

# Immunotherapy alleviates amyloid-associated synaptic pathology in an Alzheimer's disease mouse model

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Cognitive decline in Alzheimer's disease is attributed to loss of functional synapses, most likely caused by synaptotoxic, oligomeric forms of amyloid- $\beta$ . Many treatment options aim at reducing amyloid- $\beta$  levels in the brain, either by decreasing its production or by increasing its clearance. We quantified the effects of immunotherapy directed against oligomeric amyloid- $\beta$  in Tg2576 mice, a mouse model of familial Alzheimer's disease. Treatment of 12-month-old mice with oligomer-specific (A-887755) or conformation-unspecific (6G1) antibodies for 8 weeks did not affect fibrillar plaque density or growth. We also quantified densities of DLG4 (previously known as PSD95) expressing post-synapses and synapsin expressing presynapses immunohistochemically. We found that both pre- and post-synapses were strongly reduced in the vicinity of plaques, whereas distant from plaques, in the cortex and hippocampal CA1 field, only post-synapses were reduced. Immunotherapy alleviated this synapse loss. Synapse loss was completely abolished distant from plaques, whereas it was only attenuated in the vicinity of plaques. These results suggest that fibrillar plaques may act as reservoirs for synaptotoxic, oligomeric amyloid- $\beta$  and that sequestering oligomers suffices to counteract synaptic pathology. Therefore, cognitive function may be improved by immunotherapy even when the load of fibrillar amyloid remains unchanged.

**Keywords:** Alzheimer's disease pathology; immunotherapy; amyloid- $\beta$  oligomers

**Abbreviation:** CA1 = cornu ammonis 1

## Introduction

Alzheimer's disease is characterized by specific neuropathological changes, which are thought to be caused by a cascade of events (Hardy and Selkoe, 2002), initiated by an imbalance of amyloid- $\beta$  production and clearance, leading to amyloid plaque deposition, ultimately leading to neuron loss and brain atrophy. However, cognitive deficits may be present several years before the onset of neuron loss and gross brain atrophy and these can be attributed to morphological alterations and loss of synapses (DeKosky and Scheff, 1990; Scheff *et al.*, 1990). It is unclear how exactly amyloid- $\beta$  harms neurons, though the main effectors seem to be soluble, oligomeric amyloid- $\beta$  species, which were shown to have a variety of harmful effects (Gilbert, 2013).

As an imbalance between amyloid production and catabolism leads to the formation of plaques (Saido, 1998), one treatment strategy aims at sequestering amyloid- $\beta$  from the CNS. For instance, in animal studies anti-amyloid immunotherapy reduced amyloid load and improved cognitive performance (Wilcock *et al.*, 2004; Wang *et al.*, 2011). On the other hand, trials in patients were largely unsuccessful, partly because of side effects and lack of cognitive improvement (Doody *et al.*, 2014; Salloway *et al.*, 2014). Nevertheless, a number of clinical trials are ongoing using adjusted experimental or treatment paradigms (Karran and Hardy, 2014).

We studied the effects of passive immunotherapy directed against oligomeric amyloid- $\beta$  on synaptic pathology and plaque growth in an animal model of familial Alzheimer's disease. Pan-amyloid- $\beta$  antibodies as well as oligomer-specific antibodies alleviated the synaptic pathology, while amyloid deposition was unaffected by the treatment regimen.

## Materials and methods

### Animals

All animal procedures had been approved by the animal welfare official of the Ludwig-Maximilian University and the Government of Upper Bavaria.

For *in vivo* imaging experiments, female, 12–18 month old, Tg2576 mice (Hsiao *et al.*, 1996), which had been obtained from Taconic, were used and non-transgenic littermates served as controls. Detailed protocols and settings are listed in the Supplementary material.

### Antibody treatment

A weekly dose of 500  $\mu$ g antibody per animal was given eight times, starting after the first imaging session, at 12 months of age. Animals received one of the following antibodies by intraperitoneal injection: 6G1, which is pan-amyloid- $\beta$  specific and has similar binding characteristics as the diagnostic 6E10 antibody (Barghorn *et al.*, 2005); A-887755, a globulomer-derived antibody which specifically detects oligomeric, but not monomeric or fibrillar amyloid- $\beta$  (Hillen *et al.*, 2010); IgG2a as a non-amyloid specific control. A fourth cohort was not treated. The experimenter was blind to the type of antibody administered.

The brain penetration of antibodies, as tested in different cohorts, was in the range of 0.1% of plasma levels.

## Statistics

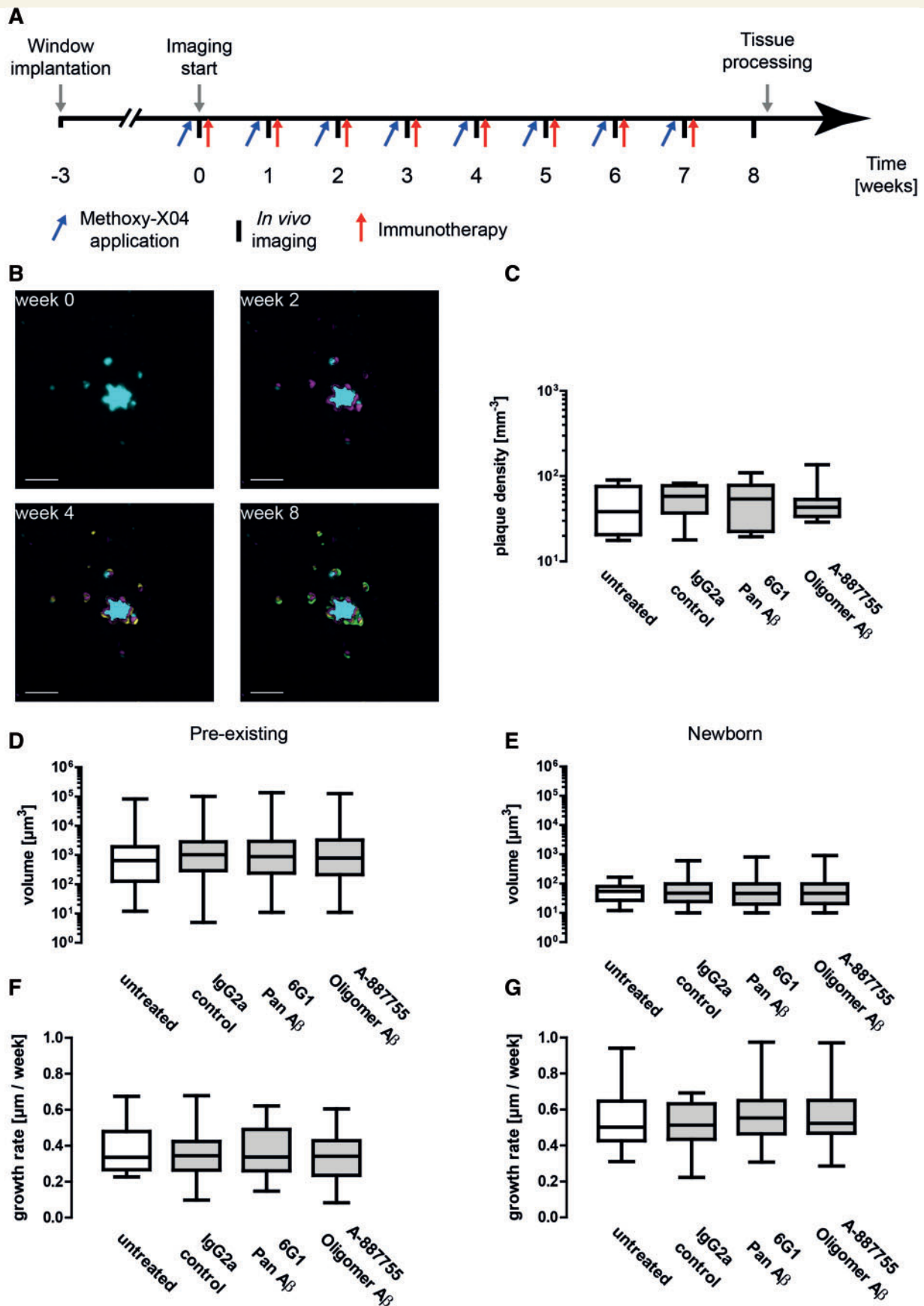
Synapse densities in relation to the distance from plaques were fit using one-phase association curves and the best-fit values for the plateaus were compared using F-tests. Synapse densities distant from plaques were compared using *t*-tests. Plaque densities, volumes and growth rates were compared using Kruskal-Wallis tests with Dunn's *post hoc* test. Unless specified otherwise, error bars show standard deviation (SD).

## Results

### Fibrillar plaques are unaffected by immunotherapy

*In vivo* imaging was performed once a week and the antibody treatment was administered at the end of the treatment session. Thereafter, the animals were sacrificed and the brains removed and processed for immunohistochemistry (Supplementary material and Fig. 1A).

We quantified the number, size and growth rates of fibrillar plaques *in vivo* by two-photon imaging of methoxy-X04 stained fibrillar deposits in the somatosensory cortex during the treatment period (Burgold *et al.*, 2014). First, we quantified the plaque densities in the volumes imaged. Because of the heterogeneity of amyloid deposition in Tg2576 mice, the resulting data showed large variances. At the end of the treatment period, overall plaque densities did not differ significantly between groups (Kruskal-Wallis test,  $n = 4-8$ ; Fig. 1C). Because amyloid deposition is a gradual process, the majority of the plaques may have been deposited before the treatment had an effect. Therefore, we analysed the volumes of pre-existing plaques, i.e. plaques that had been present before the treatment was started (Fig. 1D) and newborn plaques, i.e. plaques that appeared during the treatment period, separately (Fig. 1E). Neither group were altered by immunotherapy (Kruskal-Wallis test,  $n = 4-8$ ; Fig. 3B and C). Finally, we analysed the linear growth rate of pre-existing and newborn plaques, which is proportional to the cube root of the difference in volumes between two imaging sessions (Hefendehl *et al.*, 2011; Burgold *et al.*, 2014). Again, immunotherapy had no effect on the growth rates of fibrillar plaques (Kruskal-Wallis test,  $n = 4-8$ ; Fig. 1F and G). To assess the efficacy of anti-amyloid- $\beta$  antibodies, we first quantified the levels of total soluble and oligomeric amyloid- $\beta$  in Tg2576 mice from 4.5 to 15 months of age, using quantitative immunoprecipitation with 6E10 and A-887755 antibodies, respectively (Supplementary Fig. 1A). Then we quantified amyloid- $\beta$  levels in animals, which had been immunized at 3 months with either amyloid- $\beta_{1-42}$  monomer or with amyloid- $\beta_{20-42}$  globulomer. The latter generate a monospecific immune response resembling the antibody specificity of A-887755. At 12 months, the brain levels of total soluble and oligomeric amyloid- $\beta$  were quantified (Supplementary Fig. 1B and C). Immunization with amyloid- $\beta_{1-42}$  monomer caused a significant reduction in both



**Figure 1** Anti-amyloid immunotherapy with 6G1 and A-887755 antibodies has no effect on fibrillar plaque growth and density. 6G1 recognize monomeric, oligomeric and fibrillar amyloid- $\beta$  (A $\beta$ ), A-887755 recognizes only oligomeric amyloid- $\beta$ . (A) Timeline of

(continued)

total soluble amyloid- $\beta$  as well as oligomeric amyloid (ANOVA with Bonferroni's *post hoc* test;  $n = 11$ – $14$ ; Supplementary Fig. 1B and C), whereas immunization with amyloid- $\beta_{20-42}$  globulomer caused a significant reduction only in oligomeric amyloid (ANOVA with Bonferroni's *post hoc* test;  $n = 11$ – $14$ ; Supplementary Fig. 1B and C). Thus, immunotherapy caused a reduction in the respective amyloid isoforms.

## Synaptic pathology distant from plaques

The occurrence of fibrillar amyloid deposits is highly heterogeneous within the brains of Tg2576 mice, so that relatively large areas can be entirely free of plaques. We quantified the densities of DLG4 (previously known as PSD95) and synapsin-positive punctae in cortical and hippocampal areas which were at least  $50\mu\text{m}$  and typically  $\sim 1\text{mm}$  distant to the closest plaque to study the effects of non-fibrillar amyloid- $\beta$ . DLG4 and synapsin-positive punctae were detected using morphological image analysis routines (Dorostkar *et al.*, 2010), automatically counted and related to the tissue area after subtraction of blood vessels and neuronal cell bodies (Fig. 2A–E).

In mice treated with control antibodies, the densities of DLG4-positive punctae were significantly reduced in certain brain regions of Tg2576 compared to non-transgenic controls. In the cortex these were reduced from  $0.62 \pm 0.05\mu\text{m}^{-2}$  in controls to  $0.50 \pm 0.06\mu\text{m}^{-2}$  in Tg2576 ( $P < 0.01$ ,  $n = 4$ – $6$ , *t*-test, Fig. 2F) and in the stratum radiatum of the hippocampal field CA1 from  $0.63 \pm 0.07\mu\text{m}^{-2}$  in controls to  $0.51 \pm 0.05\mu\text{m}^{-2}$  in Tg2576 ( $P < 0.05$ ,  $n = 4$ – $6$ , *t*-test, Fig. 2F). In the stratum oriens or dentate gyrus molecular layer, densities of DLG4-positive punctae were not significantly altered (Fig. 2F). We also analysed the densities of synapsin-positive punctae in the respective brain regions. However, significant alterations were not detectable in any region (Fig. 2G).

We focused on the cortex and stratum radiatum to study whether loss of post-synapses was alleviated by immunotherapy. Indeed, animals treated with either 6G1 or A-887755 antibodies showed no significant loss of DLG4-positive punctae (Fig. 2H), compared to wild-type littermates treated with the same antibody. Taken together, these results suggest that DLG4-positive post-synapses are lost as a consequence of the toxic effects of diffusible, oligomeric amyloid- $\beta$ , whereas synapsin-positive presynapses remain unaffected.

## Plaque-associated synaptic pathology

Next, we analysed if synapses were lost in the immediate vicinity of fibrillar plaques. Tissue sections were treated with methoxy-X04 to stain fibrillar amyloid (Fig. 3A), while immunostaining against

DLG4 and synapsin marked glutamatergic post-synapses and pre-synapses, respectively (Fig. 3B and C). Plaque-associated synaptic pathology was analysed by quantifying the numbers of DLG4 and synapsin-positive punctae in  $5\text{-}\mu\text{m}$  wide bins around plaques (Fig. 3D and E).

The densities of DLG4-positive punctae around plaques followed monophasic association functions (Fig. 3D), with a plateau of  $0.43\mu\text{m}^{-2}$  [95% confidence interval (CI) 0.42–0.44]. The half-distance, a measure for the radius of influence of plaques, was  $3.02\mu\text{m}$  (95% CI 2.54–3.74 $\mu\text{m}$ ). Immunotherapy caused small, but significant, increases in synapse densities, while half-distances were not significantly altered. In animals treated with 6G1 and A-887755 antibodies, the maximal densities of DLG4-positive punctae were  $0.47\mu\text{m}^{-2}$  (95% CI 0.45–0.49,  $P < 0.001$ , F-test) and  $0.49\mu\text{m}^{-2}$  (95% CI 0.47–0.50,  $P < 0.001$ , F-test), respectively. Similar effects were seen on densities of synapsin-positive punctae (Fig. 3E): in animals treated with control antibodies, the maximal densities were  $0.59\mu\text{m}^{-2}$  (95% CI 0.56–0.61 $\mu\text{m}^{-2}$ ) and half-distances were  $5.09\mu\text{m}$  (95% CI 4.03–6.90 $\mu\text{m}$ ). In animals treated with 6G1 and A-887755 antibodies, the maximal densities of synapsin-positive punctae were  $0.63\mu\text{m}^{-2}$  (95% CI 0.61–0.66 $\mu\text{m}^{-2}$ ,  $P < 0.005$ , F-test) and  $0.64\mu\text{m}^{-2}$  (95% CI 0.62–0.66 $\mu\text{m}^{-2}$ ,  $P < 0.005$ , F-test), respectively, while the half-distances were not significantly altered ( $P > 0.05$ , F-test).

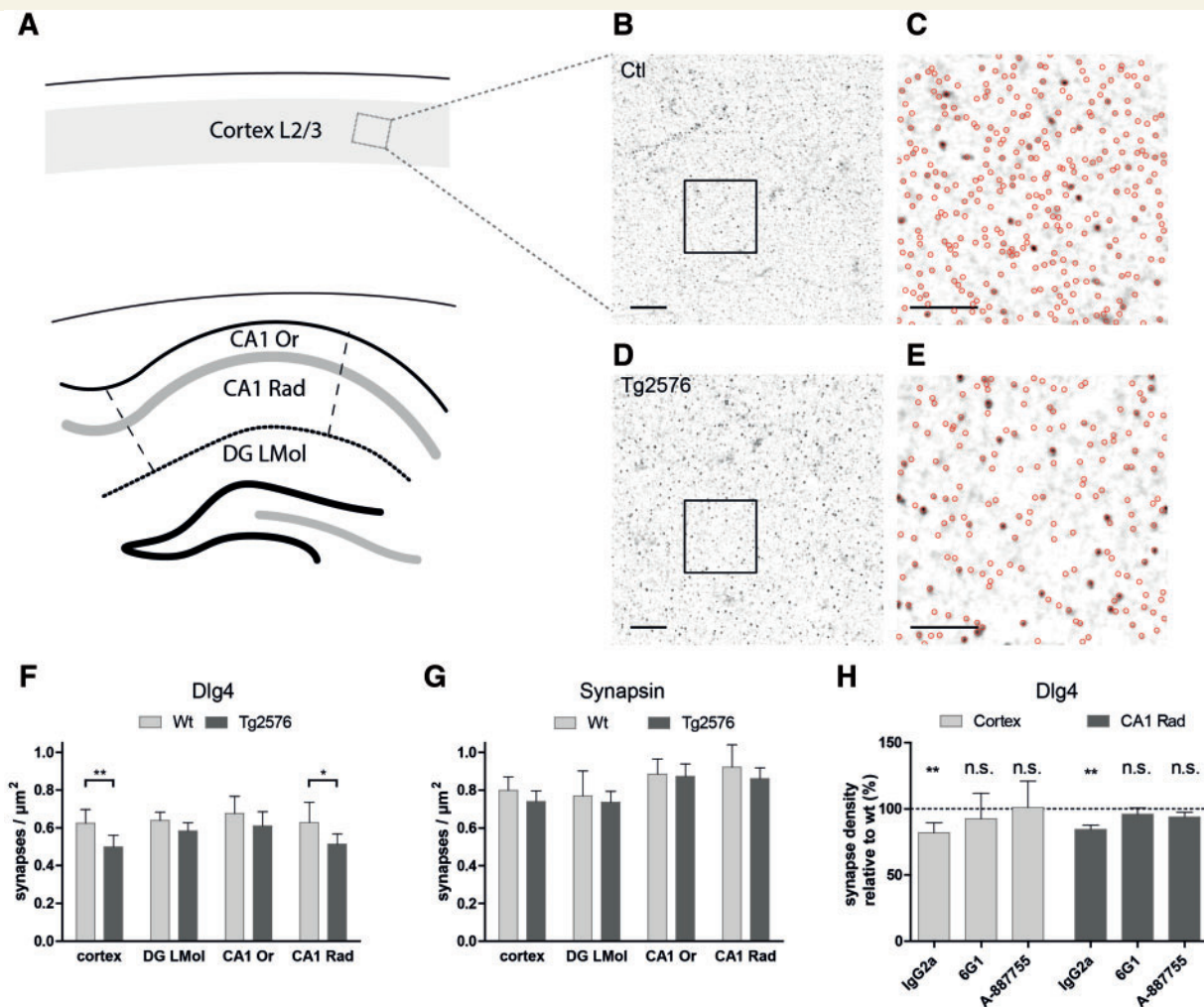
These results demonstrate that in the immediate vicinity of amyloid plaques, both pre- and post-synaptic structures are lost. Removal of amyloid- $\beta$  oligomers alleviates this loss, though only by a small degree.

## Discussion

Tg2576 mice express human amyloid precursor protein with the Swedish mutations and develop numerous amyloid plaques in the cortex and hippocampus as they age (Hsiao *et al.*, 1996). Typically, growth of amyloid plaques is observed at the age of 12 months, while at  $\sim 18$  months plaque growth tapers off (Christie *et al.*, 2001). We treated female, 12-month-old mice with one of three antibodies for 8 weeks: 6G1, which is pan-amyloid- $\beta$  specific and recognizes monomeric, fibrillar and oligomeric forms (Barghorn *et al.*, 2005); A-887755, a globulomer-derived antibody which specifically detects oligomeric, but not monomeric or fibrillar amyloid- $\beta$  (Hillen *et al.*, 2010); IgG2a as a non-amyloid specific control. Active immunization of animals in a pilot study with amyloid- $\beta_{1-42}$  globulomer, which results in a monospecific immune response resembling the antibody specificity

### Figure 1 Continued

experimental procedures. Methoxy-X04 was applied 24 h before *in vivo* imaging, immunotherapy was administered immediately afterwards. (B) Chronic *in vivo* imaging of fibrillar plaques. Maximum intensity projections of volumes are shown, while analyses were performed in 3D. Changes from the previous time points are colour-coded. Scale bars =  $10\mu\text{m}$ . (C) Overall plaque densities at the last imaging session (8 weeks). (D) Volumes of pre-existing plaques, i.e. plaques that were present at the first imaging session, and (E) volumes of newborn plaques, i.e. plaques that appeared during the treatment period. (F) Linear growth rates of pre-existing plaques. Linear growth is proportional to the cube root of the differences of plaque volumes. (G) Linear growth rates of newborn plaques. (C–G) Box-and-whiskers plots show median, 25th and 75th percentiles (box) as well as minimum and maximum values (whiskers). Groups were compared using Kruskal-Wallis tests. No significant differences were found between groups ( $P > 0.05$ ).

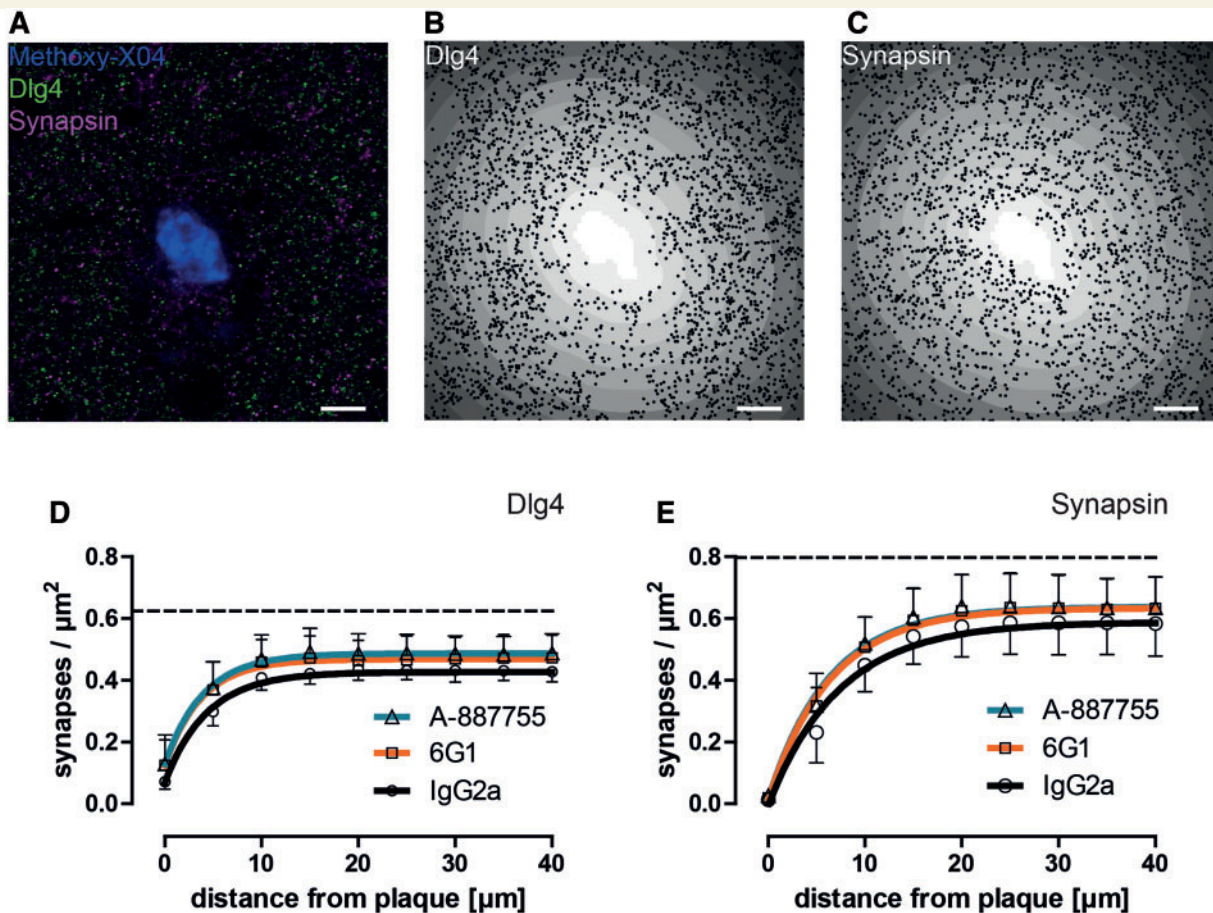


**Figure 2** Synapse density measurements distant from plaques. (A) Synapse densities were quantified in floating sections stained with antibodies against DLG4 and synapsin. The regions analysed were cortical layers 2/3, hippocampal field CA1, stratum oriens (CA1 Or) and stratum radiatum (CA1 Rad) and the dentate gyrus molecular layer (DG LMol) in areas at least 50  $\mu\text{m}$  distant from the closest fibrillar plaque. (B) DLG4 stained cortex of a wild-type mouse. Scale bar = 10  $\mu\text{m}$ . (C) Automatically detected synaptic punctae in the square region highlighted in B. Scale bar = 5  $\mu\text{m}$ . (D) DLG4 stained cortex of a Tg2576 mouse treated with control antibodies (IgG2a). Scale bar = 10  $\mu\text{m}$ . (E) Automatically detected synaptic punctae in the square region highlighted in D. Scale bar = 5  $\mu\text{m}$ . (F) Quantification of DLG4-positive punctae in the respective brain regions. (G) Quantification of synapsin-positive punctae in the respective brain regions. (H) Quantification of DLG4-positive punctae in the cortex and CA1 stratum radiatum of animals treated with control (IgG2a) antibodies, with conformation-unspecific (6G1) or oligomer-specific (A-887755) antibodies. Synapse densities were normalized to values in control animals treated with the same antibody. (F–H)  $n = 4$  (wild-type) to 6 (Tg2576). (F)  $*P < 0.05$ ;  $**P < 0.01$  ( $t$ -test). (H)  $**P < 0.01$ , one-sample  $t$ -test. Wt = wild-type; n.s. = not significant. PSD-95 is now known as DLG4.

of A-887755, had led to a significant reduction of amyloid- $\beta$  globulomer peptides after 9 months (Supplementary Fig. 1).

As loss of cognitive function in Alzheimer's disease ultimately depends on loss of functional synapses (DeKosky and Scheff, 1990; Scheff *et al.*, 1990) rather than on plaques themselves, we quantified the numbers of pre- and post-synapses as a readout to assess the efficacy of immunotherapy in a model of Alzheimer's disease. In this context, we defined presynapses as spots immunoreactive for the presynaptic marker synapsin and post-synapses as spots immunoreactive for DLG4, essentially marking dendritic spines (Sorra and Harris, 2000). These were quantified using automated algorithms, based on morphological spot detection

(Dorostkar *et al.*, 2010), thus eliminating any possible bias after image acquisition. In the immediate vicinity (up to  $\sim 10$ – $15$   $\mu\text{m}$ ) of fibrillar, methoxy-X04-positive, amyloid plaques, the densities of both pre- and post-synapses were strongly reduced (Fig. 3G and H). However, even several tens of micrometres away from plaques, the densities of pre- and post-synapses were reduced compared to non-transgenic controls. This suggests that the concentration of synaptotoxic factors is highest at the fibrillar core of plaques and diminishes with the distance from plaques. Similar results were obtained previously in different amyloid mouse models, which showed loss of DLG4 positive post-synapses or dendritic spines around fibrillar (Kirkwood *et al.*, 2013).



**Figure 3** Synapse density measurements in the proximity of fibrillar plaques. (A) Fibrillar plaques were stained with methoxy-X04, glutamatergic post-synapses were immunostained with anti-DLG4 (PSD-95) antibodies and presynapses with anti-synapsin antibodies. (B) Automatically detected DLG4-positive punctae overlaid on a distance transform highlighting the distances from the fibrillar plaque (white) in 5- $\mu\text{m}$  wide bins (grey scale). (C) Automatically detected synapsin-positive punctae overlaid on a distance transform. (D) Quantification of the densities of DLG4-positive punctae as a monophasic association function of the distance from the fibrillar plaque. (E) Quantification of the densities of synapsin-positive punctae as a monophasic association function of the distance from the fibrillar plaque. (A–C) Scale bars = 10  $\mu\text{m}$ . (D–E) Dashed lines indicate wild-type levels.

Plaques may be reservoirs of oligomeric amyloid- $\beta$ , causing loss of DLG4-positive post-synapses near plaques (Koffie *et al.*, 2009, 2011). Similarly, presynapses have been shown to be lost in various models of Alzheimer's disease (Pozueta *et al.*, 2013). Curiously, we found that in areas free of plaques, synapses were affected differently. On one hand, only DLG4-positive post-synapses, but not synapsin-positive presynapses, were reduced. Furthermore, not all brain areas were affected similarly: while we detected reductions in the densities of post-synapses in the cortex and stratum radiatum of the CA1 field, we were unable to detect any effects in the stratum oriens of the CA1 field or in the molecular layer of the dentate gyrus (Fig. 2F and G). This could mean that different types of neurons are affected to different degrees (Capetillo-Zarate *et al.*, 2006), or that different amounts of amyloid- $\beta$  oligomers (Sakono and Zako, 2010; Benilova *et al.*, 2012) are present in different brain regions.

Currently, no treatment for Alzheimer's disease is established to be safe and efficacious. Initial approaches with immunotherapy

have failed for various reasons including harmful side effects and lack of efficacy (Karran and Hardy, 2014). Nevertheless, immunotherapy seems to be one of the more promising approaches to combat Alzheimer's disease, if nothing else because several preclinical trials showed promising results: some studies showed cognitive improvement as well as increased clearing of amyloid deposits in animal models as a result of immunotherapy (Wilcock *et al.*, 2004; Wang *et al.*, 2011; DeMattos *et al.*, 2012), which was most effective when used preventatively (DeMattos *et al.*, 2012). Thus, the amyloid clearing effect may depend on a number of factors, including the epitope recognized by therapeutic antibodies, the time relative to amyloid deposition when the antibody is administered, but also the animal model itself. Neither the oligomer-specific antibody had an effect on amyloid plaques, nor the conformation-unspecific antibody (Fig. 1). This was expected for the former, as A-887755 does not recognize fibrils. However, we observed no effect of the conformation-unspecific antibody, 6G1, which has an intermediate

affinity for fibrils (Hillen *et al.*, 2010), though in contrast to most other studies we quantified only fibrillar amyloid, as only this can be visualized *in vivo*. Nevertheless, the lack of effect even on newly appearing plaques suggests that amyloid deposition was unaffected by both antibodies. However, even in the absence of an amyloid clearing effect we found that synapse loss was alleviated in animals treated with either non-conformation specific or oligomer-specific antibodies (Fig. 2). Close to fibrillar plaques, the beneficial effect was small, whereas distant from plaques synapse densities were restored to similar levels as in control animals (Fig. 2H). Beneficial effects of A-887755 antibodies on spine loss have been reported previously (Hillen *et al.*, 2010). These results suggest that synapse loss close to plaques is not reversible to the same degree as synapse loss distant from plaques. This may be the case because synapse loss at plaques occurs earlier, is more pronounced and is therefore irreversible by immunotherapy. Alternatively, fibrillar plaques may act as reservoirs for toxic amyloid- $\beta$  species. For instance, oligomeric amyloid- $\beta$ , which is widely accepted as a major neurotoxic effector of Alzheimer's disease (Benilova *et al.*, 2012; Gilbert, 2013), accumulates around plaques (Koffie *et al.*, 2009). A-887755 only recognizes oligomeric, but not monomeric or fibrillar amyloid- $\beta$ , whereas 6G1 recognizes all forms (Hillen *et al.*, 2010). The fact that A-887755 had protective effects further corroborates the hypothesis that oligomers are synaptotoxic and that removal of oligomers suffices to confer a synaptoprotective effect. Additionally, our results signify that sequestering oligomers by immunotherapy suffices to counteract synaptic pathology, thus suggesting that cognitive function may be improved even when the load of fibrillar amyloid remains unchanged.

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## Conflict of interest

This study was partially sponsored and funded by AbbVie. S Barghorn, C.K., H.H. are employees of AbbVie and may own AbbVie stocks. AbbVie contributed to the study design, research, and interpretation of data, writing, reviewing and approving the publication.

## Supplementary material

Supplementary material is available at *Brain* online.

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