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Aβ seeds resist inactivation by formaldehyde

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Abstract Cerebral β-amyloidosis can be exogenously induced by the intracerebral injection of brain extracts containing aggregated β-amyloid (Aβ) into young, pre-depositing Aβ precursor protein- (APP) transgenic mice. Previous work has shown that the induction involves a prion-like seeding mechanism in which the seeding agent is aggregated Aβ itself. Here we report that the β-amyloid-inducing activity of Alzheimer's disease (AD) brain tissue or aged APP-transgenic mouse brain tissue is preserved, albeit with reduced efficacy, after formaldehyde fixation. Moreover, spectral analysis with amyloid conformation-sensitive luminescent conjugated oligothiophene dyes reveals that the strain-like properties of aggregated Aβ are maintained in fixed tissues. The resistance of Aβ seeds to inactivation and structural modification by formaldehyde underscores their remarkable durability, which in turn may contribute to their persistence and spread within the body. The present findings can be exploited to establish the relationship between the molecular structure of Aβ aggregates and the variable clinical features and disease progression of AD even in archived, formalin-fixed autopsy material.

Keywords Amyloid · Alzheimer · Prion · Fixation · Strain

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Introduction

The deposition of aggregated Aβ peptide in the brain parenchyma is an early and obligatory event in the pathogenesis

of Alzheimer's disease (AD) [12, 14]. Studies in several laboratories have demonstrated that Aβ aggregation can be instigated in the living brain by exogenous, Aβ-rich brain extracts [8, 9, 11, 17, 19–21, 28, 30, 34, 35], and that the causative agent is an aggregated form of Aβ itself [16, 20, 30]. These Aβ seeds bear many similarities to classical prions, i.e., aberrant assemblies of misfolded prion protein (PrP) that infect by inducing other PrP molecules to misfold, aggregate, and self-propagate [16, 25].

A remarkable attribute of prions is their resistance to inactivation by formaldehyde, a fixative that has been used for decades to neutralize viruses in the preparation of vaccines [7]. In the 1930s, W.S. Gordon discovered that a concentration of formaldehyde which inactivates the virus causing louping ill in sheep fails to disable the agent causing scrapie (now known to be a prion) [10]. The extreme resistance of the scrapie agent to formaldehyde, which has been confirmed in many laboratories, was one of the earliest indications that the pathogenic agent is unorthodox [23, 24], helping to impel the development of the prion concept [25].

Given the theoretical significance of the prion paradigm for understanding a diversity of diseases [16, 26], we asked whether Aβ seeds resemble prions in their resistance to inactivation by formaldehyde. Our findings show that Aβ-rich brain extracts retain their ability to induce Aβ deposition in APP-transgenic host mice even after the tissue has spent years in formaldehyde fixative. Using luminescent conjugated oligothiophenes (LCOs), our results further indicate that formaldehyde fixation partially modifies plaque morphology but at the same time maintains the molecular architectures that define Aβ strains.

Materials and methods

Mice

Three-to-four-month-old male and female APP23 transgenic (tg) mice were used as the seed hosts for all studies [31]. The mice had been backcrossed with C57BL/6J mice for 20 generations (C57BL/6J-Tg(Thy1-APPK670N;M671L)23). All mice were kept under specific pathogen-free conditions. The experimental procedures were carried out in accordance with the veterinary office regulations of Baden-Württemberg (Germany) and with US federal guidelines, and all experiments were approved by the Institutional Animal Care and Use Committees.

Donor brain tissue

Human post-mortem brain samples (frontal cortex) were obtained at autopsy from histopathologically diagnosed AD patients (CERAD C/Braak stage VI; with extensive Aβ deposition in the frontal cortical tissue sample) and from a non-demented control patient (CERAD 0/Braak stage III–IV, with no Aβ deposition in the frontal cortical tissue sample) obtained from the brain bank affiliated with the University of Tübingen. Consent for autopsy was obtained from the legal representative in accordance with local institutional review boards. Prior to use, the human tissue was stored at 4 °C or room temperature for 1–2 years (AD1: 2 years; AD2 and Control: 1.5 years) in a PBS-neutral buffered 4.5 % formaldehyde solution (Roti®-Histofix, Carl Roth, Karlsruhe, Germany). Prior to homogenization, residual formaldehyde was removed by rinsing the tissue under water for 2 h. Mouse brains were obtained from aged, Aβ-depositing APPPS1 tg mice (20–22 months old), APP23 tg mice (25–27 months old), and from age-matched, non-tg wildtype (WT) mice [27, 31]. After removal of the brain, the cerebellum and the lower brainstem were detached at the mesencephalic flexure and the forebrain was divided midsagittally into the two hemispheres. The left hemisphere was immediately fresh-frozen (unfixed) on dry ice and stored at −80 °C until use. The right hemisphere was immersion-fixed for 48 h in PBS-buffered 4 % formaldehyde solution [prepared by de-polymerization of 4 % (w/v) paraformaldehyde in PBS] and then cryoprotected in 30 % sucrose in PBS for an additional 48 h. The right hemibrains were then frozen on dry ice and stored at −80 °C until use.

Extract preparation

Fixed and fresh-frozen tissues were homogenized at 10 % (w/v) in sterile PBS at 4 °C (4 × 10 s at 5,500 rpm, each round separated by a 10 s pause) using the Precellys 24-Dual homogenizer (Bertin, Montigny-le-Bretonneux, France; 7 ml lysing tubes with 2.8 mm ceramic beads). Homogenates were centrifuged at 3,000×g for 5 min (4 °C). The supernatants were aliquoted and immediately frozen. For all experiments, a 10 % (w/v) extract was used unless otherwise stated.

Biochemical analyses

Fixed and fresh-frozen brain extracts were analyzed on NuPage Bis–Tris mini gels using NuPage LDS sample buffer and MES running buffer (Invitrogen). For Western blotting, samples were semi-dry blotted onto a nitrocellulose membrane and then probed with antibody 6E10 to human-sequence Aβ (reactive to amino acid residues 1–16; Covance Research Products). Samples were visualized with chemiluminescence using SuperSignalWest Pico (Thermo Scientific). Synthetic Aβ from American Peptide was used for standardization.

Stereotactic injection of brain extracts

Male and female 3–4-month-old APP23 tg hosts were anesthetized using a mixture of ketamine (100 mg/kg body weight) and xylazine (10 mg/kg body weight) in saline. 2.5 µl of brain extract was then infused bilaterally into the dorsal hippocampus (AP −2.5 mm, L ± 2.0 mm, DV −1.8 mm) by stereotactic injection. Injection speed was 1.25 µl/min and the needle was slowly removed after being kept in place for an additional 2 min. The surgical area was cleaned with sterile saline and the incision was sutured. Mice were kept under infrared light for warmth and monitored until recovery from anesthesia.

Histology and immunohistochemistry

After an incubation period of 4 months, inoculated mice were perfused for 5 min with ice-cold PBS. Brains were removed and immersion-fixed in 4 % formaldehyde in PBS for 48 h, and then placed in 30 % sucrose in PBS for 48 h. Brains were frozen in 2-methylbutane cooled with dry ice and then serially cut into 25 µm-thick coronal sections using a freezing-sliding microtome. The sections were collected in cryoprotectant (35 % ethylene glycol, 25 % glycerol in PBS) and stored at −20 °C until use. For immunostaining of Aβ, polyclonal antibody CN3 was used [9]. Sections were counterstained with Congo red according to standard protocols.

Stereological analysis

Total β-amyloid load was quantified on a CN3/Congo red-stained set of every 12th systematically sampled coronal section throughout the entire hippocampus. A microscope equipped with a motorized x–y–z stage coupled to a video-microscopy system and the Stereo Investigator software (MicroBrightField, Inc., Williston, VT, USA) was used as previously described [1]. The investigators who performed the analysis were blind to the inoculation groups. The total β-amyloid load (percentage) was determined by calculating the areal fraction occupied by CN3- and Congo red-positive staining in two-dimensional sectors (20×/0.45 objective).

Histological staining with pFTAA and spectral analysis

Coronal brain sections (25 µm) from inoculated mice were washed in PBS (3 × 10 min) and subsequently mounted on Superfrost slides. Sections were allowed to dry for 2 h at RT. Staining with pentamer formyl thiophene acetic acid (pFTAA; 1.5 mM in deionized water, diluted 1:1,000 in PBS) was performed as previously described [18]. (Note that the trimeric polythiophene acetic acid (tPTAA) used in a previous study [13] is no longer produced because of stability issues, and has been replaced by pFTAA). Spectra were acquired on a Zeiss LSM 510 META (Axiovert 200 M) confocal microscope (40× oil-immersion objective, 1.3 NA) equipped with an argon 458 nm laser for excitation and a spectral detector. Emission spectra were acquired from 470 to 695 nm. The spectra were collected from three matched regions of interest (ROIs) within 40 Aβ plaques per animal. The ratio of the intensity of emitted light at the green-shifted portion (492 nm) and red-shifted peak (599 nm) was used as a parameter for spectral distinction of different plaques. The peaks were selected to maximize the spectral variations of pFTAA-stained APP23 and APPPS1 Aβ plaques obtained by this single wavelength excitation procedure to minimize exposure time.

In vitro aggregation assay

In brief, Thioflavin T was dissolved in water to generate a 200 µM stock solution. Lyophilized recombinant Aβ1-40 peptide was dissolved to a stock concentration of 5 mM in 100 % DMSO. Prior to use, this stock was freshly diluted to 0.5 mM in DMSO and sonicated for 10 min in a water bath, followed by 5 min centrifugation at $16,100 \times g$ at room temperature. The supernatant was then further diluted to a concentration of 250 µM Aβ1-40 in a 50 % DMSO stock solution. For kinetic measurement in the 96-well format, 1 µl of brain extract was incubated with 20 µM Thioflavin T, 25 µM Aβ1-40, 50 mM phosphate and 150 mM NaCl (final volume of 1 ml) at 37 °C. Using 96-well clear-bottom plates (Greiner, Bio-One), each brain homogenate was assayed in 8 sealed wells. Thioflavin T fluorescence at 480 nm was measured from the plate bottom every 30 min using a BMG Fluostar plate reader. Before each measurement, a double orbital shaking step at 500 rpm for 30 s was performed. The increase in fluorescence over time was followed until the maximum signal was reached. Raw data were fitted and lag times determined with GraphPad Prism 5 as previously described [22].

Results and discussion

Extract from formaldehyde-fixed AD brain tissue harbors β-amyloid-inducing activity

Formaldehyde-fixed brain extract from two confirmed AD cases robustly induced Aβ deposition in young APP-tg mouse brains 4 months after intracerebral inoculation (Fig. 1). No such Aβ deposits were observed following infusion of an extract from formaldehyde-fixed, non-demented human control brain. Fixed tissue extract from AD case 1 yielded more seeded Aβ deposition than did fixed extract from AD case 2 (Fig. 1c). Correspondingly,
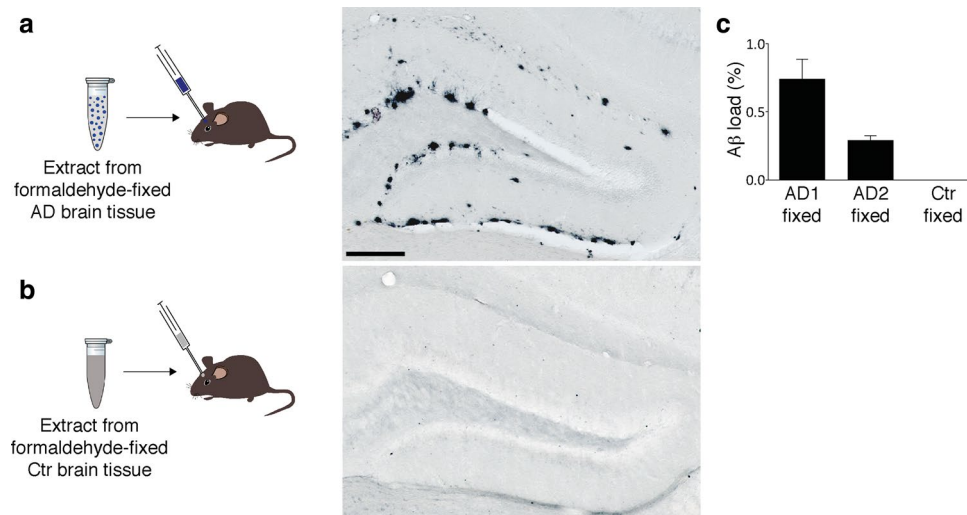
Fig. 1 Formaldehyde-fixed brain tissue from AD patients induces cerebral β-amyloidosis in APP23 transgenic mouse hosts. **a**, **b** Young 3–4 month-old APP23 transgenic (tg) mice were intracerebrally inoculated with extracts of formaldehyde-fixed tissue from two AD cases (AD1 and AD2) or from a non-demented human control (Ctr) case. Four months after intrahippocampal extract injection (2.5 µl), Aβ-deposition was induced in the hippocampus of APP23 tg mice receiving AD extract (**a**). No Aβ-deposits were induced by the control brain extract (**b**). **c** Stereological quantification of the percent area of the hippocampus occupied by immunoreactive Aβ (Aβ load) induced by extract from the two AD cases and the control case (n = 3–6 mice/group, mean ± SEM, *scale bar* 200 µm)

Aβ immunohistochemical staining indicated that AD case 1 had more Aβ-deposition (and in particular numerous dense-core plaques and significant Aβ-deposition in the vasculature) than did AD case 2. No Aβ-deposits were found in the control donor (Supplementary Figure 1).

Extract from formaldehyde-fixed, β-amyloid-laden mouse brain tissue also induces β-amyloid deposition

To substantiate the amyloid-inducing activity of formaldehyde-fixed β-amyloid-containing human brain tissue, we repeated the inoculation experiments with brain extract from an aged (22-month-old) APPPS1 tg mouse. At this age, APPPS1 tg mice exhibit extensive cerebral β-amyloidosis [27].

For this experiment, the APPPS1 brain and a brain from an age-matched wildtype (WT) mouse were midsagittally divided into two hemispheres. One hemisphere was immersion-fixed in formaldehyde while the other was fresh-frozen (unfixed) (Fig. 2a). SDS-PAGE and subsequent Aβ-immunoblotting of the 3,000×g brain homogenate supernatants revealed ~20-fold less monomeric/monomerized Aβ (and also total protein) in the extract from the formaldehyde-fixed tg hemisphere compared to the extract from the fresh-frozen tg hemisphere (Fig. 2b), presumably because less Aβ/protein was liberated from the tissue to enter the supernatant in the fixed samples (only a negligible fraction of the fixed tissue did not enter the gel). To adjust for the difference in the amount of Aβ

in the extracts, the fresh-frozen brain material was diluted 1:20 to approximate the Aβ concentration in the fixed brain extract prior to intrahippocampal injection of the extracts into APP23 tg mice. Aβ-immunostaining 4 months later revealed robust β-amyloid induction in the hippocampus of mice receiving the diluted (1:20) fresh-frozen tg brain extract (Fig. 2c). Extract from the formaldehyde-fixed tg hemisphere also induced Aβ deposition that was generally similar in pattern and morphology to that induced by the fresh-frozen extract (Fig. 2d). Stereological quantification of Aβ immunoreactivity revealed that the fixed tg mouse brain extract yielded less Aβ deposition than did the diluted fresh-frozen brain extract, although this difference was not statistically significant (Fig. 2g). No Aβ deposits were found after inoculation with extracts from fresh-frozen or fixed WT hemispheres (Fig. 2e, f).

The probable β-amyloid-inducing factor in fixed brain extracts is likely similar to that of fresh-frozen brain material, previously identified as Aβ species ranging in size from soluble oligomers to fibrillar Aβ [19, 20]. However, because the denaturing conditions in SDS-PAGE tend to disrupt the native protein state and monomerize multimeric assemblies, the precise nature of the biologically active seeds in the fixed material (and whether they differ from seeds in the native state) remains to be determined.

Next, extracts from three additional APPPS1 tg mice and WT mice (fixed and fresh-frozen, all 20–22 months old) were tested in an in vitro aggregation assay. The results confirmed both the in vivo seeding capability of
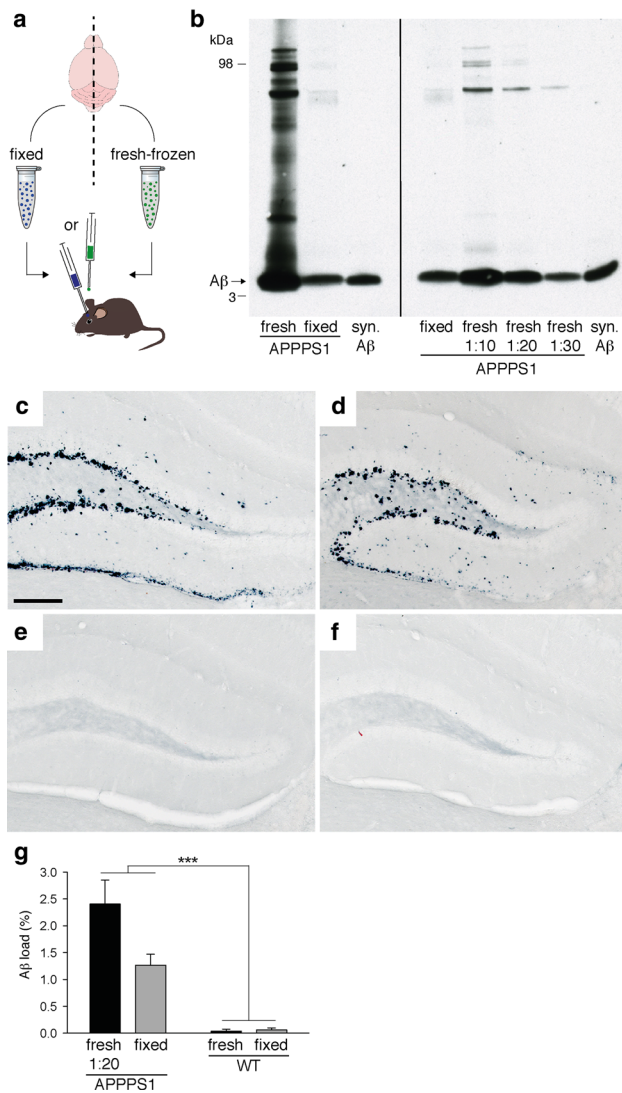
Aβ plaques in aged APPPS1 mice, while APP23 seeds injected into an APP23 host induce Aβ deposits reminiscent of Aβ plaques in aged APP23 mice [13].

To investigate whether this phenomenon of congruent templated conversion also applies to the fixed brain material, extracts from fixed and fresh-frozen APPPS1 and APP23 hemispheres were intracerebrally injected into young APP23 tg mice, respectively. Immunohistochemical analysis 4 months after inoculation revealed the expected Aβ morphotypes that result from injections of fresh-frozen brain extracts (i.e., Aβ plaques with diffuse penumbras for APP23 mice, and small, compact deposits for APPPS1 mice, Fig. 4a, b). However, fixed donor extracts from both APP23 and APPPS1 tg mice gave rise to Aβ plaques that were rather small and compact (Fig. 4c, d). Subsequently, luminescent conjugated oligothiophenes (LCOs) were used to further investigate whether the amyloid induced by extracts from fixed brains can be distinguished by their emission spectra, as previously shown for injections of fresh-frozen brain material [13]. Quantitative results revealed that pFTAA spectrally discriminates between Aβ deposits in mice that were injected either with fresh-frozen APP23 or fresh-frozen APPPS1 brain extract (Fig. 4e), consistent with previous studies [13]. Spectral analysis of plaques induced by the injection of fixed APP23 or fixed APPPS1 brain extracts also showed a significant difference (Fig. 4e). These data indicate that formaldehyde fixation partially modifies the histological appearance of seeded Aβ plaque morphology, but at the same time maintains at least some of the basic conformational properties of the aggregated Aβ.

formaldehyde-fixed brain homogenates and the reduced efficacy of the fixed material (Fig. 3). Furthermore, as with APPPS1 tissue donors, extract from fixed brains of aged APP23 tg mice (25–27 months old) also displayed seeding activity in vitro (Fig. 3).

Formaldehyde fixation preserves the strain-like properties of seeded Aβ plaques

Aβ plaques in different APP-tg mouse lines vary in appearance and molecular architecture; whereas Aβ deposits in aged APP23 tg mice are fairly large with congophilic cores and diffuse penumbras, Aβ plaques in aged APPPS1 tg mice are small, compact, and highly congophilic [13, 20]. By cross-inoculation experiments, we previously showed that such Aβ plaque morphotypes can be maintained by seeded conversion, i.e., APPPS1 seeds injected into an APP23 host induce Aβ deposits reminiscent of endogenous

An important characteristic of prions is their resistance to inactivation by physical and chemical treatments that neutralize most microbes and viruses [5, 23, 25, 29]. Although viruses vary in their sensitivity to formaldehyde treatment [2], the persistent infectivity of prions even after exposure to formaldehyde [10] for months or years [4, 23, 24] was one of the earliest indications that the causative agent of scrapie was unlike conventional infectious agents [23]. Here we demonstrate that Aβ seeds, like prions, are resistant to inactivation by formaldehyde. Specifically, brain material containing aggregated Aβ from APP-tg mice (fixed for 48 h) or from human AD cases (fixed for up to 2 years) retained the capacity to seed Aβ deposition when infused into the brains of APP-tg host mice. Aβ seeds are not known to be infectious in the sense of facile transmissibility from one organism to another [16]. However, in light of previous studies showing that Aβ seeds remain functional following boiling [20] or drying [8], these findings further underscore their molecular commonalities with prions.

The seeding efficacy of fixed tissue was less than that of comparable unfixed tissue, possibly due to reduced liberation of Aβ from fixed samples during homogenization, as shown by immunoblot analysis of the extracts (Fig. 2b). It is also possible that fixation additionally diminishes the inductive efficacy of Aβ seeds by corrupting their molecular structure, although the LCOs indicate that formaldehyde fixation largely preserves the strain-like properties of the induced amyloid. The loss of diffuse Aβ immunostaining in the penumbras of plaques that were induced with extract from the fixed compared to fresh-frozen APP23 brains may indicate that various Aβ species are differentially sensitive to formaldehyde fixation.

Aβ in fixed tissue is partially resistant to digestion by proteinase K (unpublished data), suggesting that fixation may act to immobilize and shield Aβ seeds from proteolytic degradation. Interestingly, tissue fixation by formaldehyde can protect prions against inactivation by autoclaving [5, 33], though denaturation with formic acid effectively neutralizes prions in fixed tissue [6, 32]. Formic acid also negates the amyloidogenic capability of Aβ seeds in unfixed tissue [20]. Given the effectiveness of formic acid against prion infectivity, it seems likely that formic acid also neutralizes Aβ seeds in fixed tissue, but this possibility remains to be tested. Importantly, the resistance of prions to standard methods of inactivation has prompted improvements in the sterilization of medical instruments and general handling practices for tissue suspected of harboring prions [29]. As a result, iatrogenic transmission of prion disease to humans has essentially ceased [3].
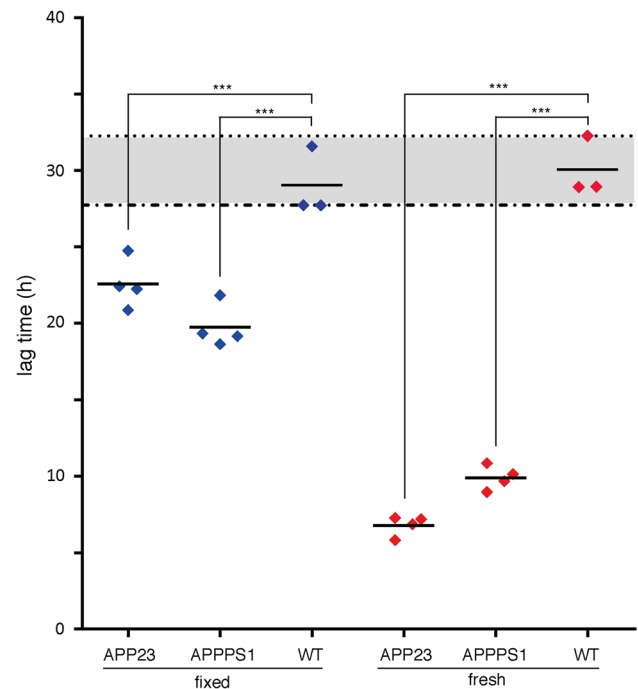

Fig. 3 Formaldehyde-fixed brain material from APP-transgenic mice harbors in vitro seeding capacity. Fibrillization kinetics of recombinant Aβ1–40 were monitored by incorporation of Thioflavin T (ThT). Lag times were determined as measures of the seeding capacity of the extracts. Both APP23 brain tissue and APPPS1 brain tissue show reduced in vitro seeding activity in response to formaldehyde fixation ($n = 4$/group). Note that the fresh-frozen extracts were not diluted in this experiment (compare also to Fig. 2). Extracts of both fixed APP23 and fixed APPPS1 mouse brains still show strong seeding activity compared to fixed (or fresh-frozen) wildtype (WT) brain extracts ($n = 3$/group). Each data point represents the mean lag time of an individual brain extract determined by measurement of eight technical replicates, with seeding by all of the extracts measured twice, with the exception of extracts from three donor mice that were measured only once. The mean of each group is indicated by a *black line*. ANOVA (genotype of donor material × tissue preparation with matched fixed vs. fresh values) revealed a significant effect of the donor genotype ($F(2, 8) = 124.8$; $p < 0.001$), tissue preparation ($F(1, 8) = 759.9$; $p < 0.001$) and interaction ($F(2, 8) = 256.1$; $p < 0.001$). Subsequent Tukey's multiple comparison tests revealed that lag times associated with both fixed APP23 and fixed APPPS1 mouse brain extracts were shorter compared to lag times in the presence of fixed WT brain extracts (all ps < 0.001). Similarly, the lag times yielded by fresh-frozen APP23 and APPPS1 mouse brain extracts were significantly shorter compared to those associated with fresh-frozen WT brain extracts (all ps < 0.001)

Conclusions

Our experiments show that Aβ seeds in brain tissue extracts retain the ability to induce β-amyloidosis even after prolonged (2 years) exposure to formaldehyde. Furthermore, the formaldehyde-fixed seeds largely retained their conformational templating capacity, as the nascent amyloid
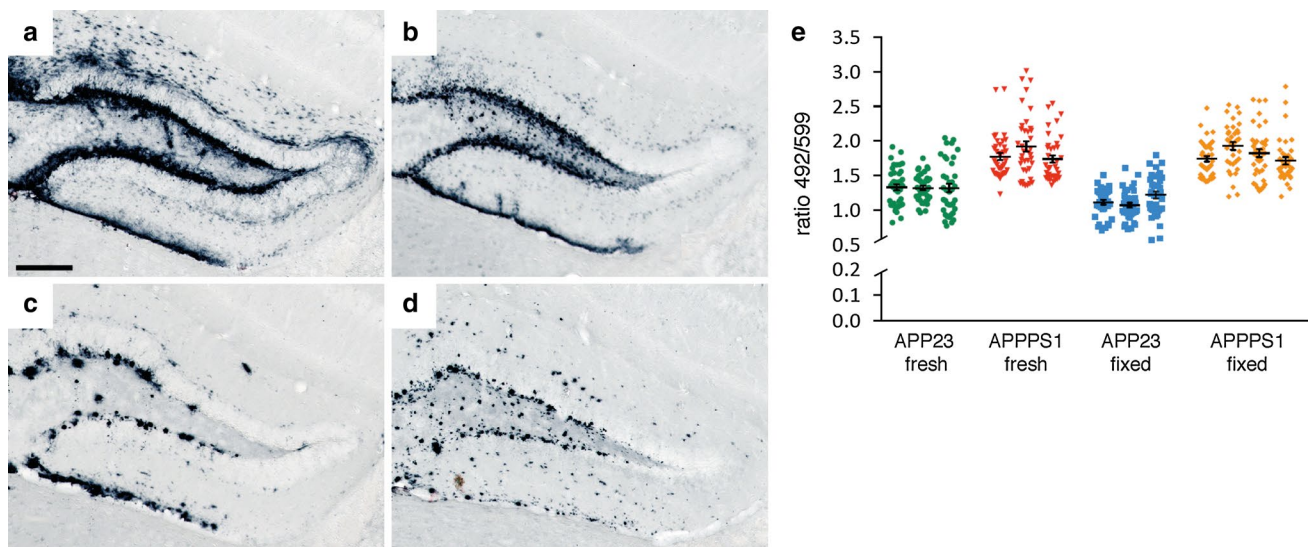
Fig. 4 Formaldehyde fixation preserves strain-like properties of seeded Aβ plaques. Brain extracts from aged APPPS1 or APP23 tg donor mice (the extracts were from two donor mice each) were intracerebrally injected into pre-depositing, 3–4 month-old APP23 tg host mice. Brains were analyzed 4 months after inoculation using Aβ immunostaining. **a–d** The extract of the fresh-frozen APP23 brain tissue induced a more diffuse pattern of Aβ deposition (**a**), whereas the extract of the fresh-frozen APPPS1 brain tissue induced a more punctate pattern of Aβ deposition (**b**). Injection of extracts from formaldehyde-fixed brain tissue induced a more punctate pattern for both APP23 (**c**) and APPPS1 (**d**) donor mice (*scale bar* 200 μm). **e** Spectral properties of the induced Aβ deposits using luminescent conjugated oligothiophenes (pFTAA). For quantitative analysis, the ratio of the intensity of the emitted light at 492 and 599 nm was calculated (see "Materials and methods"). Each dot represents one Aβ plaque. The mean and SEM are indicated for each animal ($n = 3–4$/group). ANOVA (genotype of donor material × tissue preparation) revealed a significant effect of the donor genotype ($F(1, 9) = 165.6$; $p < 0.001$) but no significant effect of the tissue preparation ($F(1, 9) = 4.856$; $p = 0.055$) or interaction ($F(1, 9) = 3.947$; $p = 0.078$)

deposits replicated the spectral properties of the parental seeds, at least at the binding sites of conformation-sensitive oligothiophene ligands. The discovery that both the self-propagating activity and strain-like features of aggregated Aβ are maintained in fixed tissue further supports the incorporation of Aβ seeds into the broad conceptual framework of prions. In addition, the stability of the seeds in fixed material can now be exploited to establish the relationship between the molecular architecture of Aβ and the variable clinical features of AD, even in archived, formalin-fixed brain samples. Although Aβ seeds can persist in fixed tissue, the implications for standard laboratory practice are uncertain. Currently the weight of evidence—albeit mostly circumstantial—argues against the exogenous infectivity of Aβ seeds in AD [15, 16]. Rather, AD appears to arise spontaneously due to the endogenous emergence and autopropagation of pathogenic protein seeds. The present findings highlight the extraordinary robustness of Aβ seeds; once they gain a foothold in the brain, their resistance to destruction facilitates the formation and endogenous spread of new seeds, thereby sustaining the disease process.

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