amyloid angiopathy (CAA) and intracerebral hemorrhages. In contrast, the Osaka (E693Δ) intra-Aβ APP mutation shows a recessive pattern of inheritance that leads to AD-like dementia despite low brain amyloid on in vivo positron emission tomography imaging. Here, we investigated the effects of the Osaka APP mutation on Aβi accumulation and deposition in vivo using a newly generated APP transgenic mouse model (E22ΔAβi) expressing the Osaka mutation together with the Swedish (K670N/M671L) double mutation. E22ΔAβi mice exhibited decreased α-processing of APP and early accumulation of intraneuronal fibrillar oligomers associated with cognitive deficits. In line with our in vitro findings that recombinant E22ΔAβi peptides form amyloid fibrils, aged E22ΔAβi mice showed extracellular CAA deposits in leptomeningeal cerebellar and cortical vessels. In vitro results from thioflavin T aggregation assays with recombinant Aβ peptides revealed a yet unknown antiamyloidogenic property of the E693Δ mutation in the heterozygous state and an inhibitory effect of E22Δ Aβ42 on E22Δ Aβ40 fibrillogenesis. Moreover, E22Δ Aβ42 showed a unique aggregation kinetics lacking exponential fibril growth and poor seeding effects on wild-type Aβ aggregation. These results provide a possible explanation for the recessive trait of inheritance of the Osaka APP mutation and the apparent lack of amyloid deposition in E693Δ mutation carriers.

Introduction

Extracellular deposition of fibrillar amyloid-β (Aβ) peptide as amyloid plaques and congophilic amyloid angiopathy (CAA) is considered a cardinal neuropathological feature of Alzheimer’s disease (AD). According to the amyloid cascade hypothesis, soluble and fibrillar Aβ species have a central role in the pathogenesis of AD and start to accumulate within the brain years before cognitive decline and dementia symptoms are observed. Evidence supporting the amyloid cascade hypothesis comes from several sources including genetic studies of familial AD cases carrying mutations in the amyloid-β peptide precursor (APP) gene and the presenilin genes. Pathogenic mutations in the APP gene have been shown to influence the metabolism of the Aβ peptide in various ways. The Swedish double mutation (K670N/M671L), located upstream of the Aβ N terminus adjacent to the β-cleavage site, results in an increased production of both Aβ40 and Aβ42 species, whereas mutations located at the γ-cleavage site of APP cause an increase of the Aβ42/Aβ40 ratio and, as a consequence of this, result in increased Aβ aggregation and deposition (for a review see Goate). The recently discovered E693Δ Osaka mutation in a Japanese pedigree is one of the six so-called intra-Aβ mutations clustered around the hydrophobic core of the Aβ sequence. Position 693 seems to be a critical site involved in pathogenic aggregate formation since mutations at or (± 1) around this site, including the Dutch (E693Q), Flemish (E692G), Italian (E693K), Iowa (D694N) and Arctic (E693G) mutations, have been reported to result in an increase in total Aβ production and—with the exception of the Flemish mutation—enhance Aβ aggregation and toxicity. Interestingly, all currently known intra-Aβ APP mutations—with the exception of E693Δ—have previously been shown to be vasculotropic and are characterized neuropathologically by prominent vascular amyloid deposition.

Although neuropathological data have not been reported to date, homozygous carriers of the recessive Osaka APP mutation are believed to develop an AD-like clinical phenotype in the absence of relevant extracellular amyloid deposition as revealed by a very low signal on amyloid positron emission tomography imaging. In vitro experiments demonstrated enhanced oligomerization but no fibrillization of synthetic...
E22Δ Aβ[40] and E22Δ Aβ[42] preparations, suggesting that AD-like symptoms may be caused by the presence of synaptotoxic Aβ oligomers, rather than fibrillar Aβ, in the affected patients.  

Consistent with these findings, synthetic E22Δ Aβ[42] potently inhibited hippocampal long-term potentiation and induced synapse loss in mouse hippocampal slices.  

Further cell culture experiments and results from the recently reported E693Δ transgenic mouse model revealed enhanced accumulation of intraneuronal Aβ oligomers as a prominent feature of the Osaka APP mutation.  

The apparent lack of extracellular amyloid deposition in these mice has been suggested to be in line with the initial in vitro findings with synthetic Aβ preparations that E22Δ-mutated Aβ peptides do not form amyloid fibrils.  

However, follow-up studies with recombinant Aβ preparations revealed that both E22Δ Aβ[40] and E22Δ Aβ[42] readily formed amyloid fibrils in vitro. Based on these findings, we hypothesized that E22Δ-mutated Aβ may, in principle, also form amyloid fibrils in vivo and generated a novel APP transgenic mouse line (E22ΔAβ) to investigate the effects of the E693Δ mutation on amyloid accumulation and deposition in vivo. In line with our recent in vitro findings, aged E22ΔAβ mice were characterized by late extracellular amyloid deposition in the leptomeningeal vasculature at 24 months of age, which was preceded by an early accumulation of intracellular oligomeric Aβ species already at an age of 3 months. The results of this study provide strong evidence that E22Δ-mutated Aβ species are fibrilogenic and can deposit extracellularly in vivo as CAA, thus placing the E693Δ Osaka mutation on the list of other vasculotropic intra-Aβ APP mutations.

Materials and methods

Animals. The newly generated E22ΔAβ mice express the human APP695 isoform containing the Swedish (K670N+M671L) and Osaka (E693Δ) mutations. The mutations were generated by site-directed mutagenesis of pGEM-9zf(-) huAPP695. The cDNA was inserted into pMoPrP-Xho, and generated by site-directed mutagenesis of pGEM-9zf(M671L) and Osaka (E693Δ) human APP695 isoform containing the Swedish (K670N+M671L) mutation on amyloid accumulation and deposition in vivo. In line with our recent in vitro findings, aged E22ΔAβ mice were characterized by late extracellular amyloid deposition in the leptomeningeal vasculature at 24 months of age, which was preceded by an early accumulation of intracellular oligomeric Aβ species already at an age of 3 months. The results of this study provide strong evidence that E22Δ-mutated Aβ species are fibrilogenic and can deposit extracellularly in vivo as CAA, thus placing the E693Δ Osaka mutation on the list of other vasculotropic intra-Aβ APP mutations.

All animal experiments were performed in compliance with Swiss national guidelines and were approved by the veterinary authorities of the Canton of Zurich.

Protein extracts and western blotting. Brain tissues were homogenized with a glass teflon homogenizer in a sixfold wet weight amount of buffer A containing 100 mM Tris-HCl, 150 mM NaCl, Complete Protease Inhibitor Cocktail (Roche Diagnostics, Rotkreuz, Switzerland) and Phosphatase Inhibitor Cocktails 1+2 (Sigma-Aldrich, Buchs, Switzerland). After centrifugation at 100 000 g for 1 h, supernatants were collected (Tris fraction) and pellets were rehomogenized in buffer A containing 1% Triton X-100. Centrifugation at 100 000 g was repeated and supernatants were again collected (Tris fraction). The remaining pellets were rehomogenized in buffer A containing 2% sodium dodecyl sulfate (SDS). After an additional centrifugation step and collection of the supernatants (SDS fraction), the resulting pellets were eventually dissolved in 70% formic acid (FA), sonicated for 30 s at 30% power, ultracentrifuged, supernatants extracted, lyophilized and reconstituted in buffer A containing 2% SDS for further analysis. Total protein concentrations were measured with the DC protein assay (Bio-Rad Labs, Gessler, Switzerland). Extracts were separated by SDS-polyacrylamide gel electrophoresis, blotted onto nitrocellulose, boiled for 5 min in phosphate-buffered saline and blocked in Tris-buffered saline containing 5% milk for 1 h at room temperature. Primary antibodies were incubated overnight at 4 °C (6E10 1:500; anti-C-terminal APP (Sigma, Buchs, Switzerland); 1:2000) and visualized by peroxidase-conjugated antibodies and ECL reactions (Amersham Biosciences, Otelfingen, Switzerland). Monoclonal anti-β-actin antibody (1:2000; Abcam, Cambridge, UK) was used as internal loading control and for normalization of densitometric analyses of the immunoreactive bands. Quantification of the immunoreactive bands was carried out by densitometry of the scanned films under conditions of non-saturated signal using the Image J software (rsb.info.nih.gov/ij/).

MSD analysis. Aβ fragments were measured in the above-mentioned brain homogenate fractions and plasma was measured using a MesoScale Discovery (MSD) 3plex multi-SPOT Aβ human kit (Gaithersburg, MD, USA) for Aβ38, Aβ40 and Aβ42, in accordance with the manufacturer’s instructions. Human sAPPα levels were determined using an MSD 2plex kit (Gaithersburg, MD, USA), and human Swedish sAPPβ levels were determined using an MSD 1plex kit (Gaithersburg, MD, USA), in accordance with the manufacturer’s instructions. All reagents were provided with the kits containing a 96-well plate with two carbon electrodes precoated with analyte-specific capture antibodies. After 1 h of blocking with bovine serum albumin, plates were washed with Tris wash buffer and samples and standards added to the wells. Plates were sealed and incubated for 1 h on an orbital shaker (750 r.p.m.) at room temperature, followed by additional washing steps and incubation with the detection antibody for 1 h. For Aβ3plex assay (Gaithersburg, MD, USA), standards and samples were added at the same time as the detection antibody, and plates were incubated for 2 h. At the end of the incubation period, plates were washed again and measured
on an MSD SECTOR Imager 6000 plate reader (Gaithersburg, MD, USA) after the addition of the MSD Read Buffer T (Gaithersburg, MD, USA, RAW). Raw data were measured as electrochemiluminescence (light) signal detected by photodetectors. The MSD DISCOVERY WORKBENCH software (Version 3.0.17) (Gaithersburg, MD, USA) with Data Analysis Toolbox was used to calculate sample concentrations by comparing them against a standard curve.

**Histological analysis.** Histological stainings were carried out on 5 μm paraffin brain sections by using standard published procedures. For the immunohistochemical detection of intraneuronal Aβ deposits, sections were boiled in 10 mM sodium citrate buffer (pH 6.0), followed by antigen retrieval with 95% FA for 5 min. The following antibodies were used for immunohistochemistry: 6E10 (1:500 dilution; Signet, Dedham, MA, USA) and β-amyloid antibody (1:200 dilution; Cell Signaling, Danvers, MA, USA) were used for the detection of pan-Aβ. Anti-β-amyloid protein (1–40) antibody (1:100; Sigma) and BA27 (Amyloid β-Protein Immunohistostain Kit; Wako, Wako Chemicals GmbH, Neuss, Germany) were used to specifically detect Aβ40. Anti-amyloid 42 Polyclonal Antibody (1:100; Signet) and BC05 (Amyloid β-Protein Immunohistostain Kit; Wako) were used to specifically detect Aβ42. Polyclonal antibodies A11 and OC (provided by C Glabe, both 1:100) were used for the detection of prefibrillar and fibrillar Aβ oligomers, respectively. 11A1 (1:100; IBL Japan, Gunma, Japan) was used to detect Aβ with a conformational turn epitope between positions 22 and 23 of the Aβ sequence. Anti-Amyloid Precursor Protein, C-Terminal antibody (1:200; Sigma) was used for the detection of full-length APP and APP C-terminal fragments. Secondary antibodies were obtained from Vector Laboratories, Burlingame, CA, USA (Vectastain ABC kits PK-6101 and PK-6102) for peroxidase diaminobenzidine stainings, and from Jackson Immunoresearch Laboratories (West Grove, PA, USA) for immunofluorescence.

Thioflavin S staining and Congo red stainings were performed according to standard protocols as described previously.

**Recombinant Aβ production.** Production of recombinant Aβ peptides (wild-type Aβ40 and Aβ42, E22Δ Aβ40 and Aβ42, and E22G Aβ40 and Aβ42) was performed as described previously. In brief, recombinant Aβ peptides were expressed under the control of the T7 promoter/lac operator in *Escherichia coli* BL21 (DE3) as fusion proteins to the peptide sequence (NANP)19 with an N-terminal hexahistidine tag. Mutagenesis at codon 22 in both Aβ1–40 and Aβ1–42 was performed with the QuikChange site-directed mutagenesis kit (Stratagene, Basel, Switzerland). The correct genetic sequences of the constructs were verified by DNA sequencing. Cleavage of the fusion proteins (100 μM) with 7.5 μM tobacco etch virus protease was performed in 10 mM Tris-HCl (pH 8.0), 0.5 mM ethylenediaminetetraacetic acid and 1 mM dithiothreitol for 1 h at room temperature, followed by incubation at 4°C overnight. The cleaved Aβ peptides precipitated during the cleavage reactions and were pelleted by centrifugation (4500 g, 20 min, 4°C), dissolved in 6 M guanidinium chloride-HCl (pH 2.0) and purified via reversed-phase high-performance liquid chromatography. Peptides were eluted with CAN, aliquoted in Protein LoBind Eppendorf tubes (Vaudaux-Eppendorf), lyophilized and stored at ~8°C. The high purity and identity of the peptides were verified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry using sinapinic acid as matrix.

**Thioflavin T aggregation assays.** Preparation of Aβ solutions and thioflavin T aggregation assays were performed as described earlier. In brief, Aβ variants were dissolved in 10 mM NaOH to concentrations of 100–150 μM. Aβ concentrations were determined via the absorbance of Aβ at 280 nm in 10 mM NaOH (extinction coefficient at 280 nm and pH 12 corresponding to a single tyrosine residue: 1730 μM⁻¹ cm⁻¹). Stock solutions were kept on ice and were used for aggregation experiments within 6 h. Aggregation reactions were performed at 37°C with 2.5 or 5 μM Aβ (final concentration) in 10 mM H2PO4-NaOH (pH 7.4), 100 mM NaCl and 35 μM thioflavin T in a volume of 1000 μl in stirred quartz fluorescence cuvettes (1–0.4 cm). The thioflavin T concentration was determined via its extinction coefficient of 36000 M⁻¹ cm⁻¹ at 412 nm. Aggregation reactions were started by a dilution of the Aβ stock solution in 10 mM NaOH (prepared and ultracentrifuged immediately before use) with an aggregation buffer mix, resulting in pH 7.4, and the final concentrations indicated above. Thioflavin T fluorescence emission at 482 nm (excitation at 440 nm; excitation and emission slit at 1.6 nm) was monitored on a Quantamaster (QM-7/2003) fluorescence spectrometer (Photon Technology International, Birmingham, NJ, USA).

**Cognitive–behavioral testing.** A battery of well-validated and carefully controlled tests was used to behaviorally assess mice for motoric and cognitive performance. At the time of testing, mice were weighed and examined for general health measures to ensure that the mice were physically able to conduct the cognitive–behavioral test. Mice expressing the Osaka intra-Aβ APP mutation

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(150 lx), thus providing motivation for the animals to avoid the open surface and escape into a small dark recessed chamber (escape box) located under the platform. The inclusion of false boxes that look the same as the target escape box, but are too small to be entered, were used to remove visual cues that might allow the mouse to discriminate the location of the escape hole from the other holes. A cylindrical plastic start chamber (10.5 cm diameter, 9.5 cm height) was used to hold mice in the middle of the maze at the start of each trial. A web camera (Logitech, Zurich, Switzerland) was placed 1.2 m above the center of the maze to record trials using the ANY-maze Video Tracking System (Stoelting). Distinct spatial cues placed in a constant location around the maze served as a reference point to learn the position of the escape hole.

Mice completed 4 days of acquisition training with four trials per day with an inter-trial interval of 10–15 min. For each trial, mice were placed in the start chamber in the middle of the maze, and after 10 s, the start chamber was raised to start the trial and the mouse was allowed to explore the maze for 3 min. During these 3 min, the latency to enter the escape box, distance traveled, speed, time spent immobile and search strategy used was measured, among other parameters. The trial ended when the mouse successfully entered the escape box or after the 3 min had elapsed. If a mouse did not successfully enter the escape box during the 3 min, it was placed back at the start position and gently guided to the escape hole, where the mouse remained in the escape box for 1 min. At the end of each trial, mice were returned to their holding cage until the next trial. To reduce intramaze odor cues, the maze surface and escape box were cleaned with 70% ethanol after each trial. Search strategies were determined by examining individual track plots for each mouse per day and classifying their ability to find the escape location as either: (1) random search strategy—search patterns that cross through the center of the maze in a completely random manner; (2) serial search strategy—in which mice search every hole or every other hole in a clockwise or counter-clockwise systematic manner; or (3) spatial search strategy—in which the mice were able to locate the escape box directly plus or minus two adjacent holes within the target quadrant only.

**Statistical analysis.** Data analysis was performed using the Statistica 10.0 (StatSoft Inc, Tulsa, OK, USA) and SPSS 19.0 (16M, Schweiz, Zurich, Switzerland) statistical software. Tests for normal distribution were performed before statistical testing, according to the results of the Shapiro–Wilk and the Kolmogorov–Smirnov test for normality, either Student’s t-test or Mann–Whitney U-test for two sample groups or analysis of variance was performed (followed by post-hoc Fischer’s least significant difference analysis). A P-value <0.05 was considered statistically significant. Error bars are s.e.m.

**Results**

**Transgene expression and APP processing.** The newly generated E22ΔAβ mice overexpress the human APP695 isoform containing the Swedish (K670N + M671L) and Osaka (E693Δ) mutations. The Swedish double mutation was introduced to increase the amount of total secreted Aβ without affecting the Aβ42/Aβ40 ratio. Several transgenic founder lines were analyzed for brain expression of the full-length human APP transgene, and a line expressing transgene levels comparable to our APP transgenic arcAβ mouse model (expressing APP with the Swedish and Arctic (E693G) mutations) and the widely established Tg2576 AD mouse model (expressing APP with the Swedish mutation alone) was chosen for further analysis (Figure 1a). Western blot analysis of SDS extracts revealed similar full-length APP and β-stub (C99) levels in the E22ΔAβ, arcAβ and Tg2576 mice (Figure 1a). β-Stub (C83) levels, however, were significantly reduced in both E22ΔAβ and arcAβ mice as compared with Tg2576 mice (Figure 1a). MSD assay analysis of soluble APP levels from SDS brain extracts revealed a two- to threefold reduction in sAPPβ levels in the E22ΔAβ and arcAβ mice. In contrast, sAPPβ levels were comparable to the Tg2576 mice (Figure 1b). These findings indicate that both intra-Aβ APP mutations at position 22 of the Aβ sequence specifically interfere with the β-secretase processing of APP in vivo.

**Age-dependent changes in Aβ levels.** As a next step, we used MSD technology to determine Aβ levels in four sequentially extracted protein fractions from the cortical brain tissue (Figure 2). In agreement with previous findings,27,31,32 Tg2576 and arcAβ mice showed an age-dependent exponential accumulation of Aβ in detergent-insoluble (FA-soluble) protein fractions (Figure 2e–k) that accompanied the occurrence of parenchymal amyloid deposits in these mouse models (data not shown). Aβ accumulation occurred earlier and was more pronounced in the arcAβ mice as compared with age-matched Tg2576 mice (Figure 2e–k), as previously reported in immunohistological findings.27,31 In contrast, most of the Aβ in the E22ΔAβ mice accumulated in the detergent-soluble (SDS) protein fraction up to the age of 15 months (Figure 2a-c). MSD analysis of Tris buffer and mild detergent (Triton X-100) extracts in the E22ΔAβ mice revealed only very low Aβ levels as compared with the Tg2576 mice (Figure 2a–c). In addition, E22ΔAβ mice (similar to the arcAβ mice) showed four- to fivefold lower plasma Aβ levels than the Tg2576 mice (Supplementary Figure 1). These results indicate that the lack of accumulation of detergent-insoluble Aβ in the E22ΔAβ mice up to the age of 15 months was not accompanied by a relative increase in soluble brain or peripheral (plasma) Aβ levels. Western blot analysis of the four protein fractions at 15 months further confirmed the MSD findings (Supplementary Figure 2). At 24 months of age, however, a significant increase in detergent-insoluble (FA-soluble) Aβ was observed in the E22ΔAβ mice (Figure 2d). This change in Aβ solubility provided the first biochemical evidence for a substantial accumulation of fibrillar amyloid deposits in aged E22ΔAβ mice, even though FA-soluble Aβ levels remained relatively low in comparison to amyloid depositing Tg2576 and arcAβ mice at 15 months (Figure 2b and c).
evidence of extracellular parenchymal amyloid deposition up to an age of 15 months (Figure 3a and b). At 24 months of age, however, extracellular vascular amyloid deposits were observed in cortical and—more pronounced—cerebellar leptomeningeal vessels (Figure 4a and b). Vascular amyloid deposits were thioflavin S (Figure 4c) and Congo red positive (Figure 4d), and were immunostained with several Aβ-specific antibodies (Figure 4a–f). Immunostainings with Aβ40- and Aβ42-specific monoclonal antibodies revealed that Aβ40 was the dominant Aβ species deposited in the vessel walls of the E22ΔAβ mice (Figure 4e and f).

Early accumulation of intracellular Aβ oligomers. Although extracellular (vascular) amyloid deposits were detectable in the E22ΔAβ mice only as late as at the age of 24 months, immunostaining of paraffin-embedded brain sections with the monoclonal antibody 6E10 revealed prominent intraneuronal accumulation of dot-like aggregates mainly in hippocampal CA1 (Supplementary Figure 3a) and cortical (Supplementary Figure 3b) neurons that were observed already at the age of 3 months (data not shown). Double immunostaining with a polyclonal antibody directed against the APP C terminus (Supplementary Figure 3c and d) showed little colocalization of 6E10 and APP immunoreactivity in the cortex and hippocampus (Supplementary Figure 3e and f), thus excluding 6E10-positive aggregates as full-length APP or APP C-terminal fragments. Immunostaining with Aβ40-specific antibodies directed against the C terminus of Aβ40 and Aβ42 confirmed that the intraneuronal deposits indeed corresponded to Aβ aggregates (Supplementary Figure 4a–f). Interestingly, immunostaining with the Aβ40-specific antibody led to a more diffuse staining of both dot-like aggregates and neuronal cell bodies and
processes (Supplementary Figure 4a, b and e), whereas the Aβ42-specific specifically stained compact intraneuronal aggregates (Supplementary Figure 4c, d and f).

Further characterization of the intraneuronal Aβ aggregates in the E22ΔAβ mice with several oligomer-specific (conformation-dependent) antibodies revealed that the dot-like Aβ
deposits were strongly stained by the polyclonal OC antibody directed against fibrillar Aβ oligomers (Figure 5a–d), but not the A11 antibody directed against prefibrillar oligomers (Supplementary Figure 4g and h). OC-immunoreactive intraneuronal deposits were observed in CA1 hippocampal and cortical neurons as early as 3 months of age and appeared to grow and become more compact as the mice aged (Figure 5a–d). Strikingly, the intraneuronal oligomeric Aβ deposits were also recognized by 11A1 (Figure 5e and f), a novel conformation-dependent monoclonal antibody specifically designed against the turn between positions 22 and 23 of the Aβ sequence. 11A1 has recently been shown to stain intraneuronal Aβ aggregates in brains of AD patients but not APP transgenic mice.26

Early cognitive deficits in transgenic E22ΔAβ mice. Based on our previous findings in the arcAβ mouse model in which intracellular Aβ deposits occurred concomitantly with robust cognitive deficits, we hypothesized that the early accumulation of intraneuronal oligomeric Aβ deposits, particularly in hippocampal brain regions of the E22ΔAβ mice, would also be accompanied by significant impairments on several cognitive tasks. Both E22ΔAβ mice and wild-type littermates displayed, across all age groups, similar general health measures, auditory–visual sensory integrity, comparable grip strength, intact righting and extension reflexes. Assessment of body weight demonstrated equivalent body weights across all experimental groups, for both males and females. Locomotor and anxiety examination using the open field test demonstrated the published phenotype of increased locomotor activity and exploratory behavior, including increased total distance traveled, less time immobile and more time spent in the center zone, in transgenic mice (data not shown). Cognitive assessment in the Y-maze demonstrated a significant decrease in the percentage spatial alternation rate for E22ΔAβ mice at 3 months of age (t(55) = −3.16, P < 0.005), 6 months of age (t(28) = −1.727, P < 0.05) and 9 months of age (t(16) = −1.772, P < 0.05), as compared with their wild-type littermates (Figure 6a). The Barnes maze was also used at 3 months of age as a second...
hippocampus-dependent cognitive task to assess spatial learning and memory. Results demonstrated that both wild-type and transgenic E22\(D\)\(A\)\(b\) mice showed successful learning, with significantly lower latencies to escape over the 4 days of training (all \(P\)'s \(<0.05\); Figure 6b). Between genotypes, a significant main effect was shown for E22\(D\)\(A\)\(b\) mice (\(F(1,340)=16.656, P<0.001\)). Fischer’s least significant difference post hoc analysis demonstrated a significantly longer latency to escape for E22\(D\)\(A\)\(b\) mice, as compared with wild-type littermates on day 3 (\(P=0.01\)) and day 4 (\(P=0.03\)), with similar but nonsignificant trends on day 1 (\(P=0.096\)) and day 2 (\(P=0.095\)) (Figure 6b). Qualitative assessment of Barnes maze search strategies revealed a decrease in spatial search strategies in 3-month-old E22\(D\)\(A\)\(b\) mice (Figure 6d), as compared with wild-type control mice (Figure 6c).

Unique aggregation properties of recombinant E22\(D\)\(A\)\(b\) peptides. In agreement with our previous findings that both E22\(D\)\(A\)\(40\) and E22\(D\)\(A\)\(42\) readily formed amyloid fibrils in vitro, we observed fibrillar (congophilic) amyloid deposits in vivo in aged APP transgenic E22\(D\)\(A\)\(b\) mice. Extracellular amyloid deposition in the E22\(D\)\(A\)\(b\) mice, however, only occurred as leptomeningeal CAA at an advanced age of 24 months (Figure 4), whereas wild-type A\(b\)-producing Tg2576 mice and E22G (Arctic) A\(b\)-producing arcA\(b\) mice accumulated fibrillar amyloid deposits at a much earlier age (Figures 2 and 3).\(^{27,31}\) To further investigate the biophysical basis of the E22\(D\) intra-A\(b\) mutation leading to early intracellular and very late extracellular amyloid deposition in vivo, we conducted in vitro thioflavin T aggregation assays of recombinant E22\(D\) A\(40\) and E22\(D\) A\(42\) peptides. E22\(D\)A\(b\) aggregation curves were compared with the aggregation curves of A\(b\)\(40\) and A\(b\)\(42\) with wild-type or Arctic sequence (Figure 7). Measurements were terminated as the thioflavin T signal reached a plateau, after which the different A\(b\) preparations were further incubated for a total of 1 h, followed by western blot analysis. Results demonstrated typical aggregation kinetics of wild-type A\(b\)\(42\) and E22G A\(b\)\(42\) peptides involving a lag phase, an exponential growth phase and a plateau phase of saturated fibril growth.

**Figure 5** Intraneuronal amyloid \(b\) (A\(b\)) consists of fibrillar oligomers bearing a ‘toxic turn’ conformation. (a–d) Immunostaining with polyclonal OC antibody directed against fibrillar A\(b\) oligomers reveals age-dependent accumulation of intracellular oligomeric deposits in cortical (a and b) and CA1 hippocampal neurons (c and d). Note the temporal change of intraneuronal oligomers from rather diffuse and smaller dot-like aggregates at 3 months of age (a and c) to bigger and more compact deposits at 15 months of age (b and d). (e and f) Conformation-dependent monoclonal antibody 11A1 directed against the turn between positions 22 and 23 of the A\(b\) sequence stains hippocampal (e) and cortical (f) intraneuronal aggregates (representative immunostaining in a 15-month-old E22A\(b\) mouse). Scale bar: 40 \(\mu\)m (a–f).
of Barnes maze search strategies reveals a decrease in spatial and relative increase in serial search strategies in 3-month-old E22 control mice. Previous findings of a highly increased fibrillogenic property of preparations that were used for the thioflavin T analysis as revealed by western blotting of the recombinant peptide weight aggregates immediately after reconstitution at pH 7.4, wild-type A formation for both E22 peptide variants revealed a dramatically accelerated fibril growth phase in comparison to the aggregation of wild-type A. 42—formed detergent-insoluble high-molecular-weight aggregates immediately after reconstitution at pH 7.4, as revealed by western blotting of the recombinant peptide preparations that were used for the thioflavin T analysis (Figure 7c). Thioflavin T analysis of recombinant Aβ40 peptide variants revealed a dramatically accelerated fibril formation for both E22A and E22G Aβ40 in comparison to wild-type Aβ40. In contrast to E22A Aβ42, E22A Aβ40 aggregation was characterized by a lag phase, an exponential growth phase and a plateau phase with unusually high absolute thioflavin T fluorescence values indicating increased thioflavin T binding capacity of the E22A Aβ40 peptide as reported previously. In summary, thioflavin T analysis of recombinant E22A Aβ42 and E22A Aβ40 aggregation kinetics confirmed our previous findings of a highly increased fibrillogenic property of single preparations of the two peptide variants.

Inhibition of E22ΔAβ aggregation in the presence of wild-type Aβ. The E693A APP mutation is one of the two currently known familial AD mutations in the APP gene with a recessive Mendelian trait of inheritance. The second recessive mutation, A673V, has recently been shown to be highly amyloidogenic in the homozygous state, but anti-amyloidogenic in the heterozygous state as revealed by an inhibition of Aβ aggregation when mutated and wild-type peptides were co-incubated. We hypothesized similar effects on the aggregation kinetics when incubating mixtures of the wild-type and E22Δ-mutated Aβ peptides. Co-aggregation of wild-type Aβ42 with E22Δ Aβ42 led to a significant extension of the lag phase and a delay of the growth phase in comparison to the aggregation of wild-type Aβ42 alone. This effect was specific for the E22Δ intra-Aβ mutation, as it was not observed with the E22G Aβ42 variant. In contrast, co-aggregation of E22G (Arctic) Aβ42 with wild-type Aβ42 revealed a similar aggregation kinetics as E22G Aβ42 alone (see Figure 8a). Similarly, the aggregation of E22Δ Aβ40 peptides was also significantly delayed in mixtures containing 50% wild-type Aβ40, whereas co-incubation of E22G Aβ40 with wild-type Aβ40 only slightly delayed fibril formation in comparison to E22G Aβ40 alone (Figure 8b). In conclusion, only the E22Δ intra-Aβ mutation (but not the E22G variant) was associated with a significant delay of aggregation in the heterozygous state, simulated by equal mixtures with wild-type Aβ peptides.

Inhibition of E22Δ Aβ40 aggregation in the presence of E22Δ Aβ42. Based on our results that co-incubation of wild-type Aβ with E22ΔAβ significantly delayed amyloid fibril formation, we hypothesized that co-incubation of E22Δ Aβ40 with E22Δ Aβ42 may have similar antifibrillogenic effects. Therefore, recombinant E22Δ Aβ40 and E22Δ Aβ42 peptides were co-incubated in a physiological 9:1 ratio at 2.5 μM, and the increase in thioflavin T fluorescence was monitored over time (Figure 8c). We found a dramatic delay of the growth phase and a remarkable loss of absolute

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**Figure 6** Early cognitive deficits in E22ΔAβ mice. (a) Cognitive assessment in the Y-maze demonstrates a significant decrease in the percentage spatial alternation rate for E22ΔAβ mice as compared with their wild-type (wt) littermates at 3, 6 and 9 months of age (P < 0.05 for all comparisons; Student’s t-test). (b) Assessment of Barnes maze spatial learning at the age of 3 months reveals that both wild-type and E22ΔAβ transgenic mice show successful learning over the 4 days of training. However, E22ΔAβ mice, as compared with wild-type littermates, show significantly higher latencies to escape on day 3 (P < 0.01) and day 4 (P < 0.03), with similar but nonsignificant trends on day 1 (P = 0.096) and day 2 (P = 0.095) (analysis of variance (ANOVA), followed by post hoc Fisher’s least significant difference (LSD) analysis). (c and d) Qualitative assessment of Barnes maze search strategies reveals a decrease in spatial and relative increase in serial search strategies in 3-month-old E22ΔAβ mice (d) in comparison to wild-type control mice (c). n = 22-24 per group. Aβ, amyloid β.
thioflavin T fluorescence when we compared the aggregation curve with the aggregation curve of E22ΔAβ40 alone (Figures 7d and 8c). Again in contrast, 9:1 mixtures of E22G Aβ40 and E22G Aβ42 resulted in rapid aggregation, similar to E22G Aβ40 alone (Figures 7c and 8c). These results again reveal the strong aggregating properties of the E22G mutation and the inhibitory effect of the E22D mutation under conditions when different peptide species coexist, as is the case in vivo.

Poor seeding of wild-type Aβ42 aggregation by E22ΔAβ42 fibrils. Based on the assumption that amyloid formation can be seeded by preformed amyloid fibrils, we finally addressed the question whether preaggregated E22ΔAβ42 fibrils were capable of (a) seeding their own growth and (b) seeding the growth of wild-type Aβ42 fibrils. As control experiments, wild-type Aβ42 and E22G (Arctic) Aβ42 fibrils were incubated with preparations of their respective monomeric peptides, which resulted in an immediate increase in
thioflavin T fluorescence without a lag phase, thus indicating good seeding properties (Supplementary Figure 4a and b). Incubation of preaggregated E22ΔAβ42 fibrils with fresh preparations of E22ΔAβ42 peptide also resulted in an immediate increase in thioflavin T fluorescence that was steeper as compared with the aggregation curve of E22ΔAβ42 in the absence of fibrillar seeds (Figure 7a and Supplementary Figure 5c). However, when E22ΔAβ42 fibrils were used for the seeding of wild-type Aβ42, a significant delay of fibril formation was observed, which indicated poor seeding properties (Supplementary Figure 5e). In contrast, E22G Aβ42 fibrils appeared only slightly inferior to wild-type Aβ42 fibrils in seeding wild-type Aβ42 aggregation (Supplementary Figure 5d).

**Discussion**

Homozygous E693A APP mutation carriers develop an AD-like clinical phenotype characterized by early memory disturbances, visuospatial deficits and executive dysfunction, followed by atypical neurological signs including cerebellar ataxia and gait difficulties during later stages of the disease.9,36 Brain amyloid imaging by Pittsburgh Compound B positron emission tomography revealed a very weak signal in E693A APP mutation carriers with advanced dementia, suggesting that AD-like clinical symptoms occurred in the absence of relevant amyloid deposition in these patients.9,36 In *vitro* experiments with synthetic E22ΔAβ40 and E22ΔAβ42 peptide preparations demonstrated an enhanced oligomerization propensity, but no fibril formation,9 and led to the hypothesis that AD-like dementia in patients can be caused by the sole presence of synaptotoxic Aβ oligomers, which was well in line with previous experimental findings.37-43 In contrast to these initial *in vitro* findings, subsequent work, including our own recent study with highly pure recombinant peptide preparations, demonstrated that both E22ΔAβ40 and E22ΔAβ42 readily formed amyloid fibrils *in vitro*.20,24,44 Based on these *in vitro* findings, we hypothesized that E22ΔAβ40 and E22ΔAβ42 would, at least in principle, also form amyloid fibrils *in vivo*. Indeed, aged E22ΔAβ mice accumulated detergent-insoluble Aβ and showed thioflavin S and Congo red-positive amyloid deposits in leptomeningeal cortical and—more pronounced—cerebellar vessels; in contrast to the recently published E693Δ mouse model, which completely lacked extracellular amyloid deposition even at an age of 24 months.22 E22ΔAβ mice overexpress human APP at levels comparable to those in the Tg2576 mouse line, and the introduction of the Swedish double mutation results in an additional increase in total Aβ levels.6 Taken together, this might explain the differences between our model and the E693A mice. Interestingly,CAA deposits in the E22ΔAβ mice were more pronounced in the leptomeningeal vasculature of the cerebellum than in cortical vessels, which is in agreement with recent amyloid positron emission tomography findings in E693A mutation carriers showing a relative increase in Pittsburgh Compound B retention in cerebellar brain regions.36 The identification of CAA as a key neuropathological feature of aged E22ΔAβ mice adds the Osaka E693A mutation to the list of vasculotropic intra-Aβ APP mutations essentially comprising all of the currently known mutations at the APOE4 region (for a review see Kumar-Singh18). Our immunohistological analysis of CAA deposits identified Aβ40 as the major E22ΔAβ species depositing in the vessel walls of the E22ΔAβ mice, which is in line with previous findings in sporadic and familial AD cases,45,46 including the Dutch E693Q APP mutation.47 Further experiments are needed to elucidate the mechanisms underlying the vasculotropism of E22Δ-mutated Aβ and whether similar mechanisms as previously reported for the Dutch mutation (that is, a reduced receptor-mediated clearance across the blood–brain barrier) have a role.48,49
E22ΔAβ mice—similar to our previously reported arcAβ mice—develop intraneuronal Aβ aggregates that coincide with cognitive deficits beginning at 3 months of age. Intraneuronal Aβ accumulation is generally believed to be an early event in AD pathogenesis, although its relevance and role in the disease process remain a controversial topic. This may be partly due to technical considerations, including the use of nonspecific antibodies for the detection of intraneuronal Aβ. The intraneuronal Aβ staining in our E22ΔAβ mouse model only partially colocalized with staining of the APP C terminus, excluding false interpretation of Aβ staining emerging from full-length APP or APP C-terminal fragments. Furthermore, intraneuronal Aβ was stained by different Aβ C-terminus-specific antibodies, which do not crossreact with APP or APP fragments, including BC05 and BA27. These results therefore indicate that the intraneuronal aggregates in the E22ΔAβ mice indeed correspond to Aβ and not accumulating APP/APP fragments. The assembly state of the intraneuronal aggregates was characterized by immunohistochemistry using several oligomer-specific antibodies. Accumulation of intracellular oligomeric Aβ species has recently been reported in other APP transgenic mouse lines, including the McGill-Thy1-APP mice, and the E693A mice. Further immunohistological characterization of the intraneuronal deposits in the E22ΔAβ mice revealed that the intraneuronal aggregates were strongly stained by OC antibody, directed against fibrillar oligomers, but not A11 antibody, which recognizes prefibrillar oligomers. Interestingly, soluble fibrillar oligomers detected by OC antibody (but not prefibrillar oligomers) have recently been shown to be elevated in multiple brain regions of AD patients and to correlate with cognitive dysfunction. Building on the hypothesis that intra-Aβ mutations at or around position 22 of the Aβ increase Aβ fibrillogenesis through a facilitation of a ‘toxic turn’ conformation between positions 22 and 23 of the Aβ sequence, Murakami et al. developed a novel monoclonal antibody (11A1) directed against this specific conformational epitope of Aβ. In human AD brains, 11A1 stained both extracellular and intracellular Aβ deposits, whereas in APP transgenic Tg2576 mice, only extracellular deposits were labeled. Interestingly, 11A1 stained intraneuronal Aβ aggregates in the brains of the E22ΔAβ mice, suggesting that the oligomeric E22ΔAβ-mutated Aβ deposits contained the ‘toxic turn’ conformation.

Western blot and MSD analysis of APP cleavage products from cortical brain extracts revealed a relative increase in amyloidogenic (β-secretase-mediated) APP processing in the two mouse models bearing the E22 intra-Aβ APP mutations, which is in accordance with previous in vitro findings. As both E22Aβ and arcAβ mice overexpress similar full-length APP, sAPPβ and C99 levels, but significantly reduced sAPPβ+ and C83 levels, as compared with the Tg2576 mice, we concluded that the two intra-Aβ mutations specifically interfered with the β-secretase cleavage of APP in vivo. Previous in vitro reports in the Alzheimer’s disease research revealed that E693G APP was not a poor substrate for β-secretase, but instead reduced APP levels at the cell surface making Arctic APP less available for β-secretase cleavage, and increasing Aβ levels, especially at intracellular locations. Similar to the Arctic APP mutation, E693Δ APP overexpression in cell culture was also associated with reduced extracellular Aβ levels in vitro. Although these results may imply similar effects of the two intra-Aβ mutations on APP processing, we currently cannot exclude E693Δ APP as an inferior substrate to α-secretase-mediated cleavage, thus leading to the relative increase in amyloidogenic APP processing, as shown previously for other intra-Aβ APP mutations, including the Flemish E692G APP mutation.

MSD analysis revealed a marked reduction of buffer- and Triton buffer-soluble brain Aβ, as well as peripheral plasma Aβ levels, in particular when E22ΔAβ mice were compared with age-matched Tg2576 mice-expressing wild-type Aβ. This indicated that the absence of extracellular amyloid deposition up to an age of 15 months was not associated with a relative increase in soluble brain or plasma Aβ levels. Instead, most of the Aβ accumulated in the SDS-soluble protein fraction likely corresponding to intraneuronal Aβ pools. These results are in agreement with recent in vitro results from cell culture experiments showing increased intracellular Aβ accumulation, but markedly reduced secreted Aβ levels in E693Δ APP-transfected cell lines. The early accumulation of intracellular fibrillar oligomeric Aβ deposits in brains of the E22ΔAβ mice is in line with the unique aggregation profiles of the recombinant E22Δ Aβ40 and E22Δ Aβ42 peptides showing accelerated β-sheet formation in thioflavin T aggregation assays as compared with the respective wild-type Aβ peptides (and this study). When aggregation curves of E22Δ, E22G and wild-type Aβ40 and Aβ42 were compared, E22Δ Aβ42 showed the highest fibrillogenesis propensity as it aggregated without a lag phase and formed SDS-resistant amyloid fibrils immediately after reconstitution in solution at pH 7.4. However, E22A Aβ42 fibril growth after reconstitution in solution occurred only in a slow, non-exponential manner in contrast to the other peptides, including E22Δ Aβ40, whose aggregation was characterized by an exponential growth phase following lag phase. This slow fibril growth and lack of exponential growth in E22Δ Aβ42 may be a crucial factor in the prevention of extracellular amyloid deposition in the E22ΔAβ mice.

Apart from A673V, a recently described familial AD APP mutation in an Italian pedigree, E693A, is the second currently known recessive APP mutation that is pathogenic only in the homozygous state. Similar to the A673V mutation, which is antiamyloidogenic in the heterozygous state, our co-aggregation experiments with recombinant E22ΔAβ peptide variants also revealed an inhibition of Aβ aggregation when mutated and wild-type peptides were co-incubated. In contrast, co-aggregation of E22G (Arctic) Aβ peptides with the respective wild-type Aβ peptides resulted in aggregation curves very similar to those of the E22G Aβ peptides alone. Moreover, E22Δ Aβ42 fibrils, in contrast to wild-type Aβ42 and E22G Aβ42 fibrils, only very inefficiently seeded wild-type Aβ42 fibrillogenesis. These results provide a possible explanation why heterozygous carriers of the E693A mutation do not develop the disease, whereas heterozygous carriers of the autosomal dominant Arctic mutation develop dementia. Hence, we propose the following hypothetical model to explain the phenotype of the Osaka E693A mutation, in which E22ΔAβ aggregation occurs primarily in intracellular compartments where peptide concentrations are high enough to allow
for aggregate formation (Supplementary Figure 6). Outside the cell, E22Δβ peptide variants may interact with each other—and possibly also other peptides—in a way that results in an inhibition of aggregation and amyloid seed formation. Moreover, the E22Aβ42 peptide shows specific aggregation properties (slow non-exponential growth, lack of exponential growth phase) that likely further prevent parenchymal amyloid plaque deposition (Supplementary Figure 6). However, late vascular amyloid deposition occurs possibly due to the vasculotropism of the E22-mutated Aβ peptide variant and age-related changes along perivascular clearing pathways (Supplementary Figure 6).

Conflict of interest

Noriaki Kinoshita is employee of Immuno-Biological Laboratories, Gunma, Japan. Luka Kulic, Jordan McAfoise, Tobias Welt, Christian Tackenberg, Claudia Späni, Fabian Wirth, Verena Finder, Uwe Konietzko, Maria Giese, Anne Eckert, Takahiro Shimizu, Kazuma Murakami, Kazuhiro Irie, Suhail Rasool, Charles Glabe, Christoph Hock and Roger M Nitsch have no competing interests to declare.

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Mice expressing the Osaka intra-AP APP mutation

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