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Multiple Factors Contribute to the Peripheral Induction of Cerebral β-Amyloidosis

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Deposition of aggregated amyloid-β (Aβ) peptide in brain is an early event and hallmark pathology of Alzheimer’s disease and cerebral Aβ angiopathy. Experimental evidence supports the concept that Aβ multimers can act as seeds and structurally corrupt other Aβ peptides by a self-propagating mechanism. Here we compare the induction of cerebral β-amyloidosis by intraperitoneal applications of Aβ-containing brain extracts in three Aβ-precursor protein (APP) transgenic mouse lines that differ in levels of transgene expression in brain and periphery (APP23 mice, APP23 mice lacking murine APP, and R1.40 mice). Results revealed that beta-amyloidosis induction, which could be blocked with an anti-Aβ antibody, was dependent on the amount of inoculated brain extract and on the level of APP/Aβ expression in the brain but not in the periphery. The induced Aβ deposits in brain occurred in a characteristic pattern consistent with the entry of Aβ seeds at multiple brain locations. Intraperitoneally injected Aβ could be detected in blood monocytes and some peripheral tissues (liver, spleen) up to 30 d after the injection but escaped histological and biochemical detection thereafter. These results suggest that intraperitoneally inoculated Aβ seeds are transported from the periphery to the brain in which corruptive templating of host Aβ occurs at multiple sites, most efficiently in regions with high availability of soluble Aβ.

Key words: Abeta; cerebral beta-amyloidosis; peripheral induction; seeding

Introduction

Cerebral β-amyloidosis, a hallmark of Alzheimer’s disease (AD), can be exogenously induced by the intracerebral infusion of β-amyloid (Aβ) containing brain extracts into young Aβ-precursor protein (APP) transgenic (Tg) mice (Kane et al., 2000; Meyer-Luehmann et al., 2006; Eisele et al., 2009; Watts et al., 2011; Hamaguchi et al., 2012; Morales et al., 2012; Rosen et al., 2012). The beta-amyloidosis inducing activity has been shown to consist of multimeric Aβ peptides (Meyer-Luehmann et al., 2006). Subsequent studies have demonstrated that aggregated synthetic Aβ is itself sufficient to induce cerebral β-amyloidosis although with lower biological activity than the Aβ-containing brain extracts (Stöhr et al., 2012). These observations are reminiscent of prion disorders such as Creutzfeldt-Jakob disease or bovine spongiform encephalitis in which it is now established beyond a reasonable doubt that these disorders can be transmitted by exogenous, conformational variants of the prion protein (PrPrP; Colby and Prusiner, 2011). It is currently hypothesized that the self-replication of pathogenic protein aggregates (prion-like misfolding of proteins) is a uniform principle responsible for the instigation and spreading of many age-related neurodegenerative disorders (Jucker and Walker, 2013).

We found previously that intraperitoneal applications of Aβ-containing brain extract can also induce cerebral β-amyloidosis in young APP23 Tg mice, albeit at a higher concentration and a longer incubation interval compared to the intracerebral inoculation route (Eisele et al., 2010). In APP23 Tg mice, expression of human APP is primarily confined to neurons in the brain (Sturchler-Pierrat et al., 1997), but murine APP is also expressed in peripheral neuronal and non-neuronal tissues and may contribute to peripheral seeding. From studies in the prion field, it is known that the cellular PrP (PrPc) in peripheral cells plays a crucial role in the transfer of the infectious prion (PrPSc) from the periphery to the CNS (Blättler et al., 1997), and PrPSc accumulates in some peripheral sites, such as lymphoid tissues, during its transmission from the periphery to the brain (Mabbott and MacPherson, 2006; Aguzzi et al., 2013).
In the present study, we characterize the nature, mechanism, and the time- and concentration-dependent intraperitoneal induction of cerebral β-amyloidosis. We compare APP23 mice as hosts with a second Tg mouse strain, R1.40, that harbors a yeast artificial chromosome containing a genomic copy of the human APP gene. In R1.40 mice, human APP is expressed not only in the brain but also in the systemic organs, such as kidney, testes, and heart, and reflects normal tissue-specific APP/Aβ expression (Lamb et al., 1997). As a complementary host system, we used APP23 mice bred on a murine App-null background (Calhoun et al., 1999), i.e., a host expressing only human APP in brain and devoid of peripheral APP expression. The present study confirms the concept of prion-like misfolding of Aβ and suggests that Aβ seeds can be transported from distant locations to the brain in which correlative templating of host Aβ occurs.

**Materials and Methods**

*Mice.* APP23 Tg mice (Sturchler-Pierrat et al., 1997) expressing a human APP751 cDNA with the K670N–M671L mutation under control of the murine Thy1.2 promoter were backcrossed with C57BL/6J mice for >20 generations and maintained on a C57BL/6J background (C57BL/6J-Tg[Thy1–APPK670N;M671L]). To generate APP23 mice on a murine App-null background, hemizygous APP23 mice were bred with C57BL/6J–App mice (Calhoun et al., 1999). The resulting APP23/23 mice were bred with APP23/23 to obtain the desired APP23/23 line. Hemizygous R1.40 APP Tg mice on a B6.129 background were purchased from The Jackson Laboratory. The mice carry a yeast artificial chromosome containing the genomic copy of human APP harboring the K670N–M671L mutation (Lamb et al., 1997). The mice were bred once with C57BL/6J mice; then homozygous R1.40 mice were generated by crossbreedings, resulting in homozygous mice on a B6.129/C57BL/6J background. C57BL/6J mice were purchased from The Jackson Laboratory. All inoculations in the present study were done with homozygous R1.40 mice and hemizygous APP23 mice. Female and male mice were used in all experiments with the exception of the results presented in Figure 3 for which only male mice were used. When males and females were used, care was taken that groups had a similar number of males and females. As indicated in the individual figure legends, male and female mice were combined because either statistical analysis did not reveal a significant difference or numbers were too small to do proper statistical gender analysis. Overall, the induction of amyloid appeared somewhat higher in females compared with males. All mice were maintained under specific pathogen-free conditions. The experimental procedures were undertaken in accordance with the veterinary office regulations of Baden-Württemberg (Germany) and approved by the local animal care and use committees.

*Brain tissue extracts.* Brains from aged (18–30 months old) male and female Tg APP23 and APPPS1 donor mice or age-matched, non-Tg (wild-type [WT]) mice of our APP23 or APPPS1 colonies were used (Sturchler-Pierrat et al., 1997; Radde et al., 2006). After the cerebellum and lower brainstem were removed, the forebrains were frozen on dry ice and stored at −80°C. Brains were individually homogenized at 10% (w/v) in PBS (Lonza), vortexed, sonicated three times for 5 s (Lab Sonic; B. Braun; 0.5-mm-diameter sonotrode, cycle 1, amplitude 80%) and centrifuged at 3000 × g for 5 min. The supernatant was aliquoted and stored at −80°C until use. Aβ levels in extracts prepared from individual brains were determined using electrochemiluminescence-linked immunoassay and/or immunoblotting as described previously and revealed total Aβ concentrations of 10–20 ng/μl in the Ig extract (Eisele et al., 2009). Aliquots of several extracts (from at least three animals, Tg or WT, respectively) were thawed just before intraperitoneal injections and mixed to yield large batches of Tg or WT seeding extracts. All injected extracts were derived from aged APP23 Tg mice but occasionally also contained a minor amount (<10% v/v) of extract derived from aged APPPS1 Tg mice. [Note that adding such a minor amount of APPPS1 extract was shown to yield similar induction of cerebral β-amyloidosis as pure APP23 seeding extract in APP23 Tg mice (Eisele et al., 2010) and has no major influence on the Aβ40/Aβ42 ratio of the seeding extract mix.]

**Intraperitoneal inoculation.** Mice received two intraperitoneal injections of 100 μl of Tg extract or WT extract 1 week apart, unless indicated otherwise. For the concentration-dependent study, mice received a single intraperitoneal injection of 200 μl of Tg extract or 1:10, 1:100, or 1:1000 PBS dilutions thereof. All mice were randomly assigned to the inoculation groups.

*Mixture of anti-Aβ antibodies and Tg brain extract.* Before the intraperitoneal inoculation, Tg extract was 2:1 mixed with 4.5 mg/ml of the anti-Aβ antibody β1 (monoclonal, mouse IgG2a; Meyer-Luehmann et al., 2006). The mixing procedure results in a 6.7% extract. A total of 200 μl of this mixture was intraperitoneally injected into APP23 hosts.

**Preparation of inoculated mice.** APP23 Tg mice were prepared 1, 4, 6, 7, and 8 months after inoculation without perfusion. APP23 Tg mice on an App-null background (APP23/23) were prepared 8 months after inoculation. Brains were removed, and right hemispheres were directly frozen on dry ice for biochemical analysis and stored at −80°C. Left hemispheres were immersion fixed in 4% paraformaldehyde in PBS for 48 h, cryoprotected in 30% sucrose, frozen in 2-methylbutane, and stored at −80°C until analysis. R1.40 mice, which normally develop Aβ pathology later in life than do APP23 mice (Hamaguchi et al., 2012), were prepared 1, 6, 8, 10, and 12 months after intraperitoneal injections. R1.40 mice were killed under deep ketamine/xylazine anesthesia (400 mg/kg ketamine, 40 mg/kg xylazine) and transcardially perfused with PBS. The brains were harvested and hemi-dissected. Right hemispheres were again directly frozen, and the left hemispheres were immersion fixed. Peripheral organs were also taken when indicated and immersion fixed in 4% paraformaldehyde, followed by paraffin embedding.

**Histology and immunohistochemistry.** Fixed frozen brains were cut into 25-μm-thick coronal sections using a freezing-sliding microtome. Paraffin-embedded tissue was cut into 6-μm-thick sections on a rotary microtome (Microm HM 325), and a previously published enhanced antigen retrieval technique was used (Kai et al., 2012). All sections were Aβ immunostained using polyclonal antibody CN3 (1:1000; raised against residues 1–16 of human Aβ) and visualized using standard immunoperoxidase procedures with the Vectastain Elite ABC kit (Vector Laboratories). After immunohistochemistry, some sections were additionally stained with Congo red and visualized between cross-polarizers and/or counterstained with nuclear fast red to analyze the integrity of the blood–brain barrier, sections were immunostained with antibodies against mouse IgG (1:250; Vector Laboratories) and mouse albumin (rabbit polyclonal, 1:1000; Thermo Fisher Scientific).

**Immunoblot and immunoassay for APP and Aβ.** Right brain hemispheres were homogenized at 10% (w/v) in homogenization buffer [in mM: 50 Tris, pH 8.0, 150 NaCl, 5 EDTA, and Complete Mini protease inhibitor mixture (Roche)]. To assess Aβ and APP levels, 4–12% NuPage Bis-Tris mini gels using NuPage LDS sample buffer were used (Invitrogen), followed by protein transfer onto nitrocellulose membranes and probing with antibody 6E10 (Covance). Monoclonal anti-GAPDH antibody (Hy Test) was used to detect the housekeeping protein GAPDH. Densitometric values of band intensities were analyzed using NIH Image 1.44 (http://rswebw.nih.gov/ij/).

For electrochemiluminescence-conjugated immunooassay, 500 μl of homogenized brain sample (10% w/v in PBS) was extracted with an equivalent volume of 0.4% diethylamine (DEA) and centrifuged at 135,000 × g for 1 h at 4°C. A total of 850 μl of the supernatant was neutralized with 85 μl of 0.5 M Tris-HCl, pH 6.8, to obtain the DEA-soluble fraction. The DEA-insoluble pellet was resuspended to 500 μl volume with 70% formic acid (FA), incubated at 4°C for 12 h, and then sonicated for 35 s at 4°C. The samples were analyzed on a Sector Imager 6000 using the MSD 96-well Multi-Spot Human 6E10 Aβ Triplex Assay (Meso Scale Discovery).

**Quantification of Aβ load on brain sections.** The areal density of Aβ-immunoreactive lesions (CN3 staining) and Congo red staining was
quantified in random-systematic sets through the neocortex according to four categories: (1) CN3-positive vascular; (2) CN3-positive parenchymal; (3) Congo red-positive vascular; or (4) Congo red-positive parenchymal. For APP23 Tg and APP23/App−/− mice, every 24th section was assessed. The Aβ load (% Aβ-immunostained area) of R1.40 Tg mice was lower compared with the APP23 mice, and thus every 12th section was assessed. Stereological analysis was performed using a Zeiss Axioskop microscope equipped with a motorized x–y–z stage coupled to a video-microscopy system and the Stereo Investigator software (MicroBrightField). Quantification was performed by researchers who were blinded to the inoculation groups.

Table 1. Classification of plaque size according to area on section

<table>
<thead>
<tr>
<th>Area (μm²)</th>
<th>Minimum circular diameter (μm²)</th>
<th>Class</th>
</tr>
</thead>
<tbody>
<tr>
<td>A &gt; 14000</td>
<td>133.5</td>
<td>Large</td>
</tr>
<tr>
<td>14000 ≥ A &gt; 4000</td>
<td>71.4</td>
<td>Medium</td>
</tr>
<tr>
<td>4000 ≥ A &gt; 800</td>
<td>31.9</td>
<td>Small</td>
</tr>
<tr>
<td>800 ≥ A ≥ 20</td>
<td>5.04</td>
<td>Very small</td>
</tr>
<tr>
<td>A &lt; 20</td>
<td></td>
<td>Excluded</td>
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A, Area.

Results

Earlier and stronger amyloid induction in APP23 compared with R1.40 Tg mice

APP23 and R1.40 mice at the age of 1–2 months were inoculated with the same brain extracts derived from either aged non-Tg WT mice (WT extract) or Aβ bearing brain extract derived from APP23 and APPPS1 Tg mice (the extracts of the individual mice were pooled; see Materials and Methods; Tg extract). All mice received two intraperitoneal injections of 100 μl each 1 week

electrochemiluminescence-linked immunoassays and immunoblotting, the blood samples were separated into plasma and the cellular pellet by centrifugation for 5 min at 16,000 x g at room temperature. The blood pellets were resuspended in the plasma-equivalent volumes of 1× homogenization buffer [in mm: 50 Tris, pH 8.0, 150 NaCl, 5 EDTA, and Complete Mini protease inhibitor (Roche)]. Samples were snap frozen and stored at −80°C until analysis.

Aβ quantification in blood. For electrochemiluminescence-linked immunoassay, blood plasma was subjected to measurement without additional treatment. The resuspended blood pellet (see above) containing the cellular components was treated with FA (minimum of 95%), sonicated for 35 s on ice, and centrifuged at 25,000 x g for 1 h at 4°C. Supernatants were neutralized (1× Tris base, 0.5× NaHPO4, and 0.05% NaN3). Aβ levels were determined in duplicates using the MSD 96-well MULTI-ARRAY Human (6E10) Aβ40 and Aβ42, Ultra-Sensitive Assay or MULTI-SPOT Human (6E10) Aβ Triplex Assay (Meso Scale Discovery) and read on a Sector Imager 6000 according to the instructions of the manufacturer. Each sample was measured twice, and the mean was taken. Levels below detection (depending on the experiment; ~5 pg/ml) were taken as zero. Resuspended cells isolated by peritoneal lavage were likewise subjected to FA treatment. For Aβ detection by immunoblotting, the resuspended blood pellet was subjected to immunoprecipitation using anti-Aβ antibody 6E10 (Covance) and Dynal paramagnetic beads with sheep anti-mouse IgG (Invitrogen) as described previously (Meyer-Luehmann et al., 2006).

Histochemical and immunohistochemical staining of cells from the blood and peritoneal cavity. Blood films on Superfrost slides (R. Langenbrink, Emmendingen, Germany) were air dried for 24 h at room temperature. Cells were fixed using methanol/acetic (1:1) for 90 s and air dried again. Cells obtained by peritoneal lavage were resuspended in PBS and subjected to cytospin (Cytospin J; Thermo Shandon) using disposable single cell funnels (JC320; Tharmac). Pappenheim’s stain (Giema’s solution, May-Grünewald’s solution; Merck) was used for the differentiation of cells isolated from the blood and peritoneal cavity. For immunohistochemical analysis, slides were preincubated in PBS containing 0.1% Triton X-100 (Sigma). In combination with Pappenheim’s stain, Aβ immunostaining (CN3 antibody) was performed using standard immunoperoxidase procedures with the Vectastain Elite ABC kit (Vector Laboratories). Double labeling for Aβ (CN3 antibody) and CD11b, CD45, or CD68 (Serotec) was performed using standard immunofluorescence procedures with goat anti-rabbit Alexa Fluor 568 and goat anti-rat Alexa Fluor 488 secondary antibodies (Invitrogen). For staining with pFTAA [pentamer formyl thiophene acetic acid (sodium salt), part of the amyloid-binding class of luminescence conjugated oligothiophene (LCO) dyes; Klingstedt et al., 2011], cytospins from peritoneal lavage were dried at room temperature for 2 h and incubated with PBS for 10 min. Staining with pFTAA (1.5 nm in deionized water, diluted 1:1000 in PBS) was performed as described previously (Klingstedt et al., 2011). The sections were mounted with Vectashield mounting medium for fluorescence with DAPI (Vector Laboratories). Images were acquired on a Zeiss LSM 510Meta laser scanning confocal microscope using a 63×/1.4 numerical aperture oil-immersion objective (Axiovert 200M; Zeiss). The amyloid-binding dye pFTAA was excited with the 488 nm laser line, and its emission was collected in the range of 505–550 nm. The dyes were imaged sequentially.

Statistical analysis. All values are expressed as means ± SEMs. The statistical analyses were performed using GraphPad Prism (version 6.0) and/or JMP 8, and results are detailed in the figure legends.
Figure 1.  Time course reveals earlier and stronger induction of cerebral β-amyloidosis in APP23 versus R1.40 Tg mice with the same peripherally applied Aβ-containing seeding extract. A, Experimental design: 1- to 2-month-old male and female APP23 and R1.40 Tg mice were intraperitoneally injected (i.p. inj.; two times with 100 μl, 1 week apart) with brain extracts derived from either aged non-Tg mice (WT extract) or aged Aβ-depositing APP Tg mice (Tg extract). Brains of inoculated APP23 mice were analyzed 1, 4, 6, 7, and 8 months and brains of inoculated R1.40 mice at 1, 6, 8, 10, and 12 months after inoculation (incubation (inc.)). Male and female mice were combined for the analysis (see Material and Methods). B–E, Immunohistochemical analysis for Aβ deposition. B, In intraperitoneally inoculated APP23 mice, the first Aβ deposits were noted at 6 months and increased sharply with the longer incubation times of 7 or 8 months. In contrast, mice that received the WT extract and were incubated for 8 months showed no or very few Aβ deposits, which are typical for APP23 Tg mice at 8–10 months of age, which is the age at analysis. Stereological quantification of neocortical Aβ load is shown in C. In R1.40 Tg mice, the first Aβ deposits occurred 8 months after inoculation in all mice that received the Tg extract albeit to a minor extent and increases with longer incubation times of 10 or 12 months. In contrast, mice that received the WT extract showed no or very little Aβ deposits after a 12 month incubation (D) that is a typical finding for R1.40 Tg mice at 10–14 months of age, which is the age at analysis. Stereotactical quantification of neocortical Aβ load is shown in E. n = 5–9 for APP23 and R1.40 Tg mice per incubation time and extract; ***p < 0.001, ****p < 0.0001, one-way ANOVA, Bonferroni’s post hoc test. Scale bar, 200 μm. F, Neocortical Aβ deposits in both lines were further differentiated into vascular and parenchymal deposits, and results revealed that, in APP23 Tg mice, the majority of deposits (measured by area occupied) are parenchymal deposits with a high variability for vascular deposits. Interestingly, in R1.40 mice, vascular deposition seemed prominent during early time points but decreased thereafter and parenchymal deposits became more dominant. G, Differentiation of the neocortical Aβ deposits into congophilic (Congo red positive) and non-congophilic deposits revealed that, in APP23 Tg mice, 5–15% of induced deposits are congophilic, whereas in R1.40 mice, almost exclusively diffuse deposits are induced at least up to 12 months incubation. (However, note that Congo red stains only the core of a plaque and thus the area fraction is low compared with the Aβ stain of a given amyloid plaque.) H–K, Biochemical analysis of brains 1 month after inoculation. Representative immunoblot for APP, differentiated into mature APP (APPm) and immature APP (APPim) as well as C99 levels, both recognized by the 6E10 antibody specific to human APP (H). Relative APP levels (APPm and APPim combined) were quantified from immunoblots and normalized to the housekeeping protein GAPDH. Mean APP value for WT extract-injected APP23 Tg mice was set to 100%. Quantification shows no difference between APP23 mice injected with WT or Tg extract. Similarly, no difference was found in R1.40 mice. However, APP levels in R1.40 Tg mice were lower than in APP23 Tg mice representing ~30% of APP levels of APP23 mice (J). Overall, similar results were found for C99 levels (I). Human Aβ levels in brain homogenates were assessed with electrochemiluminescence-linked immunosassay, and again, similar to APP and C99 levels, human Aβ levels did not differ between mice of the same Tg line treated with either WT or Tg brain extract. Between the two lines, human Aβ levels in APP23 mice are significantly higher and are approximately threefold to fourfold in APP23 compared with R1.40 mice (K); n = 8 for APP23; n = 5 for R1.40. Note that the same animals (1 month incubation) were used for biochemical analysis and histology. *p < 0.05, **p < 0.001, ****p < 0.0001, one-way ANOVA, Sidak’s multiple comparison test. nctx, Neocortex.

For each mouse line, five different time points were chosen for the analysis: 1, 4, 6, 7, and 8 months after inoculation for APP23 mice and 1, 6, 8, 10, and 12 months for R1.40 Tg mice (Fig. 1A). For the APP23 line, the time points were chosen based on our previous work in which we found induction of cerebral β-amyloidosis after 6–7 months in this line (Eisele et al., 2010). For R1.40 mice, we found in pilot experiments (data not shown) that amyloid induction after intraperitoneal inoc-
Differentiation of neocortical amyloid in congophilic (Congo red positive) and non-
endogenous Aβ deposits in APP23 mice were combined for the analysis (see Material and Methods). Shown are representative coronal sections (scale bar, 500 μm), and higher-magnification images of the frontal cortex are shown below (scale bar, 100 μm). Immunohistochemical analysis revealed a similar, albeit somewhat lower, induction of Aβ-deposition by the Tg extract in APP23/ Appl− mice compared with the APP23 mice. Mice that received the WT extract showed no induced Aβ deposits. Note that 9- to 10-month-old APP23 and APP23/ Appl− mice (age at preparation) do exhibit the first endogenous Aβ deposition at this age. B, Stereological quantification indeed revealed that the amyloid load of APP23/ Appl− was 29% less compared with APP23 mice, but the difference did not achieve significance (n = 4 for APP23 mice and APP23/ Appl− mice injected with WT extract and n = 8 for APP23/ Appl− inoculated with Tg extract; mean ± SEM; one way ANOVA, F2,131 = 6.136, p = 0.013, Tukey’s multiple comparison, *p < 0.05; n.s., not significant). C, Neocortical Aβ load was differentiated into vascular and parenchymal deposits. No difference was noted between APP23 and APP23/ Appl− mice. D, Differentiation of neocortical amyloid in congophilic (Congo red positive) and non-congophilic Aβ lesions also revealed a similar distribution of deposits in both APP23 and APP23/ Appl− Tg mice. ncts, Neocortex.

For the inoculated APP23 mice, no induced Aβ deposits in brain were found at 1 and 4 months after inoculation. However, robust induction of cerebral β-amyloidosis with the Tg extract was found 6 months after inoculation with a time-dependent increase thereafter (Fig. 1B, C). Notably, WT extract did not induce cerebral β-amyloidosis at any time points (Fig. 1C). In the intraperitoneally inoculated R1.40 mice, cerebral β-amyloidosis was detectable for the first time at 8 months after inoculation with the Tg extract and further increased with longer incubation times (Fig. 1D, E). Similar to APP23 hosts, WT extract did not induce Aβ deposits in R1.40 mice (Fig. 1E). Although the histological quantification revealed 1.3-fold more induced Aβ deposition in the APP23 host (after the 8 months incubation) compared with the R1.40 host (after the 12 months incubation; Fig. 1C,E), a quantitative immunoassay detected 45-fold more insoluble Aβ (Aβ40 and Aβ42 combined) in the inoculated APP23 compared with the inoculated R1.40 mice [2877.9 ± 473.0 vs 61.6 ± 14.6 ng/ml 10% (w/v) brain homogenate]. The apparent discrepancy is likely related to the essentially diffuse nature of the induced deposits in the R1.40 mouse line, whereas the induced Aβ deposits in the APP23 host were more frequently compact and congophilic (Fig. 1G).

In addition to parenchymal deposits, vascular Aβ deposits were induced in both lines (Fig. 1F). Although the relative percentage of induced amyloid around the vasculature was greatest at early time points in the R1.40 mice and decreased thereafter, this observation was not evident for the APP23 mice in the present experiment, in contrast to our previous study (Eisele et al., 2010). Interestingly, both lines showed prominent and early Aβ deposits in leptomeningeal vessels when inoculated with the Tg extract in contrast to inoculations with WT extract in which such deposits were absent.

We next analyzed human APP levels and APP processing 1 month after inoculation in APP23 and R1.40 mice to assess potential short-term effects of intraperitoneal inoculations with the Tg extract compared with the WT extract. Moreover, this allowed for a direct comparison of human (Tg) APP protein expression in both lines and thus may explain the different seeding efficacies between the two host lines. Immunoblot analysis revealed no difference in APP levels or levels of its C99 fragment between Tg- and WT-inoculated mice for both mouse lines (Fig. 1F). When comparing APP23 with R1.40 Tg mice, APP and C99 levels were twofold to threefold higher in APP23 Tg mice compared with R1.40 mice (Fig. 1F). This is in line with the previous work reporting a fivefold to sevenfold overexpression of human APP over murine APP in hemizygous Tg32 Tg mice (Sturchler-Pierrat et al., 1997) and a twofold to threefold overexpression of human versus murine APP in homozygous R1.40 Tg mice (Lamb et al., 1997). Consistent with the expression of APP and C99, an immunoassay did not reveal any difference in “total” Aβ (here defined as Aβ40 plus Aβ42) between WT and Tg extract inoculated APP23 or R1.40 mice at this early time point (Fig. 1K). In comparison, human Aβ levels in APP23 Tg mice were approximately threefold higher than in R1.40 Tg mice, paralleling the difference in APP and C99 levels (Fig. 1K). These results suggest that the stronger amyloid induction in APP23 compared with R1.40 mice after intraperitoneal inoculation with the same Aβ-containing brain extract is related to the higher APP overexpression and higher Aβ levels in APP23 brains.

To exclude that blood–brain barrier disruption was responsible for the observed phenomenon, we next analyzed whether we could detect mouse IgG, mouse albumin, or microhemorrhages in brains of intraperitoneally seeded APP23 and R1.40 mice. Results did not reveal evidence for blood–brain barrier damage at any of the investigated time points after intraperitoneal inoculation, with the exception of albumin staining associated with the induced (congophilic) amyloid deposits. However, the latter also prominently occurs in aged APP23 mice and to a lesser extent in aged R1.40 mice because most of the amyloid deposits in R1.40 mice are of a diffuse nature (data not shown).

Cerebral beta-amyloidosis induction does not require peripheral APP expression

Both Tg mouse lines used express endogenous murine APP in brain and in the periphery (R1.40 additionally express peripheral human APP). To study a possible effect of peripheral APP/Aβ on...
Concentration-dependent induction of cerebral β-amyloidosis and efficient blocking with anti-APP antibody

Although our standard inoculation protocol consisted of two injections of 100 μl of brain extract given 1 week apart, we wondered whether a single inoculation of 200 μl of Tg brain extract and dilutions thereof were also sufficient for the induction of Aβ deposition. Moreover, to sustain that Aβ in the Tg extract is key for the amyloid-inducing activity, Tg extract was mixed with an Aβ-specific antibody (B1) that has been shown previously to block seeding during intracerebral injection of the Tg extract (Meyer-Luehmann et al., 2006). The APP23 host was selected, and the inoculated mice survived for an 8 month incubation period. Results revealed that the one-time inoculation was comparable in all aspects with the inoculations separated by 1 week (Fig. 3 compared with Fig. 1). Although the 1:10 dilutions revealed approximately one-sixth of the induction observed with the undiluted extract, no detectable amyloid deposits were induced by the 1:100 and 1:1000 dilutions within the observation period, respectively (Fig. 3C). Strikingly, the 1:10 extract dilution induced predominantly vascular Aβ deposits (Fig. 3B, F), a phenomenon also observed in our previous study in intraperitoneally seeded APP23 Tg mice after 6–7 months of incubation (Eisele et al., 2010). Tg extract mixed with the B1 antibody primarily prevented amyloid induction (Fig. 3A, B), suggesting that the induction of cerebral β-amyloidosis is dependent on the concentration of Aβ seeds in the intraperitoneally inoculated Tg extract.

Distribution characteristics of the induced cerebral Aβ deposits

In APP23 mice, intraperitoneally induced cerebral Aβ deposits were much more abundant in the frontal cortex, followed by the entorhinal cortex, parietal cortex, hippocampus, and then other brain regions (Fig. 4A). This was also true for the R1.40 line (data not shown) matching the normal endogenous distribution of Aβ deposition in aged APP23 and R1.40 mice (Sturchler-Pierrat et al., 1997; Kulnane and Lamb, 2001).

Closer examination revealed that, for all intraperitoneally inoculated mice, independent of inoculation time and host line, there was appreciable clustering of parenchymal Aβ deposits throughout the brain (Fig. 4B). Typically large Aβ plaques, in most cases congophilic, were surrounded by a myriad of smaller Aβ deposits (Fig. 4C). On numerous occasions, vascular elements were observed in the center of the clusters.
To sustain this qualitative observation of clustering of the induced Aβ deposits, the localized distribution of Aβ deposits between intraperitoneally seeded and aged (nonseeded) APP23 Tg mice was investigated. A nearest-neighbor algorithm was applied on sections calculating the Euclidean distances between surfaces of plaques (Fig. 4D–F). The resulting quantification confirmed that, in intraperitoneally seeded APP23 mice, larger plaques had a substantially higher number of smaller plaques/Aβ deposits in their close vicinities compared with the endogenous plaques in nonseeded aged APP23 Tg mice (Fig. 4D–F).

Thus, despite the similar overall regional distribution of Aβ plaques throughout the brain, quantification of the microdistribution revealed more clustering of the amyloid deposits after intraperitoneal induction compared with aged nonseeded APP23 Tg mice.

**Peripherally injected Aβ seeds are detectable shortly after injections in macrophages in the peritoneal cavity, blood, liver, and spleen**

To get additional hints toward the mechanism by which intraperitoneally applied Aβ-containing brain material induces cerebral β-amyloidosis, APP23 mice were given single intraperitoneal injections of 200 µl of Tg or WT extract and killed within the first 24 h, one week, or 1 month after injection with subsequent analysis of the peritoneal cells, blood, peripheral organs, and brain.

Analysis of the cellular fraction isolated by peritoneal lavage by immunoassay revealed that ~2% of the injected human Aβ (Tg extract) could be recovered within peritoneal monocytes at 3 and 24 h after inoculation (Fig. 5A, B). After 1 week, some of the injected Aβ was still detectable (Fig. 5A, B). Similarly, human Aβ was detected in blood monocytes (CD11b and CD45 positive) in the Tg but not WT extract-injected mice, again peaking within the first 24 h after inoculation (Fig. 5C–E). In agreement, immunoblotting of the cellular blood fraction confirmed the presence of human Aβ (Fig. 5F). No change in blood plasma human Aβ levels was found at any examined time points (data not shown).

Interestingly, Aβ taken up by peritoneal monocytes stained with the amyloid-specific dye pH-TAA, indicating that at least some of it is aggregated and in β-sheet structure (Fig. 5G). Aβ-positive macrophages were also detected in the liver 1 d after the inoculation with the Tg extract (Fig. 5H) and only occasionally after 7 d. Aβ-positive staining of cellular and extracellular elements was found at 1 d after inoculation also in the spleen (data not shown). Consistent with previous work (Eisele et al., 2010), no Aβ deposition was found or induced in the examined peripheral tissues of APP23 mice (and also R1.40 mice) at 8 and 12 months after inoculation, respectively.

**Discussion**

We recently made the surprising observation that intraperitoneal inoculation of Aβ seed-containing brain extract induced cerebral β-amyloidosis in APP23 Tg mice (Eisele et al., 2010). A similar observation has just been described for Tau, i.e., the intraperitoneal administration of Tau aggregates was found to induce Tau lesions in Tau Tg mice (Clavaguera et al., 2014). In the present study, we further characterized the peripheral induction of cerebral β-amyloidosis and provide evidence for multiple brain entry sites of the Aβ seeds and self-propagation in brain. No evidence for peripheral amyloid formation was found even in mice expressing human APP systemically.

Intraperitoneal inoculation with Aβ-containing brain extract induced cerebral β-amyloidosis in all three APP Tg mouse lines used as hosts, i.e., APP23 mice, APP23 mice on App-null back-
intraperitoneally injected $A\beta$ seeds are detectable shortly after the injection in macrophages in the peritoneal cavity, blood, liver, and spleen. A. Electrochemiluminescence-conjugated immunoassay for human $A\beta$ ($A\beta_{25}$ and $A\beta_{42}$ combined) in the cellular fraction of the peritoneal fluid 3 h, 24 h, 1 week, or 1 month after intraperitoneal inoculation with Tg or WT extract. Male or female 4- to 8-month-old APP23 mice, $n = 5$ per group and time point except $n = 4$ for the 1 month time point. Separate statistical analysis did not indicate a gender or age difference. ANOVA revealed significant main effects and a significant extract × post-inoculation time interaction ($F_{1,20} = 14.02; p < 0.001$). Post hoc Tukey’s tests showed significantly more $A\beta$ in the Tg extract-treated mice at 24 h compared with 3 h ($p < 0.05$) and 1 week ($p < 0.001$). Wilcoxon’s comparisons revealed similar significances. Indicated is the mean $\pm$ SEM. B, Pappenheim’s stain (purple) combined with $A\beta$ immunostaining (dark blue) of lavaged peritoneal cells from a Tg extract-inoculated mouse disclosed $A\beta$-positive monocytes (arrowheads). No $A\beta$ immunoactivity was found in WT extract-inoculated mice. C. Electrochemiluminescence-conjugated immunoassay for human $A\beta$ in the blood cellular fraction 1–24 h, 1 week, or 1 month after intraperitoneal inoculation. Male or female 4- to 8-month-old APP23 mice, $n = 16$ (Tg extract) and $n = 11$ (WT extract) for the 1–24 h time point and $n = 5$ per group for other time points. (Animals were added to the 1–24 h time point in an unsuccessful effort to reduce the high variability of $A\beta$ measurements.) Separate statistical analysis did not indicate a gender or age difference. ANOVA indicated no significant differences, but Wilcoxon’s test revealed a significant difference between Tg extract-treated and WT extract-treated mice in the 1–24 h post-inoculation group (median, 0.495 vs 0; $p < 0.05$). D, $A\beta$-positive monocytes (arrowheads) in a blood film from a Tg extract-inoculated mouse (Pappenheim’s stain, purple; $A\beta$ immunostain, dark blue). E, Double-immunofluorescence staining revealed that $A\beta$-positive (red) cells are positive for CD11b and CD45 (green; maximum projection of 10 μm z-stack). Scale bars, 10 μm. The same cells were also positive for CD68 (data not shown). F, Immunoprecipitation of the blood cellular fraction with an antibody against human $A\beta$ followed by immunoblot revealed a robust 4 kDa $A\beta$ band in Tg extract-inoculated mice, as well as weaker oligomeric $A\beta$ bands (indicated by asterisks) and APP, very similar as seen in the Tg extract (Langer et al., 2011). Only a weak signal corresponding to monomeric $A\beta$ was seen in WT extract-inoculated mice, possibly because of residual human endogenous $A\beta$. G, The amyloid-specific dye pFTAA stains $A\beta$ in peritoneal monocytes isolated from Tg extract-inoculated mice. DAPI is used as a nuclear counterstain (scale bar, 5 μm). H, I, Peripheral organs of representative mice measured and stained in (A–D) were immunostained for $A\beta$ and counterstained with nuclear fast red. $A\beta$-positive cells with the morphological characteristics of macrophages were found in the liver of Tg extract-inoculated mice ($n = 3$ analyzed mice) but not WT inoculated mice ($n = 2$ analyzed mice) 1 d after inoculation. Scale bars: 50 and 10 μm. J. Intraperitoneally injected $A\beta$ seeds are more abundant in the brain than in other peripheral organs, possibly because of the activation of the immune system by the injection. It has also been observed that intraperitoneal injection greatly diminishes induction of cerebral $\beta$-amyloidosis, and no induction was observed with intraperitoneally inoculated WT extract. These data are consistent with previous observations for intracerebral seeding (Meyer-Luehmann et al., 2006) and show that $A\beta$ peptides are essential for the induction of cerebral $\beta$-amyloidosis in contrast to generalized reactions to the inoculation (e.g., inflammation, stress response). The present findings also indicate that relatively large amounts of $A\beta$ seeds are needed to initiate cerebral $\beta$-amyloidosis by intraperitoneal seeding. Positive results were obtained after injection of extracts containing ~400 ng of total $A\beta$ (1:10 dilution of Tg extract), indicating that intraperitoneal seeding is >1000-fold less potent than intracerebral seeding (Eisele et al., 2009; Langer et al., 2011). However, it should be noted that peritoneal inoculation starts plaque seeding in all cortical areas, whereas intracerebral seeding acts locally at the site of injection, e.g., the hippocampus or other brain regions (Eisele et al., 2009).

No induction of $A\beta$ deposits in any peripheral tissue of the R1.40 mice was found despite the fact that R1.40 mice overexpress human APP in the periphery (Lamb et al., 1997). No amyloid positive cells with macrophagic appearance were found 1 h, 1 d, 1 week, and 1 month later. Again, $A\beta$-positive cells with a macrophagic appearance were found after 1 h and 7 d but not 1 month after inoculation (results not shown).
loid induction in peripheral organs was also reported in APP23 mice (Eisele et al., 2010). Moreover, we observed efficient intraperitoneal seeding in APP23 mice on App-null background in which even peripheral mouse APP is missing. These data suggest that peripheral APP/Ab expression may not be required for transmission of Ab seeds from the peritoneal cavity to the brain. For Scappie and other forms of transmissible spongiform encephalopathies or prion diseases, replication of misfolded PrPSc in peripheral tissues can occur before neuroinvasion (Brown et al., 1999) and play a major role in the prion spread throughout the infected organism (Aguzzi et al., 2013). In fact, peripheral PrPSc expression was found to be essential for clinical infection and transmission of Scappie to the brain of mice (Blättler et al., 1997). However, more recently, diverse other routes have been reported depending on the concentration or nature (conformation) of the prions (Bett et al., 2012; Chen et al., 2014).

We aimed to follow the fate of the injected seeds and detected traces of the injected Ab material in monocytes/macrophages in the peritoneal cavity, as well as in blood and tissue macrophages of liver and spleen shortly after injection. A significant decrease of this material was observed within 1 week, and it was below the detection limit at 1 month after inoculation. The uptake and degradation of Ab aggregates by cells of the monocyte lineage is well documented (Majumdar et al., 2008; Zaghni et al., 2009; Lai and McLaurin, 2012; Michaud et al., 2013). We have shown recently that Ab seeds are partly proteinase-K resistant (Langer et al., 2011), and insufficient clearance of Ab seeds within cells has been shown to contribute to cell-to-cell spread of the seeds (Domert et al., 2014). These observations may explain the persistence of Ab-immunoreactive monocytes for at least 1 week after intraperitoneal inoculation. Because peripheral phagocytes can be recruited to enter the brain (Rezaei-Zadeh et al., 2011; Varvel et al., 2012) it is conceivable that they could serve to transport Ab seeds from the periphery to the brain. However, it is also possible that Ab seeds in blood plasma at levels below detection reach the brain (Mackie et al., 2002; Deane et al., 2003). Of note, it has been shown previously that AA amyloidosis can be transferred by blood monocytes (Sponarova et al., 2008).

A closer look at the distribution of the induced deposits revealed more clustering of intraperitoneally induced deposits compared with normally aged mice with similar amyloid load in APP23 Tg mice. This suggests that, in normally aged APP23 Tg mice, possibly because of the high overexpression of human APP, spontaneous aggregation occurs in many locations. In contrast, the pattern of Ab aggregation in intraperitoneally seeded mice suggests hot spots of induction with subsequent spreading to areas in close proximity. It is tempting to speculate that the observed prominent vascular Ab-amyloidosis at early time points and with the 1:10 diluted extract reflects the route of transport of Ab seeds. The formation of clusters may suggest that Ab seeds enter the brain at various vascular locations in which local Ab misfolding is initiated that subsequently spreads within the brain parenchyma. It is in agreement with the notion that corrupative templating of host Ab occurs mainly in brain. Such a scenario may also explain why we found in our initial work a striking dominance of vascular amyloidosis after intraperitoneal inoculation (Eisele et al., 2010). It underlines that the nature and location of the induced Ab deposits may vary with incubation time, Ab seeds, and potentially also between host mice.

In summary, the peripheral induction of cerebral Ab-amyloidosis in APP Tg mice is dependent on the amount of inoculated Ab-containing brain extract and on the level of APP/Ab expression in the brain. No amyloid induction was found in peripheral organs. The induced Ab deposits in brain occurred in a characteristic pattern consistent with the entry of Ab seeds at multiple brain locations. Our results suggest that intraperitoneally inoculated Ab seeds are transported from the periphery to the brain in which corrupative templating of host Ab occurs at multiple sites, most efficiently in regions with high availability of soluble Ab.

References


