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Amyloid-β in the Cerebrospinal Fluid of APP Transgenic Mice Does not Show Prion-like Properties

Zhiva Skachokova[1,2], Frederik Sprenger[1,2], Karin Breu[1,2], Dorothee Abramowski[3], Florence Clavaguera[1], Jürgen Hench[1], Matthias Staufenbiel[3,4], Markus Tolnay[1] and David T. Winkler[1,2,*]

[1]*Institute of Pathology, University Hospital Basel, Basel, Switzerland;* [2]*Department of Neurology, University Hospital Basel, Basel, Switzerland;* [3]*Institute of Biomedical Research, Novartis Pharma AG, Basel, Switzerland;* [4]*Department of Cellular Neurology, Hertie Institute for Clinical Brain Research, University of Tübingen, Tübingen, Germany*

Abstract: Early diagnosis of Alzheimer`s disease (AD) is currently difficult and involves a complex approach including clinical assessment, neuroimaging, and measurement of amyloid-β (Aβ) and tau levels in cerebrospinal fluid (CSF). A better mechanistic understanding is needed to develop more accurate and even presymptomatic diagnostic tools. It has been shown that Aβ derived from amyloid-containing brain tissue has prion-like properties: it induces misfolding and aggregation of Aβ when injected into human amyloid precursor protein (APP) transgenic mice. In contrast, Aβ in the CSF has been less studied, and it is not clear whether it also exhibits prion-like characteristics, which might provide a sensitive diagnostic tool. Therefore, we collected CSF from APP transgenic mice carrying the Swedish mutation (APP23 mice), and injected it intracerebrally into young mice from the same transgenic line. We found that CSF derived Aβ did not induce increased β-amyloidosis, even after long incubation periods and additional concentration. This suggests that Aβ present in the CSF does not have the same prion-like properties as the Aβ species in the brain.

Keywords: Alzheimer's disease, amyloid-beta, Aβ, prion, cerebrospinal fluid, diagnostics, transgenic mouse models.

1. INTRODUCTION

Alzheimer's disease (AD) is the most common neurodegenerative disorder, and while its prevalence is increasing [1], curative treatment options are still lacking. In the course of AD, amyloid-β (Aβ) aggregates to form extracellular plaques, while hyperphosphorylated tau protein constitutes intracellular neurofibrillary tangles. The aggregation of Aβ is thought to be an early event that drives AD pathogenesis and begins at least a decade before clinical symptoms emerge [2-5].

In routine clinical practice, diagnosis of AD is often late and of limited accuracy [6]. At present, levels of Aβ and tau in the cerebrospinal fluid (CSF) are used as biomarkers of AD within a multimodal diagnostic approach [7, 8]. However, the validity of CSF biomarkers and their use for early or even presymptomatic diagnosis of AD is still a matter of debate. At the same time, an increasing number of experimental studies suggest that the aggregation of the proteins involved in AD pathogenesis occurs by a self-propagating process during which misfolded proteins act as templates, inducing misfolding and aggregation of native molecules [9-12]. As this self-propagating propensity is shared with prions, these proteins have been termed prion-like [13], even though they substantially differ from prions in their pathomechanisms [14, 15]. Aβ present in brain homogenates derived from AD patients or plaque-bearing amyloid precursor protein (APP) transgenic mice induces and/or accelerates Aβ deposition when injected into susceptible mouse models, in a prion-like manner [16-20]. This suggests that the pathological protein conformation can be transmitted and locally propagated a process we refer to here as 'seeding'.

Transgenic mice overexpressing human APP are widely used to model aspects of AD *in vivo*, since they develop Aβ plaques progressively with the age and have been established as a seeding model [16-18]. At the same time, little is known about the presence of pathological Aβ variants in their CSF. Such molecules could serve as sensitive early AD diagnostic markers. In a recent study, Fritschi *et al.* [21] demonstrated that CSF from both AD patients and APP transgenic mice lack *in vivo* seeding activity. Here we investigate this question further by injecting murine CSF into young APP mice and analyzing them after very long seeding times and inoculation of highly concentrated CSF. Even under these conditions, we didn't observe a significant increase in Aβ aggregation, neither at the injection site nor within the injected hippocampus, suggesting a low to absent prion-like activity of the Aβ forms present in the CSF.

*Address correspondence to this author at the Institute of Pathology, University Hospital Basel, Basel, Switzerland; Tel: 0041 61 265 25 25; Fax: 0041 61 265 41 00; E-mail: david.winkler@usb.ch

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2. METHODS

2.1. Mice

We used heterozygous APP23 transgenic mice [22], expressing human APP (751-aa isoform) containing the Swedish mutation under the control of the Thy-1.2 promoter. All experiments were approved by the University of Basel Ethics and Animal Care and Use Committees. Initial qualitative data of a subset of the mice used for the present analysis has been included in a previous study [21]. An overview of all the mice included in the study is provided in Table **S1**.

2.2. Stereotaxic Injections

Three months old APP23 mice (n=20) and non-transgenic C57BL6 (n=10) control mice were anaesthetized with a mixture of ketamine (10 mg/kg) and xylazine (20 mg/kg) and placed on a heating pad to maintain body temperature during surgery. Mice were injected in the right hippocampus (A/P, −2.5 mm from bregma; L, − 2.0 mm; D/V, −1.8 mm) using a Hamilton syringe, as previously reported [23]. Each received a unilateral stereotaxic injection of 5 µl at a speed of 1.25 µl/min. Mice were monitored until recovery from anesthesia and checked regularly following surgery.

2.3. CSF Sampling

For CSF sampling we used 3, 18 and 24 months old APP23 and C57BL6 mice (n=45). CSF was collected by puncturing the cisterna magna after deeply anesthetizing the animals, as previously described [24]. Next it was spun down at 2500 rpm for 2 min and the supernatant was collected and immediately frozen. Visibly blood contaminated CSF was discarded. For injections, CSF from 3 mice at the same age was pooled. For concentration, pooled CSF from 5-7 mice was lyophilized and later resuspended with a reduced volume of sterile H_2O.

2.4. Immunohistochemistry

Following 11 to 21 months from the date of injection, mice were deeply anaesthetized with pentobarbital (100 mg/kg) and killed by transcardial perfusion with cold PBS, followed by 4% paraformaldehyde in PBS. The brains were dissected and post-fixed overnight. 4 µm coronal sections were prepared following paraffin embedding. For immunohistochemical analysis, sections were deparaffinized, pretreated with 100% formic acid for 5 min, blocked with 10% normal horse serum and incubated overnight with an anti-Aβ 82E1 antibody (human N-terminal specific, 1:1000, *Demeditec Diagnostics GmbH*). For detection we used the Vectastain ABC Peroxidase kit (*Vector Laboratories*). Slides were counterstained with hematoxylin and eosine, and pictures were taken using Olympus DP73 microscope.

2.5. Quantification and Statistical Analysis

For quantification, we used 8 to 10 82E1 stained brain sections, depending on the tissue quality. Sections were selected for each animal from the injected right (R) and lateral left (L) hemisphere at corresponding Bregma levels, spanning the hippocampus (starting anterior to the injection site at -1.8 mm and extending to -3.1 mm posterior), with at least 50 µm distance in between. The hippocampal Aβ-plaque burden (the area occupied by all plaques as percent of the total area) was estimated for each section using ImageJ software [25]. In order to analyze the effect of CSF inoculation on Aβ-plaque-burden, linear mixed-effects models were used. We thereby compared the injected to the non-injected hippocampal side. Aβ-plaque burden served as dependent variables, independent variables were hippocampal sides (injected vs. non-injected). Subject was treated as a random factor. Side was nested within time point and group. To achieve approximately normal distribution, Aβ-plaque burden values were log-transformed (zeros were replaced by half the smallest value). Results were expressed as geometric mean ratios (GMR) with corresponding 95% confidence intervals and p-values. A p-value <0.05 was considered as significant. All analyses were done using R version 3.0.1 (R Foundation for Statistical Computing, Vienna, Austria).

3. RESULTS

3.1. Prolonged Seeding with APP23 CSF Did not Induce Increased Aβ Pathology

To test the prion-like properties of CSF derived amyloid-β, we inoculated CSF from aged APP23 or C57BL6 mice into the hippocampus of young APP23 mice. APP23 mice show robust plaque development starting at the age of 8-9 months [22], and seeding studies are mostly terminated close to this stage. To allow longer inoculation periods, we analyzed the seeding effect of CSF from aged APP23 mice 14 or 21 months post inoculation. In order to control for the endogenous pathology present at this age, we compared Aβ plaque densities of the injected with the non-injected hippocampus for each mouse. However, we did not observe a significant difference in hippocampal Aβ pathology in mice injected with transgenic CSF (Fig. **1A, B**, Fig. **2A, B**), showing that additional inoculation time did not contribute to a possible local increase of the pathology. As expected, Aβ plaque burden was unchanged after inoculation of wild type CSF (Fig. **1C**, Fig. **2C**). Seeding with APP23 brain homogenates resulted in a significant enhancement of the endogenous Aβ pathology in the right hippocampus and visible focal aggregation around the injection site, similar to previous reports (Fig. **1D**, Fig. **2D**) [17, 18]. No Aβ pathology was induced up to 21 months post inoculation of APP23 CSF into C57BL6 host mice (Fig. **1E**, Table **S3**).

3.2. Seeding with Concentrated APP23 CSF Did not Induce Increased Aβ Pathology

In order to test the hypothesis that Aβ concentration in the CSF might be too low to induce a seeding effect, we injected APP23 mice with concentrated APP23 CSF comprising a 22 fold higher amount of $A\beta_{40}$ (Table **S2**). This highly concentrated CSF did not cause tissue necrosis at or in proximity to the injection site. Because of the high number of donor mice required for the collection of a sufficient amount of mouse CSF for concentration, we were able to inject only a very limited number of mice. A quantitative analysis of the Aβ plaque load, comparing the injected versus non-injected hippocampus did not show a significant seeding effect after 11 (Fig. **3A**, Table **S3**) or 20 months (Fig. **3B**, Table **S3**). No focal Aβ aggregation was visible at or close to the injection

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site. In addition, inoculation of concentrated C57BL6 CSF did not result in any seeding effect after 11 months, as expected (Fig. **3C**, Table **S3**).

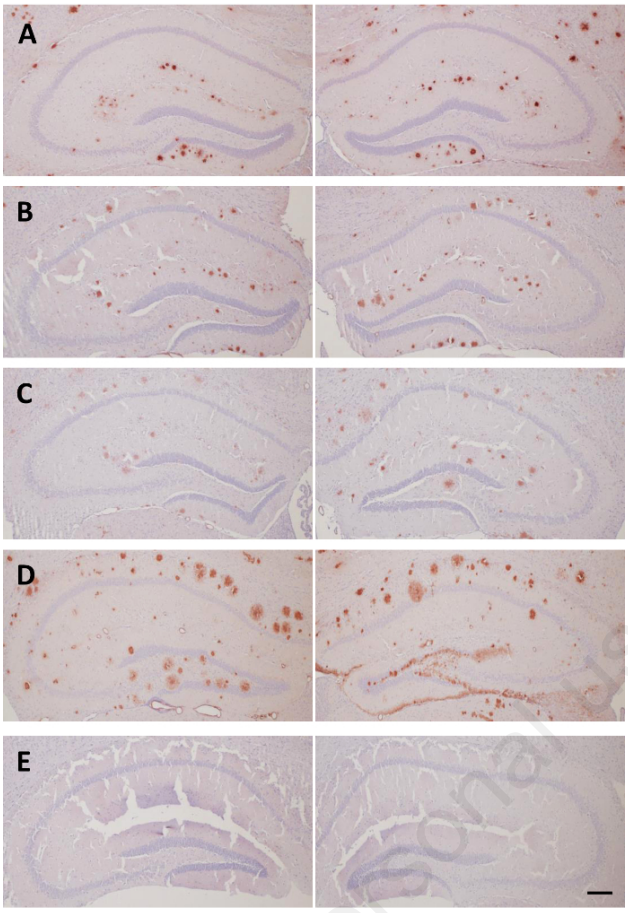

Fig. (1). Immunohistochemical anaylsis of injected mice using 82E1 antibody. APP23 mice analyzed 14 (**A**) or 21 months (**B**) after inoculation with CSF derived from aged APP23 mice (18 and 24 months old, respectively) did not show an increase in Aβ plaque burden in the injected hippocampus (right images), compared to the non-injected side (left images). Inoculation of wild-type mouse CSF did not provoke any seeding effect (**C**), while mice seeded with forebrain homogenate derived from aged APP23 mice exhibited enhanced Aβ deposition in the injected hippocampus (**D**). C57BL6 mice injected with APP23 CSF did not demonstrate any pathology (**E**). Scale bar equals 200 μm.

DISCUSSION/CONCLUSION

This study extends the findings recently reported on CSF Aβ seeding [21]. Our analysis includes mice injected with concentrated CSF for very long incubation time, and confirms the absence of relevant *in vivo* seeding effect of CSF derived Aβ.

In the present study, we used APP23 mice as a seeding model to test whether CSF Aβ exhibits prion-like properties. APP23 mice were inoculated unilaterally into the hippocampus with forebrain homogenate or CSF. This allowed an intra-individual comparison of the pathology between the injected and non-injected hippocampus. APP23 mice seeded with forebrain homogenate for 20 months exhibited a significantly higher Aβ plaque load and focal seeding effects in the injected hippocampus, confirming previous reports [16-20]. In contrast, APP23 mice injected with CSF derived from plaque-bearing APP23 mice for a period of up to 21 months did not show any increase in Aβ load at or close to the injection site. This suggests a lower seeding effect of the Aβ in the CSF, as compared to the one in the brain.

The lack of seeding activity of Aβ containing CSF could be attributed to different factors. Previously, it has been reported that seed-induced Aβ deposition increases significantly with time [16]. For this reason, we have extended our seeding time to the maximum possible with regard to the mouse life span. Our seeding periods by far exceed those reported previously (6-9 months) in studies using brain homogenates [16-20]. The time factor is thus not very likely to be responsible for the observed lack of seeding by CSF compared to brain extract.

Induction of Aβ seeding is furthermore dependent on the amount of Aβ present in the injected extract [17]. Indeed, the Aβ concentration is much lower in the CSF compared to that in standard diluted brain homogenates from plaque-bearing mice. However, only minimal amounts of brain-derived Aβ are required to induce seeding. Langer and co-workers have previously demonstrated that Aβ within the soluble fraction of ultracentrifugated brain homogenates harbors a very high seeding potential even at low concentrations [26]. In this light, concentrated AD patients` CSF was intra hippocampally injected into APP transgenic mice in a recent study, but also failed to demonstrate any Aβ seeding activity after 6-8 months [21]. In contrast, diluted brain extracts containing comparable amounts of Aβ did increase amyloid deposition [21]. We here used concentrated CSF comprising a much higher Aβ concentration than the one used in the aforementioned study (0.46 vs 0.008 ng/μl), and extended the seeding time up to 20 months. The absence of quantitative and qualitative seeding effects even under these conditions suggests that seeding competent Aβ species are absent in CSF, or their fraction is much smaller in the CSF than in the brain, and thus not detectable in a standard *in vivo* seeding model as the APP23 mice. As a limitation, we can not exclude a reduction of the seeding competence of the concentrated CSF due to freeze-thawing and/or lyophilization [27].

Small and soluble Aβ containing assemblies constitute the most potent Aβ seeds in brain extracts derived from APP23 transgenic mice [26]. Such small aggregates may be almost absent in the CSF, even if oligomeric Aβ forms have recently been detected in human CSF [28-31]. The lower level of Aβ seeds in CSF compared to brain tissue could be attributed to a reduced transport rate to the CSF compartment, possibly caused by their binding to cerebral amyloid-plaques, or an increased degradation within the CSF compartment. Additionally, the rapid turn-over of CSF might in parallel prevent a de novo assembly of prion-like Aβ strains within the CSF compartment. Aβ species present in brain homogenates or CSF may differ in their seeding potential due to structural varieties. As an example, N-truncated Aβ have been detected in the brain, but found largely absent in the CSF of AD patients [21]. Although it remains difficult to study the conformational state of Aβ *in vivo*, studies so far suggest the occurrence of conformationally distinct Aβ

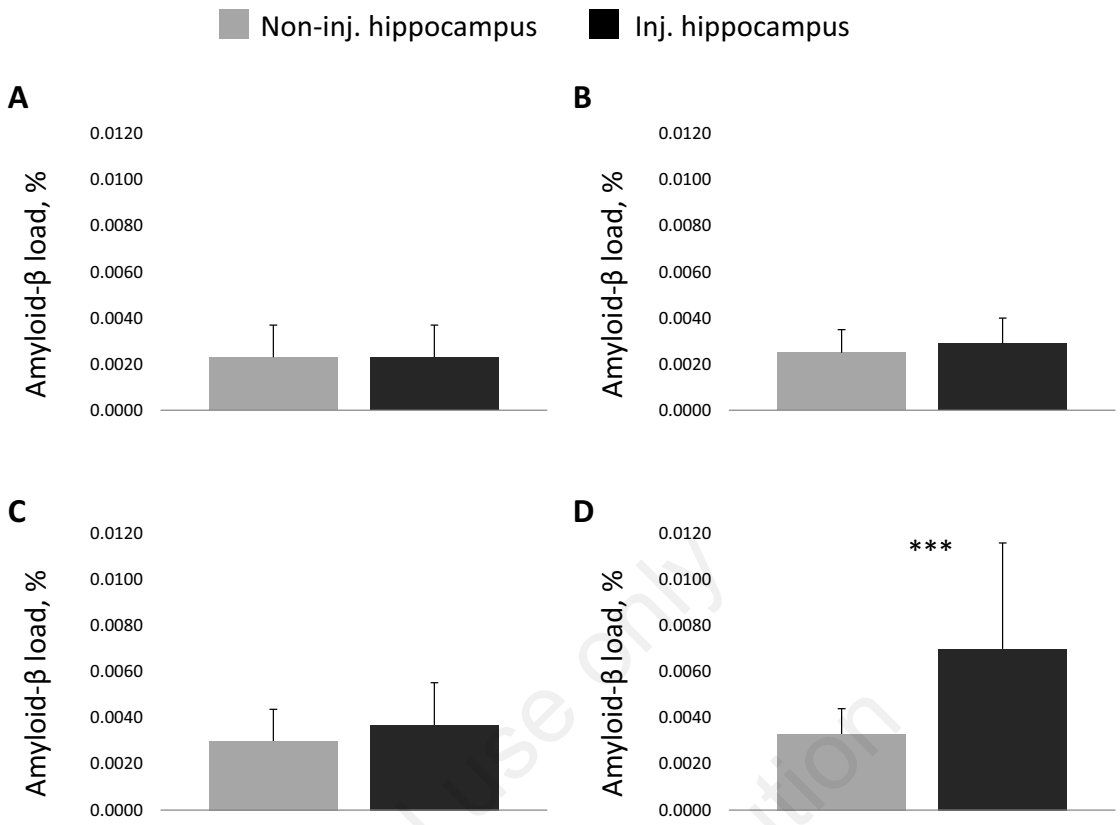

Fig. (2). Quantitative analysis of seeded mice. To assess a possible seeding effect we compared Aβ plaque burden between the non-injected (non-inj.) and injected (inj.) hippocampus in a mixed effect model for APP23 mice injected with transgenic CSF for 14 (**A**, n=4) or 21 months (**B**, n=5). In addition, we analysed APP23 mice seeded with wild type CSF (**C**, n=3) and with transgenic forebrain homogenates (**D**, n=2), as only in the last group there was a seeding effect present. Aβ plaque burden was calculated as a mean from all assessed hippocampal sections of mice in a group; error bars represent SD; *** indicates $p<0.001$. All p-values are listed in Table **S3**.

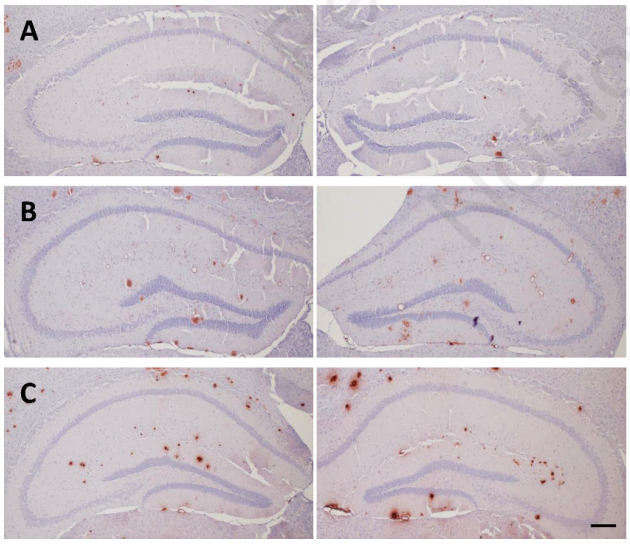

Fig. (3). Immunohistochemical analysis of mice injected with concentrated mouse CSF using 82E1 antibody. APP23 mice injected with concentrated APP23 CSF and analyzed 11 (**A**) or 20 (**B**) months later did not show an increased Aβ deposition in the inoculated hippocampus (right images), as compared to their non-injected sides (left images). The same was observed for littermates seeded with concentrated C57BL6 CSF (**C**). Scale bar equals 200 μm.

deposits in the brain [32, 33]. Different Aβ morphotypes may indicate that local factors may influence Aβ aggregation [34, 35]. Given its very low concentration in CSF, the structure of Aβ present there has still remained undescribed. This precludes us from drawing specific conclusions on the Aβ structure required for its seeding competence.

In addition, Aβ seeding may be dependent on brain components that are absent in the CSF compartment. For instance, the synaptic variant of acetylcholinesterase (AChE) has been shown to facilitate Aβ fibril formation [36]. In return, inhibitory cofactors may preferentially reach the CSF, such as the monomeric read through variant of AChE, which has been shown to delay aggregation [37].

Toxicity and seeding competence of Aβ may furthermore be influenced by RNA metabolism. miRNAs may be transmitted from one cell to another via exosomes, and RNA binding proteins have furthermore been shown to promote fibril formation [38-40]. This may contribute to the observed difference in seeding competence of brain-derived material versus CSF.

Further analysis of the potentially selective transport of particular Aβ species to the CSF and the possible co-factors involved might help to explain the apparent lack of *in vivo* active Aβ seeds in AD CSF. This knowledge could also help the development of sensitive early AD diagnostic tools.

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CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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DTW designed the study, KB, FC, JC, DA, and DTW developed the methodology, ZS, KB, FS and DTW collected the data and performed the analysis, and ZS, MS, MT, and DTW wrote the manuscript.

SUPPLEMENTARY MATERIAL

Supplementary materials are available on the publishers website along with the published article.

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