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A single dose of passive immunotherapy has extended benefits on synapses and neurites in an Alzheimer's disease mouse model

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Abstract

Alzheimer's disease (AD) is a neurodegenerative disorder that impairs memory and cognition. One of the major neuropathological hallmarks is the accumulation of the extracellular senile plaques that are mainly composed of amyloid beta (A β) protein. Plaques are associated with synapse loss, dystrophic neurites and altered neurite trajectories. A reversal of such morphological changes has been observed days after single dose anti A β immunotherapy. In this study we investigated the extended effects of a single dose of passive anti A β immunotherapy on morphological changes associated with senile plaques. We found that although plaque burden was not reduced 30 days after immunotherapy, there were fewer dystrophic neurites around each plaque, a recovery of synapse density, and normalization of neurite curvature near plaques. Taken together these results suggest that single dose immunotherapy is sufficient to cause lasting benefits to the morphology of cortical neurons, implying substantial plasticity of neural circuits despite the continued presence of plaques.

Keywords

Alzheimer's; immunotherapy; amyloid; senile plaque; synaptic plasticity

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder affecting more than 4 million Americans and >30 million individuals worldwide (Kawas, et al., 2003). It results in memory loss, personality changes and cognitive decline. The main neuropathological hallmarks of AD are intracellular neurofibrillary tangles consisting of hyperphosphorylated tau and extracellular senile plaque deposition consisting mainly of amyloid beta (A β) peptide. Plaques have been shown to disrupt synaptic integration in mice (Stern, et al., 2004) hence possibly affecting cognition. In addition, plaques are also associated with altered neuritic trajectories, dystrophic neurites and dendritic spine loss (D'Amore, et al., 2003, Knowles, et al., 1998, Knowles, et al., 1999, Spires, et al., 2005, Tsai, et al., 2004). Neurites have been shown to be more curved inside and close to plaques compared to plaques and that has been suggested to interrupt

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neuronal networks (Knowles, et al., 1999, Le, et al., 2001). Swollen dystrophic neurites appear around plaques in AD and may interfere with cell transport (Spires and Hyman, 2004). Work in several mouse models has shown that dendritic spine loss is most profound around plaques (Moolman, et al., 2004, Spires, et al., 2005, Spires-Jones, et al., 2007). The cause for this spine loss could be the concentration of toxic oligomeric A β species (or other toxic molecules) around plaques (Koffie, et al., 2009, Meyer-Luehmann, et al., 2008, Shankar, et al., 2008).

Removing A β plaques and oligomeric species could prove to be a valuable treatment (Schenk, et al., 1999). So far it has been shown that with passive immunotherapy it is possible to remove A β plaques in mice (Bacsikai, et al., 2001, Bard, et al., 2000, Lombardo, et al., 2003, Morgan, et al., 2000). Along with clearing plaques, immunotherapy restores neurite architecture, increases synapse density and improves behavior (Dodart, et al., 2002, Kotilinek, et al., 2002, Lombardo, et al., 2003, Wilcock, et al., 2006, Thakker, et al., 2009). These events have been shown to occur over several days. Indeed, early improvements in neuritic morphology occur even prior to amyloid clearance, within 24 hours (Brendza, et al., 2005, Spires-Jones, et al., 2008). However, no extended effects of plaque associated neuritic changes have yet been investigated. It is essential to observe how long the recovery of neuritic morphological changes after a single dose of anti A β treatment persists. In this study we performed a single dose passive immunotherapy and quantified morphological changes 30 days later. We hypothesize that single-dose immunotherapy can have lasting beneficial effects on plaque-associated anatomical degeneration.

2. Results

After acute anti A β immunotherapy treatment it has been shown that A β plaques have been cleared within a few days (Bacsikai, et al., 2001). To determine whether A β plaques are present 30 days after a single dose of anti-A β antibody treatment, we labeled plaques with R1282 immunostaining and calculated the plaque burden in treated and untreated areas of cortex. We found that plaques were present in Tg2576 cortex but not non-transgenic controls as expected. Treatment with anti-A β antibody 3D6 did not lead to a statistically significant reduction in plaques observed 30 days post-treatment (Fig 1). Plaque burden was not significantly reduced in anti-A β treated mice (mean plaque burden = $0.99\% \pm 0.99$ in region treated with antibody) either in comparison to animals treated with an irrelevant antibody (mean plaque burden = $1.42\% \pm 1.54$ in treated area) or when compared to a region within the anti-A β treated brain that was not exposed to antibody treatment (mean plaque burden in untreated area of anti-A β treated mice = $1.01\% \pm 1.06$ ($p > 0.05$)). These data indicate that despite early clearance of plaques with passive immunotherapy as has been observed previously, there is a probable re-deposition within 30 days after a single dose of monoclonal antibody in this model.

Dystrophic neurites are often seen around plaques in AD and are an indication of neurodegeneration (Spires and Hyman, 2004). A reduction in the number of dystrophies has been observed in the first several days after anti A β immunotherapy treatment (Brendza, et al., 2005). We wanted to investigate whether the effect would persist 30 days after a single dose of passive immunotherapy treatment. In the antibody treated area there was a reduction in the number of axonal dystrophies per plaque in mice treated with anti A β (3D6) antibody (mean number of dystrophies per plaque = 2.85 ± 0.64), compared to the control anti human tau antibody (mean number of dystrophies per plaque = 4.51 ± 1.75 , t-test $p < 0.001$) (Fig 2). The effect was only seen in the area treated with anti A β antibody. There was a reduction in axonal dystrophies when anti A β treated cortex was compared to the area of cortex untreated with antibody in the same animals ($p < 0.01$). No such effect was seen in Tg2576 animals treated with control antibody. These results together with previous findings suggest that dystrophic

swellings can be rapidly reversed and the recovery can persist for at least 30 days despite the presence of A β plaques with a single dose of anti A β treatment.

Synapse loss is the strongest correlate of cognitive decline in Alzheimer's disease (Terry, et al., 1991). We wanted to determine whether improvements in synapse density with a single dose of anti A β immunotherapy treatment persisted for an extended period (30 days). Untreated Tg2576 cortex had a trend toward synapse loss compared to controls (Mann-Whitney test $p=0.05$) of 55% loss in synaptophysin immunoreactivity. We detected a significant 56% increase of presynaptic terminal (synaptophysin) optical density in Tg2576 cortex treated with anti A β antibody compared to the control treatment, so that synaptophysin optical density in the treated area is the same as in non-tg animals ($p<0.01$ Mann-Whitney test, Fig 3). In non-Tg animals, treatment with anti A β antibody had no effect compared to treatment with control antibody. These results suggest that synapse loss can be rescued by single dose passive immunotherapy and persist 30 days after the treatment despite the presence of fibrillar A β .

It has been shown that neurites have altered trajectories around plaques in AD (D'Amore, et al., 2003, Knowles, et al., 1998, Knowles, et al., 1999, Le, et al., 2001, Lombardo, et al., 2003) and with passive immunotherapy this can be rescued in several days (Lombardo, et al., 2003). In Tg2576 cortex treated with the control antibody, axons have an average curvature ratio of 1.026 ± 0.027 . Anti-A β treated areas average curvature ratio was 1.034 ± 0.038 showing no effect of treatment on average curvature when distance from a plaque was not taken into account (t-test $p>0.05$). When phantom plaques were created and distance to axons measured we found no difference between axons close ($<50 \mu\text{m}$) and far ($>50 \mu\text{m}$) from phantom plaques as expected. We calculated the ratio of close to far and found it to be 1.0057 and used that as a control baseline (Fig. 4). However, in transgenic animals treated with control antibody, axons were more curved close ($<50 \mu\text{m}$) to plaques compared to far ($>50 \mu\text{m}$) from plaques (curvature ratio 1.05 ± 0.065 and 1.034 ± 0.042 , respectively, ratio close to far 1.011 t-test, $p<0.05$) (Fig. 4). 30 days after the treatment with anti-A β antibody there was no difference between axons that were close (curvature ratio 1.056 ± 0.042) to those that were far (curvature ratio 1.045 ± 0.057 ratio close to far 1.007) from plaques (t-test, $p>0.05$) (Fig. 4), indicating some normalization of axon trajectory with anti-A β treatment, although the curvature does not approach control levels.

Together, these data show lasting morphological benefits of a single dose of passive anti-A β immunotherapy.

3. Discussion

The amyloid hypothesis of AD suggests that the onset and progression of pathogenesis of AD is influenced by the accumulation of A β in the brain (Hardy and Selkoe, 2002). However, plaques have been shown to poorly correlate with the progression of the disease (Arriagada, et al., 1992). A much better indicator of cognitive decline is synaptic density, neurofibrillary tangles and dystrophic neurites around plaques (Ingelsson, et al., 2004, McKee, et al., 1991, Terry, et al., 1991), indicating that prolonged recovery of these markers is possibly more important than plaque removal which could theoretically release more synaptotoxic soluble species of A β .

In our study we showed that plaques were present 30 days after a single treatment with anti-A β antibody similar to findings by Oddo et al. in another mouse model of AD (Oddo, et al., 2004). Since immunotherapy is a well established way of clearing plaques (Bacskai, et al., 2001, Bard, et al., 2000, Schenk, et al., 1999), it is likely that in our mice, plaques were cleared quickly, then re-emerged during the 30 days post-treatment as seen in the triple transgenic model (Oddo, et al., 2004).

We find that despite the persistent A β plaques 30 days after treatment, the density of presynaptic markers was increased. This indicates that it is possible to reverse synapse loss - one of the strongest correlates with cognitive decline. It has been recently shown that dendritic spine plasticity can be improved after 1 hour of immunotherapy treatment (Spires-Jones, et al., 2008) and here we have shown that similar effects in presynaptic markers can persist for at least 30 days.

Another important correlate with stages of dementia and a pathological hallmark of AD are dystrophic neuritic swellings (McKee, et al., 1991, Spires and Hyman, 2004). It has been shown that dystrophic neurites can recover 3 days after an anti A β treatment (Brendza, et al., 2005). In the current study we showed that axonal dystrophies around plaques remain reduced 30 days after the treatment despite the presence of A β plaques. This suggests that the presence of fibrillar aggregates of A β does not preclude the recovery of either synapses or axonal dystrophies. These results taken together with previous results indicate that dystrophic swellings can appear within a matter of days (Meyer-Luehmann, et al., 2008), clear with passive immunotherapy within several days (Brendza, et al., 2005), and the current study suggests that this recovery can persist for 30 days.

It has been reported that dendrites and axons are more curved close compared to far from A β plaques (D' more, et al., 2003, Garcia-Alloza, et al., 2006, Knowles, et al., 1998, Knowles, et al., 1999) and that dendrites (labeled with SMI32) can straighten within 4 days after treatment, an effect which persists for 32 days (Lombardo, et al., 2003, McKee, et al., 1991). In this study we found that, similar to the results with dendrites, 30 days after treatment, the curvature of axons near plaques was the same as those farther away, while in animals treated with an irrelevant antibody, curvature was still more pronounced near plaques.

It can be inferred from our study that anti A β treatment is sufficient to induce the recovery of synapses and neurite morphology persists for at least 30 days despite the presence of fibrillar plaques at this time point. It is possible that toxic oligomeric species being produced in the Tg2576 animals are being sequestered into new plaques after an initial clearance of both soluble and fibrillar species explaining the lingering beneficial effects on neurite morphology. We presume that once the pre-treatment equilibrium of fibrillar and soluble species is reached, another dose anti A β antibody would be necessary to continue the beneficial effects. This could prove beneficial in patient immunotherapy trials as only one dose passive immunotherapy treatment could provide lasting recovery of neurite morphology and by extension neural system integrity.

4. Experimental Procedures

Animals and Surgery

All animal work conformed to National Institutes of Health and institutional guidelines. Tg2576 mice expressing human amyloid precursor protein with the AD-associated Swedish mutation (Hsiao, et al., 1996) between ages 18 to 26 month and non-transgenic littermate controls were used for this study. A craniotomy was performed as described previously (Bacskai, et al., 2002, Klunk, et al., 2002, Lombardo, et al., 2003, Skoch, et al., 2004). After dura resection animals were treated with topical application of (25 μ L volume) of either anti A β antibody (3D6) (1mg/mL concentration) (Elan Pharmaceuticals) or an irrelevant human tau antibody (12E8 or 16B5) (1 mg/mL concentration) (Elan Pharmaceuticals). Following the treatment, the craniotomy was sealed with an 8mm diameter glass coverslip and animals were left to recover. 30 days post treatment animals were euthanized using an overdose of Avertin (400mg/kg, i.p.). Brains were fixed using 4% Paraformaldehyde and 15% glycerol for cryoprotection for 48h then one hemisphere was sectioned at 50 μ m using freezing microtome while the other was paraffin embedded and sectioned at 16 μ m, both were sectioned coronally.

Plaque burden

Every 15th section (selected on systematic random basis) from the paraffin-embedded hemisphere was deparaffinized and blocked in 5% Normal Goat Serum (NGS) for 1h. Sections were then immunostained with A β antibody R1282 (1: 500 in 1 % NGS in Tris-buffered saline, gift from Dr. D. Selkoe, Brigham and Women's Hospital, Boston, MA) and Alexa488 (Invitrogen, Eugene, OR) was used as a secondary antibody (1:100 in 1 % NGS in Tris-buffered saline, Invitrogen, Eugene, OR). A β fluorescent immunostaining was detected by scanning stained sections in a scan array reader (Perkin Elmer, Downers Grove, IL) using a 488 laser for excitation. Scanned images were analyzed using ImageJ software. Regions of interest were outlined in the superficial 200 μ m of cortex under the craniotomy (the area that was exposed to antibody treatment) and in the superficial 200 μ m of an untreated region (piriform cortex). The total area occupied by plaques was divided by the total area of the cortex sampled to obtain the percentage of cortex covered by A β immunoreactivity (plaque burden).

Axon curvature

Every 10th PFA fixed (free floating) section (first section selected on systematic random basis) was permeabilized using 0.5% TritonX-100 in TBS for 20 minutes followed by 1h blocking in 5% NGS. To visualize axons the tissue was incubated with a SMI 312 (1:1000 in 1 % NGS in Tris-buffered saline, Covance Research Products, Inc., Berkeley, CA) antibody that recognizes pan axonal neurofilament and conveniently labels axons and axonal dystrophies associated with plaques. A secondary antibody anti-mouse IgG conjugated to Cy3 dye (1:200 in 1 % NGS in Tris-buffered saline, Jackson ImmunoResearch, West Grove, PA) was used and then sections were counterstained with Thioflavine-S (0.05% in 50% ethanol) (Sigma, St. Louis, MO).

Images for axon analysis were taken on upright Olympus BX51 (Olympus, Denmark) fluorescence microscope with a DP70 camera sampling 100% of the 200 μ m of the area treated with the antibody. Only axons longer than 20 μ m were chosen for analysis and their curvature ratio was calculated in Image J by dividing the curvilinear length of the axon by the straight line length of the process (D' more, et al., 2003, Garcia-Alloza, et al., 2006, Knowles, et al., 1999, Lombardo, et al., 2003). Distance from the measured axon segment to the closest senile plaque (if present) was measured at three points – from each end and the midpoint of the segment and the average distance was taken from these three measurements. Axons that were closer than 50 μ m to the plaque were defined as being close to the plaque and others as far from the plaque.

Dystrophies per plaque

Every 15th section (selected on systematic random basis) was immunolabeled with SMI312 antibody as described above using Alexa488 (Invitrogen, Eugene, OR) to visualize axons and dystrophies. Plaques were immunolabeled using Amyloid beta antibody R1282 (gift from Dr. D. Selkoe, Brigham and Women's Hospital, Boston, MA) as described above and visualized using Cy3 (Jackson ImmunoResearch, West Grove, PA). SMI312 positive dystrophies within the plaque area that were larger than 2.5 μ m² were classified as dystrophic neurites and counted for each plaque using an upright Olympus BX51 (Olympus, Denmark) fluorescence microscope. The average number of dystrophies per plaque was calculated.

Synapse quantification

Every 15th paraffin section (selected on systematic random basis) was deparaffinized and incubated in 1 mg/ml pepsin in 0.2N HCl for 10 min and blocked in 5% NGS for 1h. Sections were immunolabeled with presynaptic marker antibody Sy38 (1:10 in 1 % NGS in Tris-buffered saline, Synaptic Systems) with an anti-mouse Alexa 546 secondary antibody (1:100 in 1 %

NGS in Tris-buffered saline, Invitrogen, Eugene, OR) and plaques were labeled with R1282 and an anti-rabbit Alexa 488 secondary antibody as described earlier. Optical density of fluorescent immunostaining was determined using a Scan Array Express (Perkin Elmer, Boston MA). A 568nm laser was used to excite the fluorophore and scans of each section were acquired at 3 focal planes. In Image J software (free from the NIH), the three images of each section were projected into one composite. Regions of interest were outlined in the superficial 200 μm of cortex under the craniotomy (the area that was exposed to antibody treatment) and in the superficial 200 μm of an untreated region (piriform cortex). Mean grayvalues for each group were calculated.

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Abbreviations

AD	Alzheimer's disease
Aβ	amyloid-beta peptide
NGS	normal goat serum

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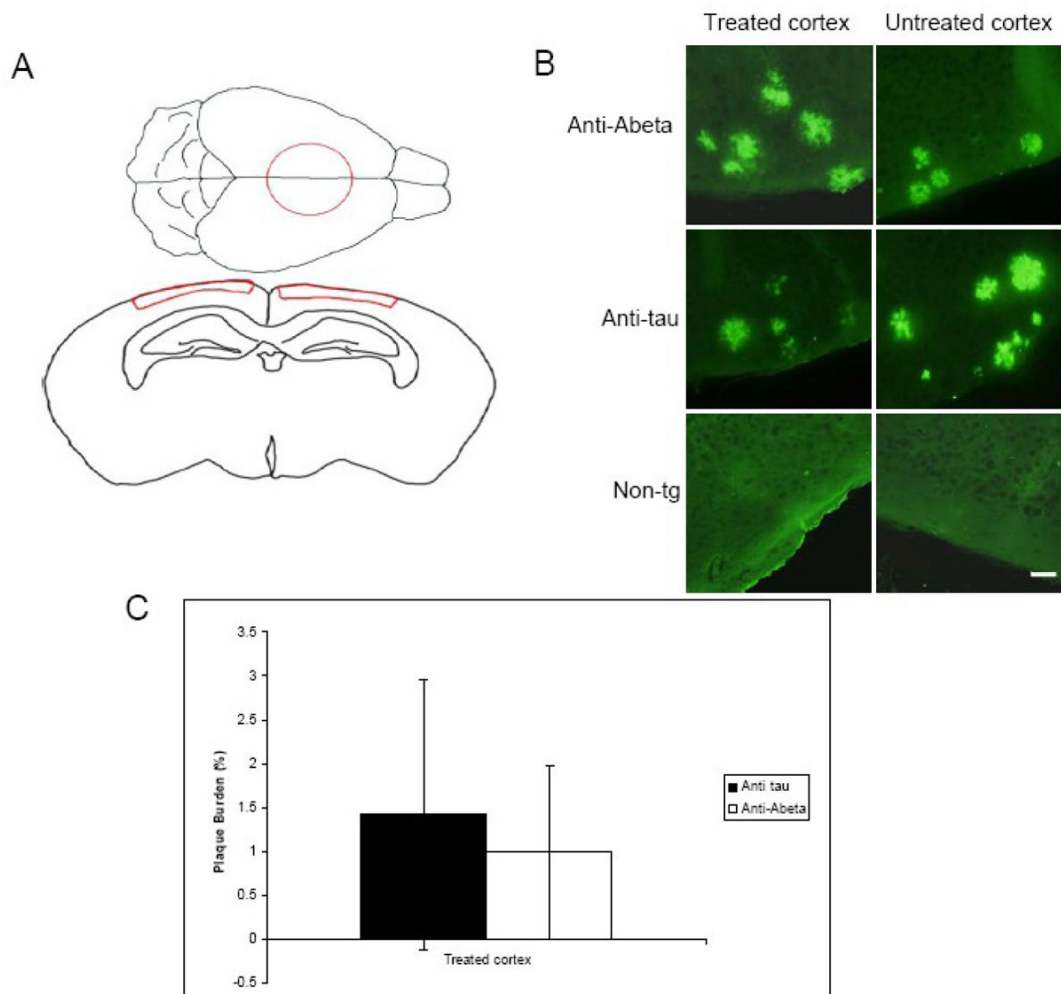


Fig 1. Plaque burden 30 days after anti A β immunotherapy treatment. Tg2576 and age-matched non-transgenic animals were treated with topical application of either anti-A β antibody or anti human tau antibody. The area treated with antibody is outlined in red in panel (A) and is referred to in text as the treated cortex. (B) Plaques were immunolabeled with R1282 primary antibody and visualized with Alexa488. (C) There was no difference in cortical plaque burden between animals treated with an anti A β antibody 3D6 and those treated with control antibody 12E8 ($p < 0.05$). Control non-transgenic animals had no plaques as expected. Scale bar 50 μ m. Data presented as mean \pm SD. Panel with the coronal section was modified from the high resolution mouse brain atlas (<http://www.hms.harvard.edu/research/brain/atlas.html>).

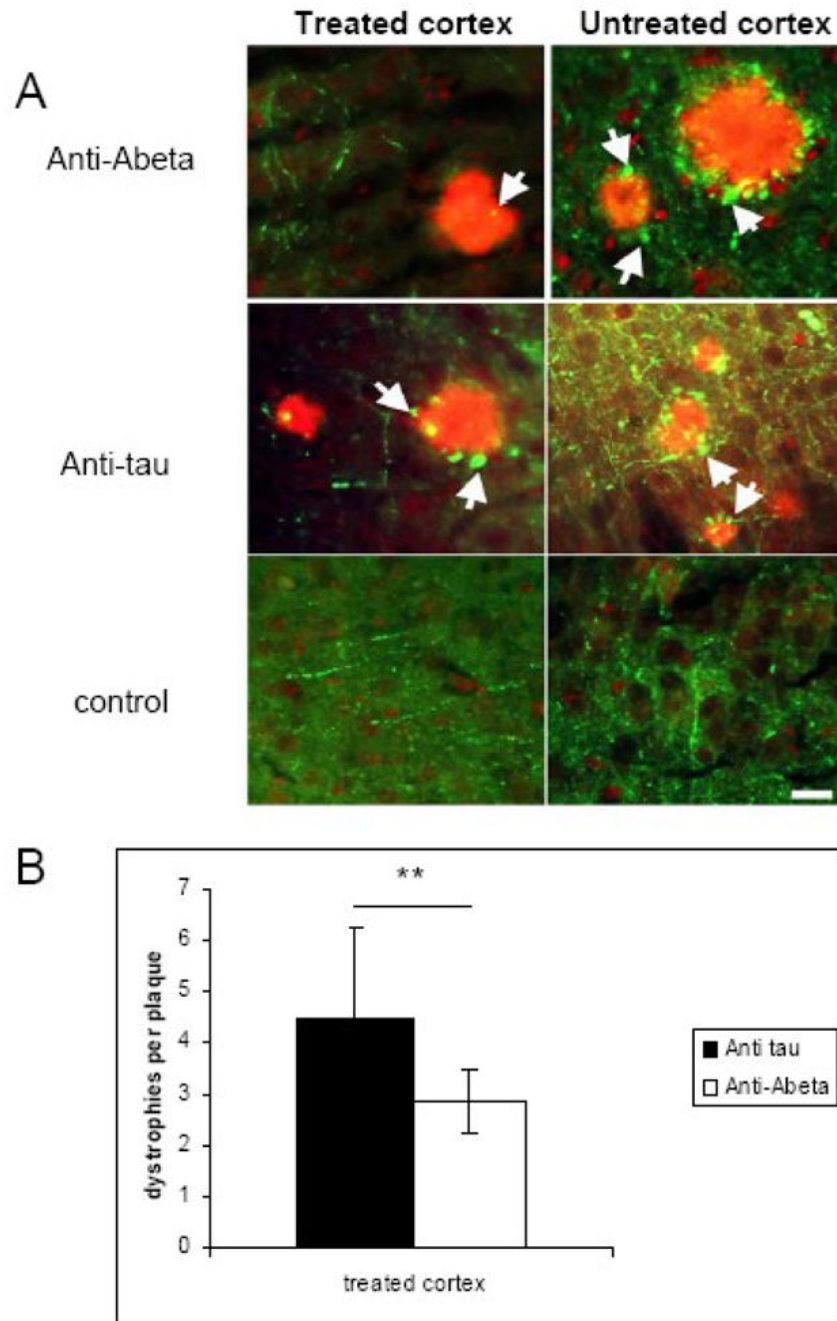


Fig 2. Effects of passive immunotherapy on axonal dystrophies. (A) Plaque-associated axonal dystrophies (arrows) were analyzed on sections stained with SMI312 (green) and R1282 to label plaques (red). (B) Quantification shows that a single topical treatment with an antibody to A β caused a reduction in the average number of dystrophic axonal swellings per plaque 30 days after the treatment (** $p < 0.001$). The effect on axons was observed only in the area treated with anti A β antibody and was not seen in the untreated area or in animals treated with irrelevant anti human tau antibody (12E8). Scale bar 20 μ m. Data presented as mean \pm SD.

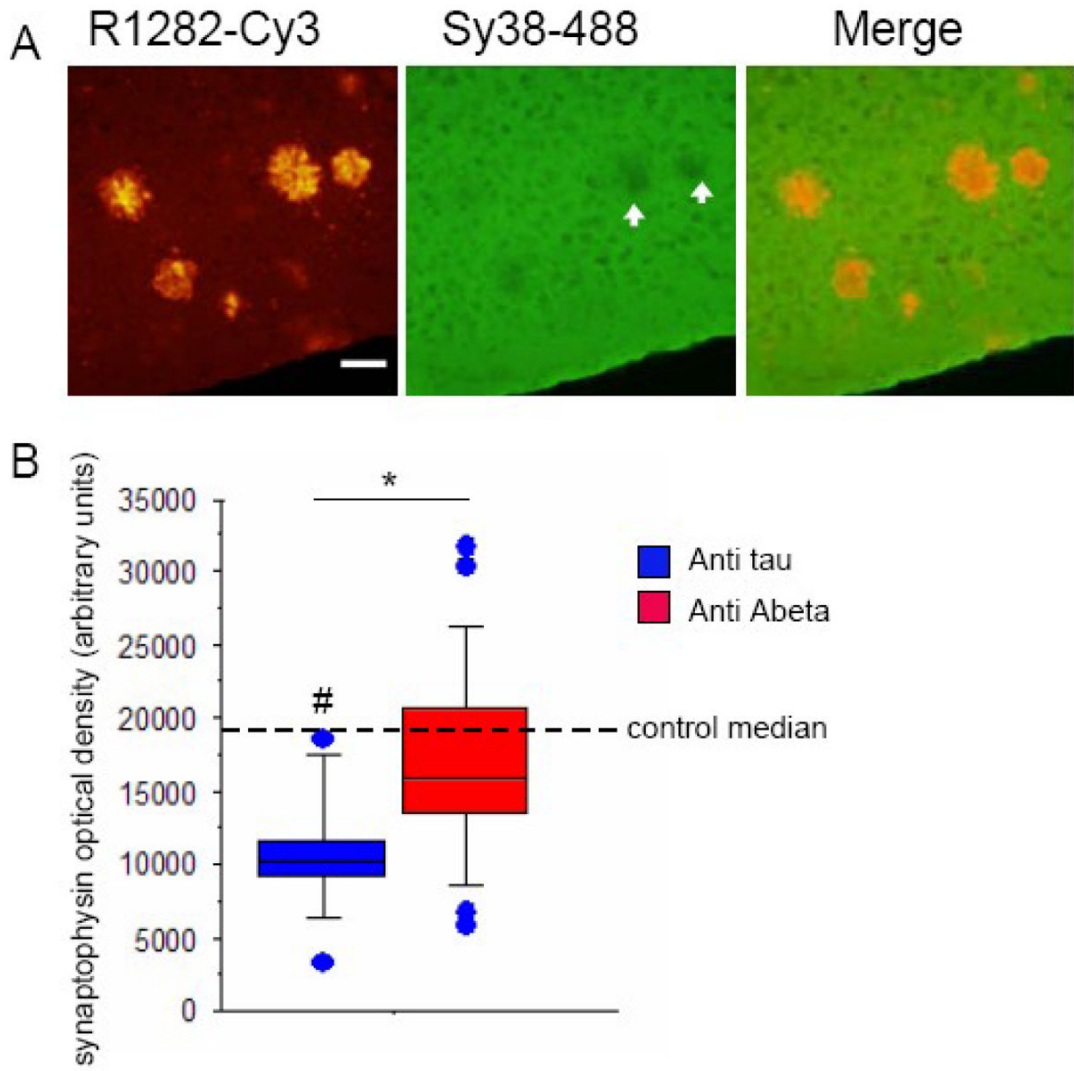
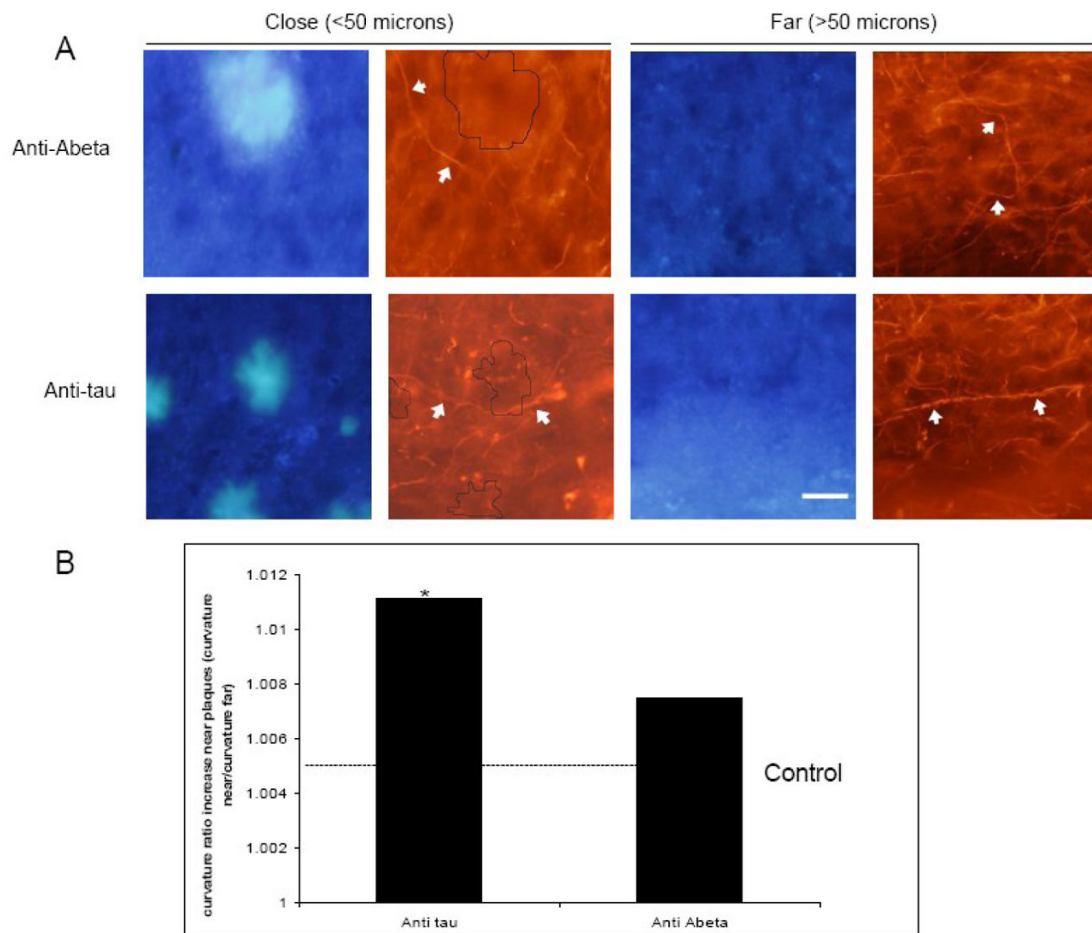


Fig 3. Presynaptic terminal density increases 30 days after a single dose of passive immunotherapy treatment. (A) SY38 synaptophysin staining (green) shows presynaptic terminal loss in the area of cortex occupied by R1282 labeled (red) plaques (arrows). (B) A box plot of synaptophysin optical density data shows that there is a trend towards 55% presynaptic terminal loss in animals treated with the control anti tau antibody compared to non-transgenic animals (# Mann-Whitney test $p=0.0527$). Presynaptic terminal density recovered in tissue treated with anti $A\beta$ antibody (3D6) compared to tissue treated with anti tau antibody (* Mann-Whitney test $p<0.01$). The recovery occurred despite persistent plaque pathology. Scale bar 50 μm . Optical density data were not normally distributed thus are presented in box plots with median, maxima, and minima shown.

**Fig 4.**

Effects of single dose passive immunotherapy treatment on axon curvature ratios. (A) Axon curvature ratios were measured on SMI312 labeled axons (red) in proximity to Thioflavin S positive plaques (blue). (B) After single dose topical application of antibody to $A\beta$ (3D6) axons close ($<50\ \mu\text{m}$) to plaque were as curved as axons far ($>50\ \mu\text{m}$) from plaque ($p>0.05$), however without reaching the level of non-transgenic animals. Axons of animals treated with anti tau antibody were significantly more curved close to plaques compared to far from plaques (* $p<0.05$). Control animals had significantly straighter axons compared to transgenic animals ($p<0.05$). Scale bar $10\ \mu\text{m}$. Data presented as mean \pm SD.