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Aβ Immunotherapy Leads to Clearance of Early, but Not Late, Hyperphosphorylated Tau Aggregates via the Proteasome

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Summary

Amyloid-β (Aβ) plaques and neurofibrillary tangles are the hallmark neuropathological lesions of Alzheimer's disease (AD). Using a triple transgenic model (3xTg-AD) that develops both lesions in AD-relevant brain regions, we determined the consequence of Aβ clearance on the development of tau pathology. Here we show that Aβ immunotherapy reduces not only extracellular Aβ plaques but also intracellular Aβ accumulation and most notably leads to the clearance of early tau pathology. We find that Aβ deposits are cleared first and subsequently reemerge prior to the tau pathology, indicative of a hierarchical and direct relationship between Aβ and tau. The clearance of the tau pathology is mediated by the proteasome and is dependent on the phosphorylation state of tau, as hyperphosphorylated tau aggregates are unaffected by the Aβ antibody treatment. These findings indicate that Aβ immunization may be useful for clearing both hallmark lesions of AD, provided that intervention occurs early in the disease course.

Introduction

Alzheimer's disease is a progressive neurodegenerative disorder that is the leading cause of dementia among the elderly. The two hallmark neuropathological lesions of this disorder include the aberrant accumulation of the amyloid β-peptide (Aβ) and intracellular inclusions consisting of hyperphosphorylated tau protein. Because Aβ, either as extracellular aggregates or perhaps an intracellular form, appears to be an early and critical event in the pathogenesis of AD, many therapeutic-based strategies are aimed at reducing its formation or facilitating its clearance.

Several immune-based strategies, including both active and passive approaches with anti-Aβ antibodies, have been shown to dramatically prevent or reduce Aβ accumulation in the brains of APP transgenic mice (Bard et al., 2000; Schenk et al., 1999; Sigurdsson et al., 2001). These approaches protect transgenic mice from cognitive decline (Dodart et al., 2002; Janus et al., 2000; Morgan et al., 2000), and likewise also appear to slow cognitive decline in human AD patients (Hock et al., 2003). It is presently unclear, however, whether lowering the Aβ burden in the brain will also effectively halt the progression of other key components of AD neuropathology such as those that involve the tau protein. Unfortunately, the human clinical trial was halted due to the unforeseen development of encephalitis in a small proportion of patients (Check, 2002). Nevertheless, one brain sample from an immunized patient was evaluated neuropathologically, and it appeared that neurofibrillary tangles were unaffected (Nicoll et al., 2003)—although clearly it is difficult to draw firm conclusions from a single case report. Thus, it remains unresolved whether preventing or clearing Aβ accumulation will also affect other aspects of AD pathology such as neurofibrillary tangles. Because tau can cause neurodegeneration in the absence of Aβ (e.g., frontotemporal dementia with parkinsonism-17) (Hutton et al., 1998), it is critically important from a clinical perspective to determine whether the tau pathology in the AD brain will also be impacted by anti-Aβ interventions.

The occurrence of both plaques and tangles in our 3xTg-AD mice allows us to directly assess whether the onset and development of the tau pathology is modulated following therapies designed to clear Aβ (Oddo et al., 2003b). Toward this end, we administered anti-Aβ antibodies into the hippocampus of the 3xTg-AD mice; this approach was shown by Morgan and colleagues to effectively and rapidly reduce Aβ deposits, with a discernible reduction in the Aβ load by 24 hr (Wilcock et al., 2003, 2004). Here we show a reduction not only of extracellular Aβ deposits, including thioflavin-S-positive structures, but also that the intracellular Aβ load is rapidly and markedly diminished. Notably, we find that intrahippocampal Aβ immunization also reduces early tau pathology. Whereas Aβ deposits are cleared within 3 days, the tau lesions require a slightly longer time and are not reduced until 5 days postinjection. Thus, Aβ is cleared first, followed by the clearance of tau localized in the somatodendritic compartment. Conversely, by 30 days postinjection, Aβ deposits have reemerged, although the tau pathology is not apparent at this time point.

Using an alternative approach, we show that administration of the γ-secretase inhibitor, N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT), leads to similar results. Therefore, the finding that Aβ-based interventions successfully clear the tau pathology in these 3xTg-AD mice provides compelling evidence in support of the amyloid cascade hypothesis. The mechanism underlying the effect on tau is dependent on the activity of the proteasome, as blocking its function prevents the immunotherapy-mediated clearance of tau. In addition, we find that the clearance of tau is critically dependent on its phosphorylation state, as late-stage, hyperphosphorylated tau aggregates appear unaffected by the Aβ antibody treatment. Our results indicate that Aβ-targeted therapies may be useful for clearing both hallmark lesions of AD, provided that the intervention is administered early in the disease course.

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Table 1. Age, Number, and Genotype of Mice Receiving Immunotherapy

Mice	Age (Months)	Number of Mice Used	Antibody Injected	Time Sacrificed Post Antibody Administration (in Days)
h3xTg-AD	12	3	Isotype control	7
h3xTg-AD	12	3	PBS	7
h3xTg-AD	12	3	HT7	7
h3xTg-AD	12	3	4G8	7
h3xTg-AD	12	11	1560	5–7
h3xTg-AD	12	3	1560	3
h3xTg-AD	12	6	1560	30
h3xTg-AD	12	3	1560	45
h3xTg-AD	12	5	1560 + Epoxomicin	7
H3xTg-AD	6	3	PBS	7
H3xTg-AD	6	3	1560	7
H3xTg-AD	12	5	1560	7

Hemizygous and homozygous 3xTg-AD mice are indicated as h3xTg-AD and H3xTg-AD, respectively.

Results

Clearance of Intracellular and Extracellular Aβ

Antibodies against Aβ were administered by intrahippocampal injections into hemizygous and homozygous 3xTg-AD mice (Table 1). The 3xTg-AD mice develop both plaques and tangles in an age-progressive and region-specific manner that closely mimics the hierarchical pattern observed in human AD (Oddo et al., 2003b). The neuropathology that develops in the hemizygous and homozygous 3xTg-AD mice is indistinguishable, although the onset is slower in the hemizygous group (Oddo et al., 2003a). Consequently, the outcome of the immune-based treatments can be evaluated in mice with different degrees of neuropathology, but where the effect of age and genetic background is not a confounding variable.

In the first experiment, a single, intrahippocampal injection of the anti-Aβ monoclonal antibody 1560 or 4G8 was administered to 12-month-old hemizygous 3xTg-AD mice (Figure 1). 7 days postinjection, there was a marked reduction in extracellular deposits as determined by Aβ immunostaining in the ipsilateral hippocampus in mice receiving the Aβ antibody (Figure 1A). Moreover, thioflavin-S-positive structures were also diminished, further demonstrating clearance of extracellular plaques (cf. Figures 1B and 1C) and also establishing that the lack of Aβ detection in the ipsilateral hippocampus was not due to competition between the injected antibody and the secondary antibody used during the immunostaining procedure. Not only were extracellular Aβ deposits reduced, but we also found that the antibody treatment led to a diminution in the number of Aβ-immunoreactive neurons around the site of injection, indicating that intracellular Aβ was also cleared (Figures 1D–1G). Clearance of both extracellular and intracellular Aβ occurred to the same extent using monoclonal antibodies 4G8 or 1560 (Figures 2J and 2K), indicating that the effect was not limited to a specific antibody. Finally, it is important to note that Aβ pathology remained unaffected in the contralateral hippocampi (Figures 1A, 1B, 1D, and 1F), and injections of anti-tau antibody (Figures 2C, 2D, and 2I), isotype control antibody, or PBS (Figure 3) had no apparent effect on Aβ pathology in either the ipsilateral or contralateral hippocampi.

Aβ Clearance Reverses Tau Aggregates

Because the 3xTg-AD mice develop both hallmark lesions of AD neuropathology and because anti-Aβ antibody treatment effectively clears extracellular and intracellular Aβ deposits, we assessed whether administration of an anti-tau antibody could reduce the tau burden in the CA1 pyramidal neurons of the hippocampus. We found that the tau pathology was unaffected following intrahippocampal administration of the anti-tau monoclonal antibody HT7, which recognizes all isoforms of human tau (Figures 2A, 2B, and 2I). Recall that the HT7 antibody treatment had no effect on the Aβ pathology (Figures 2C, 2D, and 2I). Finally, the tau pathology was also unaffected by intrahippocampal administration of an anti-protozoan isotype control antibody or by PBS (Figure 3). Therefore, tau immunotherapy had no discernible effect on the tau pathology.

Notably, administration of the anti-Aβ monoclonal antibodies 4G8 or 1560 effectively cleared the early tau pathology in the somatodendritic compartment of neurons within the injected hippocampi (Figures 2E–2H, 2J, and 2K). No clearance of tau was observed in the contralateral, uninjected hippocampi (Figures 2E and 2G). In the Aβ-injected hippocampi, tau staining was absent in regions immediately surrounding and extending from the injection site, but the effect was not due to damage to the CA1 neurons from the injection cannula as they appeared morphologically unaltered by hematoxylin and eosin staining (Figures 8A–8C). In some cases, a near total lack of tau immunoreactivity occurred in the injected hippocampi, though the contralateral hippocampi retained extensive tau pathology. These results indicate that the clearance of the tau load from the somatodendritic compartment, indicative of early tau pathology, by the anti-Aβ antibody is a selective process that neither depends on a generalized IgG response nor is a consequence of an intracerebral injection artifact. Moreover, both antibodies 4G8 and 1560 effectively cleared the tau pathology, indicating that these results are not unique to a specific anti-Aβ antibody. These findings further suggest that there is direct relationship between Aβ and tau pathology and demonstrate that clearance of Aβ can lead to a subsequent reduction in the tau burden.

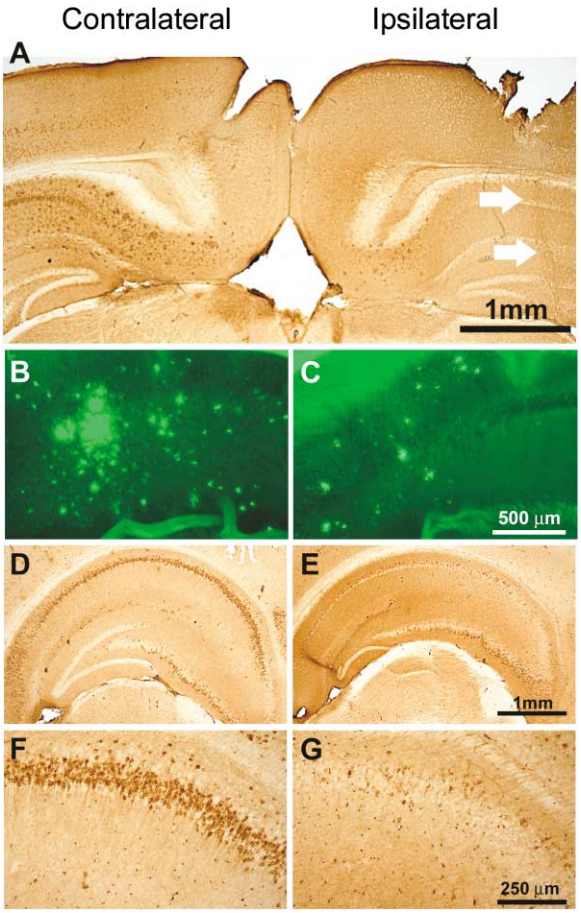

Figure 1. Intrahippocampal Administration of Anti-Aβ Antibodies Clears Both Intracellular and Extracellular Aβ Aggregates

The anti-Aβ monoclonal antibody, 1560, was administered via intrahippocampal injections into 12-month-old hemizygous 3xTg-AD mice (n = 11).

(A) Low-magnification view of the hippocampus/neocortex showing the contralateral (uninjected) and ipsilateral (injected) sides. Arrows on the right side denote the cannula track. Note the reduction in the number of Aβ immunoreactive deposits in the ipsilateral hippocampus surrounding the site of injection.

(B and C) Intrahippocampal anti-Aβ injection reduced the number of thioflavin S-positive structures.

(D–G) Low- and high-magnification views of the hippocampus/CA1 pyramidal cell layer showing that a single administration of Aβ antibody is sufficient to markedly reduce intracellular Aβ immunoreactivity.

Temporal Pattern of Aβ and Tau Clearance

The reduction of the tau pathology by utilization of an anti-Aβ antibody suggests a direct relationship between the development and expression of these two neuropathological lesions. Indeed, if such a relationship exists, it may be possible to find a time point following administration of the anti-Aβ antibody when clearance of Aβ precedes the removal of the tau pathology. To determine the time course of the antibody-mediated clearance of the Aβ and tau pathology, we delivered anti-Aβ antibody 1560 into the hippocampus of 12-month-old hemizygous 3xTg-AD mice and sacrificed them 3, 5, 7, 30, or 45 days later (Figures 4 and 5). After only 3 days postinjection, Aβ was cleared from CA1 pyramidal neurons, yet a rich pattern of human-specific tau immunostaining remained in *both* the ipsilateral and contralateral hippocampi (cf. Figures 4A–4D with 4E–4H; Figure 5A). By 5 days postinjection, both the Aβ and tau burden were cleared (data not shown), paralleling the results observed at 7 days postinjection (Figures 1 and 2). Therefore, these results established that there is a hierarchical pattern to the antibody-mediated clearance of the two hallmark lesions, with Aβ clearance initiating the removal of tau pathology.

We next investigated the time course by which both pathologies reemerged following the antibody treatment. Accordingly, 3xTg-AD mice were sacrificed 30 and 45 days after a single anti-Aβ antibody injection. By 30 days, the Aβ pathology had reemerged and the level of staining was comparable between the ipsilateral and contralateral hippocampi (cf. Figures 4K and 4L; Figure 5B). Notably, the tau pathology had not yet reemerged at this time point in the ipsilateral hippocampus, despite dense tau staining in the contralateral hippocampus (cf. Figures 4O and 4P; Figure 5B). By day 45 after antibody treatment, the level of tau staining between the ipsilateral and contralateral hippocampi is comparable, indicating that the tau pathology reemerges between days 31 and 45 (cf. Figures 4W and 4X; Figure 5C).

These findings indicate that there is a hierarchical pattern to the clearance and reemergence of the Aβ and tau neuropathology following antibody treatment. The Aβ load is reduced prior to the tau burden, and conversely, the Aβ pathology reemerges well in advance of the tau pathology. Further corroborating a hierarchical relationship between Aβ and tau is our previous finding that, despite the equivalent expression levels of the APP and tau transgenes in this model, the Aβ pathology precedes the development of the tau pathology (Oddo et al., 2003b). Taken together, these data provide compelling evidence for the amyloid cascade hypothesis, that Aβ lies upstream of tau in the neurodegenerative cascade leading to AD, and further suggest that Aβ-based therapeutic approaches may be efficacious in removing both hallmark lesions.

Clearance of Tau Is Dependent on Its Phosphorylation State

Because hyperphosphorylation of tau is a critical step in the pathway leading to neurofibrillary pathology, we determined whether the Aβ-mediated clearance of tau was dependent on its phosphorylation state. At 12 months of age, homozygous 3xTg-AD mice show extensive tau hyperphosphorylation in CA1 pyramidal neurons and are immunopositive for tau antibodies MC1, AT8, and AT180 (Oddo et al., 2003b); MC1 recognizes conformational changes in tau, an early requirement for tangle formation (Weaver et al., 2000), whereas the phospho-specific tau antibodies, AT8 and AT180, recognize tau phosphorylated at serine 202/threonine 205 and at threonine 231, respectively. In contrast, comparable age-matched hemizygous mice are immunonegative for AT8 and AT180 (Figure 6H; Oddo et al., 2003a). Consequently, we assessed whether hyperphosphorylated tau aggregates were diminished by the Aβ-based immune approach. Although the Aβ deposits were effectively

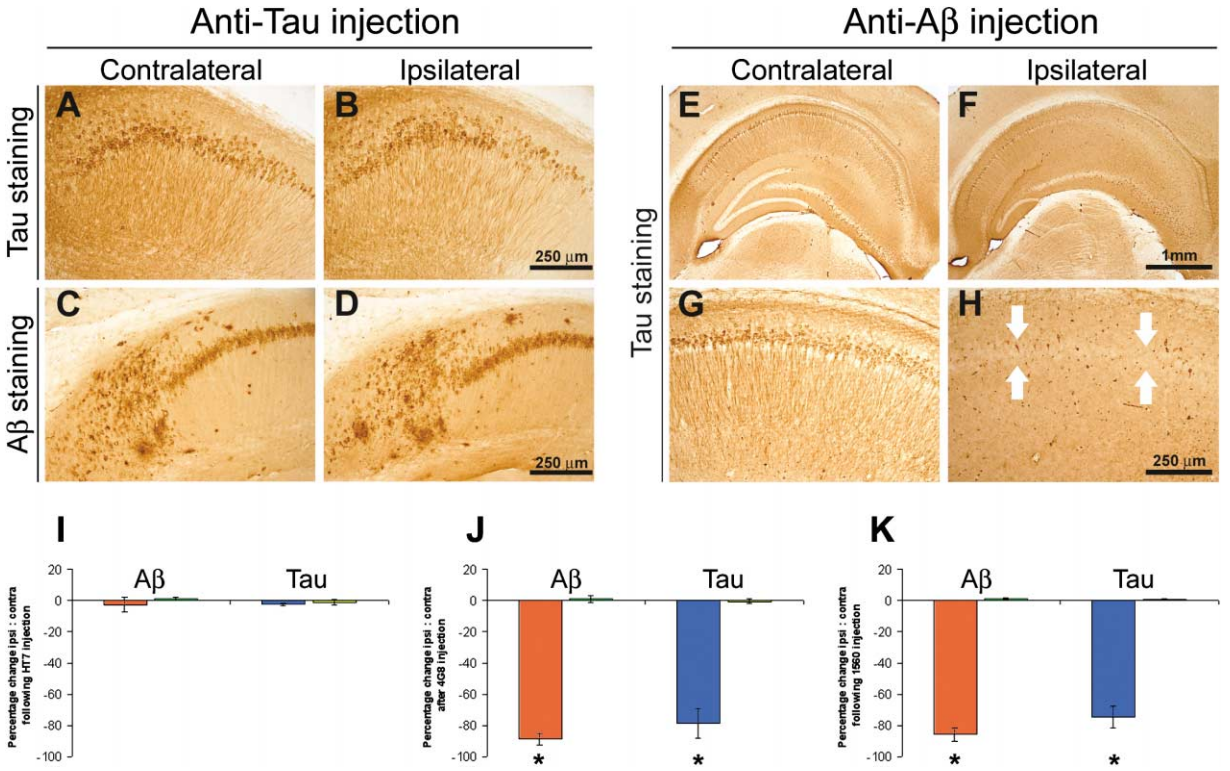

Figure 2. Clearance of Tau Aggregates in CA1 Pyramidal Neurons after Administration of Anti-Aβ Antibodies

(A–D) Intrahippocampal injection of the human-specific anti-tau antibody, HT7, had no observable effect on either tau, as determined by HT7 immunostaining (cf. A and B), or Aβ load, as determined by 1560 immunostaining (cf. C and D).

(E–H) Low- and high-magnification views of the hippocampus/CA1 pyramidal layer demonstrating that a single intrahippocampal administration of the anti-Aβ antibody, 1560, effectively reduced the tau burden in the ipsilateral hippocampus (F and H), compared to the contralateral hippocampus (E and G). Arrows in (H) denote the CA1 pyramidal neuron layer.

(I–K) Quantitative assessment of the percentage change in the Aβ and tau load after intrahippocampal injection of the tau antibody HT7 (I) or the Aβ antibodies 4G8 (J) or 1560 (K). Following administration of the anti-tau antibody (I), no significant difference was apparent between the percentage of decrease of the ipsilateral:contralateral site within the injection site and the percentage decrease of the ipsilateral:contralateral site 10 sections away from the injection site for both Aβ and tau ($p = 0.4815$ and $p = 0.3182$). In contrast, following administration of either anti-Aβ antibody (J and K), the percentage of decrease of the ipsilateral:contralateral site within the injection site and the percentage decrease of the ipsilateral:contralateral site 10 sections away from the injection site was significant for both Aβ and tau (for both antibodies 1560 and 4G8, $p < 0.0001$ for Aβ and tau). Red and blue bars denote the Aβ and tau load, respectively, in areas surrounding the injection site compared to the same region in the contralateral site. Green and yellow bars denote the Aβ and tau load, respectively, in comparable areas 10 sections away from the injection site.

cleared by a single Aβ antibody injection in the 12-month-old homozygous mice, only a small, but significant, reduction in the tau load was observed, likely due to the clearance of nonhyperphosphorylated tau (Figures 6A–6G). Note that the Gallyas silver method reveals that the tau load is comparable between the ipsi- and contralateral sections, indicating that aggregated tau was unaffected by the antibody treatment (Figures 6E and 6F).

Two factors could account for the differential clearance of tau in the hemizygous versus homozygous mice: (1) hyperphosphorylation renders the tau aggregates resistant to Aβ-mediated removal, or (2) there is a higher tau load in the homozygous mice, which express twice as much tau transgene compared to the hemizygous mice. To distinguish between these possibilities, we administered Aβ antibody into the hippocampus of 6-month-old homozygous 3xTg-AD mice. At 6 months of age, the homozygous mice do not show evidence of tau hyperphosphorylation (similar to the 12-month-old hemizygous 3xTg-AD mice), yet steady-state levels of the human tau transgene product are comparable between 6- and 12-month-old homozygous mice (Figures 6J and 6K). Following intrahippocampal administration of the anti-Aβ antibody in 6-month-old homozygous mice, both Aβ and tau pathology were cleared (Figure 7). In contrast, 12-month-old homozygous mice show only a small decrease in the tau load (Figures 6C–6G). Therefore, we conclude that the removal of pathological tau from the somatodendritic compartment is critically dependent on its phosphorylation state: hyperphosphorylated tau aggregates appear to be resistant to Aβ-mediated clearance. By extension, our results suggest that Aβ-based immune approaches may effectively clear both Aβ and tau lesions provided that it is administered early during the disease course.

Mechanism of Immune-Mediated Clearance of Tau Is Dependent on the Proteasome

While novel, the finding that the tau pathology was cleared by antibodies targeted against Aβ raised the question as to the mechanism underlying this process.

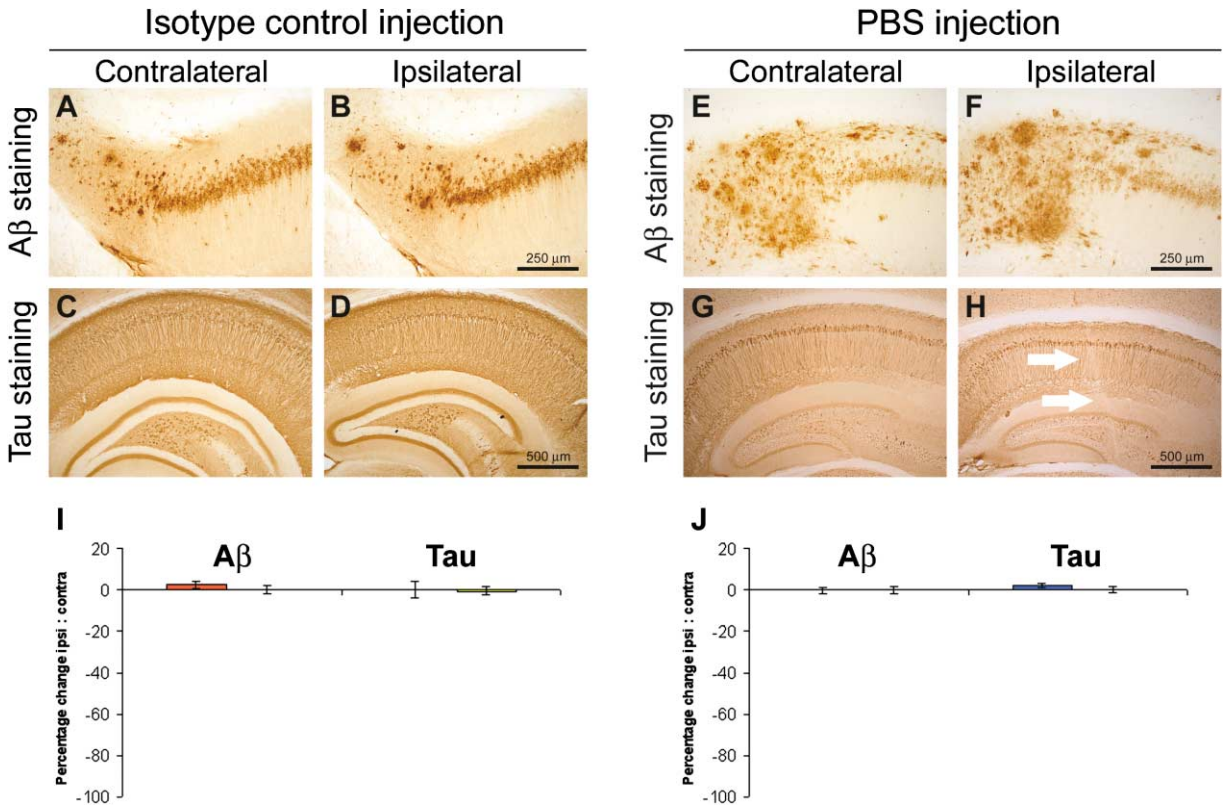

Figure 3. Aβ and Tau Load Were Unaffected after Intrahippocampal Injection of an Isotype Control Antibody or PBS

(A–D) Aβ (A and B) and tau (C and D) immunoreactive structures were labeled to the same extent in the contralateral and ipsilateral hippocampus following administration of an anti-protozoan antibody (isotype-control).

(E–H) Intrahippocampal administration of PBS had no effect on the Aβ (E and F) or tau (G and H) load. Arrows in (H) denote the cannula track.

(I and J) Quantitative assessment of the percentage change in the Aβ and tau load after intrahippocampal injection of an isotype control antibody (I) or PBS (J). Red and blue bars denote the Aβ and tau load, respectively, in areas surrounding the injection site compared to the same region in the contralateral site. Green and yellow bars denote the Aβ and tau load, respectively, in comparable areas about 10 sections away from the injection site. No significant changes were denoted between the percentage of decrease of the ipsilateral:contralateral site within the injection site and the percentage decrease of the ipsilateral:contralateral site 10 sections away from the injection site for both Aβ and tau ([I], $p = 0.4407$ and $p = 0.8901$ for Aβ and tau, respectively; [J], $p = 0.9692$ and $p = 0.3503$ for Aβ and tau, respectively).

To address this issue, we first determined whether the Aβ antibody treatment affected the steady-state levels of the APP holoprotein in the injected hippocampi. As shown in Figures 8D–8G, the levels of the APP holoprotein were comparable between the injected and uninjected hippocampus, indicating that the Aβ antibody treatment lowered the Aβ burden without affecting APP transgene levels.

Several reports indicate that the activity of the proteasome can be adversely affected by Aβ and hyperphosphorylated tau (Gregori et al., 1995, 1997; Keck et al., 2003). Consequently, we investigated whether proteasome activity was essential to the clearance of the tau pathology in the 3xTg-AD mice by injecting the proteasome inhibitor, epoxomicin (Lindsten et al., 2003), concomitantly with the Aβ antibody, 1560. As expected, this dual treatment had little effect on the clearance of the Aβ pathology (Figures 9A–9D and 9I), as its clearance is mediated by an immune-based reaction. However, inhibiting proteasome activity adversely affected the clearance of tau (Figures 9E–9I). Note that the level of tau staining is comparable between the uninjected contralateral side and the ipsilateral side. Quantitative assessment indicated there was no difference in the level of tau immunoreactivity between the contra- and ipsilateral sides, despite a significant difference for Aβ (Figure 9I). Although we cannot exclude that tau may be removed by other mechanisms, we conclude that Aβ interferes with proteasome activity and that its removal via an antibody-mediated process alleviates this impairment, allowing for the clearance of the tau pathology.

Inhibiting Aβ Formation Also Leads to Clearance of Tau Pathology

We demonstrated that Aβ immunotherapy leads to the clearance of pathological tau from the somatodendritic compartment of neurons. The effect is specific for anti-Aβ antibodies as anti-tau and isotype control antibodies and vehicle-only injections failed to have any overt effect. Nevertheless, we utilized an unrelated approach to determine if reducing the Aβ load also affected the pathological tau burden. The γ-secretase inhibitor, DAPT, has previously been shown to lower Aβ levels within 12 hr following a single injection into transgenic mice (Dovey et al., 2001; Lanz et al., 2003). We intrahippocampally injected this compound and found that there was a marked reduction in the Aβ and tau burden in the ipsilateral side, compared to the contralateral hippocampi

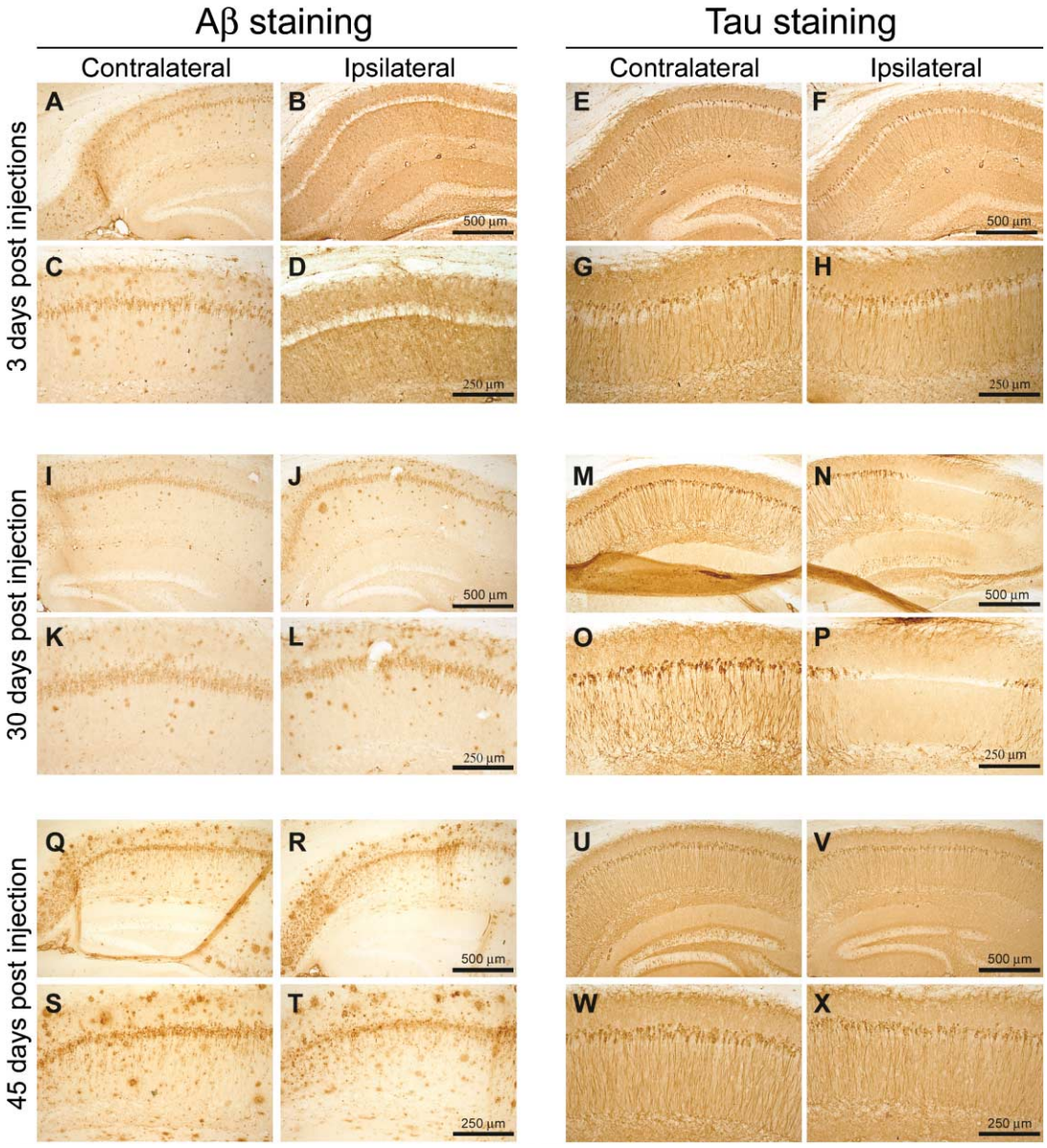

Figure 4. Clearance and Reemergence of Aβ and Tau Pathologies Is Hierarchical

(A–H) Aβ clearance occurs prior to the removal of the tau aggregates in 12-month-old hemizygous 3xTg-AD mice. 3 days after anti-Aβ antibody administration, Aβ staining was reduced in the ipsilateral hippocampi (B and D), whereas the contralateral hippocampi were unaffected (A and C). The tau burden was not cleared at 3 days postinjection as the contralateral and ipsilateral sides were stained to comparable levels (cf. G and H). The tau burden, however, was markedly reduced in anti-Aβ injected hippocampi at 5 and 7 days postinjection (data not shown and Figures 2F and 2H).

(I–X) Aβ pathology reemerges prior to tau pathology. 30 days after a single anti-Aβ antibody injection, reemergence of Aβ immunostaining is apparent in the ipsilateral hippocampus (cf. K and L), whereas tau immunostaining is still not apparent in areas surrounding the injection site (cf. O and P). At 45 days after a single anti-Aβ antibody injection, the level of Aβ (Q–T) and tau (U–X) immunostaining are comparable between the ipsilateral and contralateral sides.

(Figure 10). These results show that blocking the formation of Aβ leads to the clearance of early tau pathology. These data provide strong corroborating evidence of the immunotherapeutic results presented earlier and further support a direct relationship between Aβ and tau pathology.

Discussion

In this study, we demonstrate that a single intrahippocampal injection of an anti-Aβ antibody rapidly and markedly reduces extracellular as well as intracellular Aβ. To our knowledge, this is the first report to document clearance of intracellular Aβ pools following Aβ-based immunotherapy. Although it remains to be established if intracellular Aβ is the precursor to the extracellular deposits (see review article by Tseng et al., 2004), the antibody-mediated clearance of intracellular Aβ from pyramidal neurons suggests that there is a dynamic relationship between the intracellular and extracellular pools. It is important to note that the reduction in intracellular Aβ was not caused by a decrease in the levels

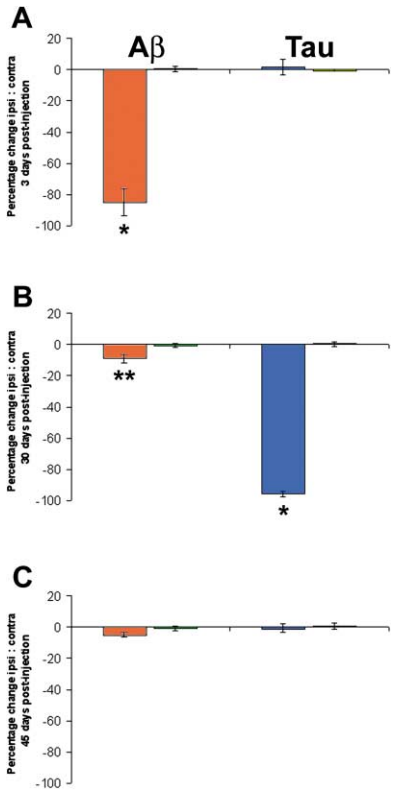

Figure 5. Quantitative Assessment of Aβ and Tau Pathology at 3, 30, and 45 Days after Antibody Administration

(A) At 3 days after antibody injection, there is a significant decrease in the Aβ load ($p = 0.0006$), whereas tau load was not significantly different ($p = 0.7149$).

(B) At 30 days after antibody administration, the difference between percentage of decrease of the ipsilateral:contralateral site within the injection site and the percentage decrease of the ipsilateral:contralateral site 10 sections away from the injection was still significant for Aβ ($p = 0.0135$) but greatly attenuated compared to the difference observed 3 days (A) and 7 days after antibody administration, indicating that Aβ pathology is reemerging.

(C) At 45 days after antibody administration, neither the Aβ or tau burden appeared significantly different between the ipsi- and contralateral sides ($p = 0.1332$ and 0.6572, respectively).

Red and blue bars denote the Aβ and tau load, respectively, in areas surrounding the injection site compared to the same region in the contralateral site. Green and yellow bars denote the Aβ and tau load, respectively, in comparable areas about 10 sections away from the injection site.

of the APP holoprotein. Overall, the finding that intracellular Aβ may be cleared may have important therapeutic implications for the degenerating muscle disorder inclusion body myositis, which is characterized by the occurrence of intracellular (but not extracellular) Aβ accumulation in affected myofibers (Mendell et al., 1991). The findings presented here demonstrate that an Aβ-based immunotherapeutic approach may clear intracellular Aβ in muscle cells as effectively as was shown for neurons.

The clearance of early tau pathology from the somatodendritic compartment of CA1 pyramidal neurons of the 3xTg-AD brain, following Aβ immunotherapy or γ-secretase inhibitor treatment, is the most significant finding to emerge from these studies, and to our knowledge represents the first report of their clearance from the mammalian brain. This finding is significant because the mislocalization of tau from the axon to the somatodendritic compartment represents a critical and presumably early event in the development of tauopathies. It is notable that tau immunotherapy was ineffective at reducing the tau burden, whereas effective clearance occurred following administration of anti-Aβ antibodies or a γ-secretase inhibitor. These data provide strong evidence supporting a direct link between Aβ and tau pathology in AD. The mechanism underlying the Aβ-mediated clearance of tau is dependent on proteasome function, as inhibiting its activity prevents tau clearance.

Proteasome Dysfunction in Alzheimer's Disease

The proteasome is a multiunit catalytic complex that is responsible for degrading misfolded or damaged proteins (Goldberg, 2003). Dysfunction of the proteasome system in the CNS may lead to the intraneuronal accumulation of potentially toxic aggregates, which subsequently may cause neuron degeneration and death. Several studies have reported a decrease in proteasome activity in the AD brain (Keller et al., 2000; Lopez Salon et al., 2000). The reason for diminished proteasome activity is unclear, although several authors have suggested that Aβ or tau-paired helical filaments may underlie this effect (Gregori et al., 1995, 1997; Keck et al., 2003). Based on the hierarchical pattern of clearance reported here, we suggest that intraneuronal accumulation of Aβ impairs proteasome function and allows the accumulation of potentially toxic aggregates such as aggregation-prone tau protein. Clearance of Aβ (including the intracellular species) presumably alleviates this impairment, allowing tau to be cleared by the proteasome. Furthermore, the results presented here show that the Aβ-mediated clearance of tau is critically dependent on its phosphorylation state, with hyperphosphorylated tau aggregates resistant to clearance. This finding agrees well with other in vitro studies demonstrating that nonhyperphosphorylated tau can be degraded by the proteasome system (David et al., 2002; Goldbaum et al., 2003).

Aβ and Tau Interactions In Vivo

These two hallmark lesions are cleared in a hierarchical fashion, with the clearance of Aβ preceding the abrogation of the tau burden. Likewise, the Aβ deposits reemerge in advance of the tau pathology. Taken together, these results strongly suggest a link between Aβ and tau. These findings also provide strong supporting evidence for the amyloid cascade hypothesis, which stipulates that Aβ accumulation triggers the onset of AD and that tau hyperphosphorylation, subsequent neurofibrillary tangle formation, and cell death are downstream consequences of Aβ aggregation (Hardy and Selkoe, 2002). Mounting evidence, including reports from other in vivo studies, suggests that the Aβ and tau pathologies are linked. Lewis et al. (2001) reported enhanced tau pathology after crossing a transgenic line overexpressing mutant APP with another, independent line overexpressing mutant tau. Likewise, Gotz et al. (2001) showed that injection of fibrillar Aβ into the brains of transgenic mice overexpressing tau_{P301L} markedly increased the number of neurofibrillary tangles in the amygdala. Our laboratory has also shown that overexpression of human APP in transgenic mice can modulate the neurofibrillary

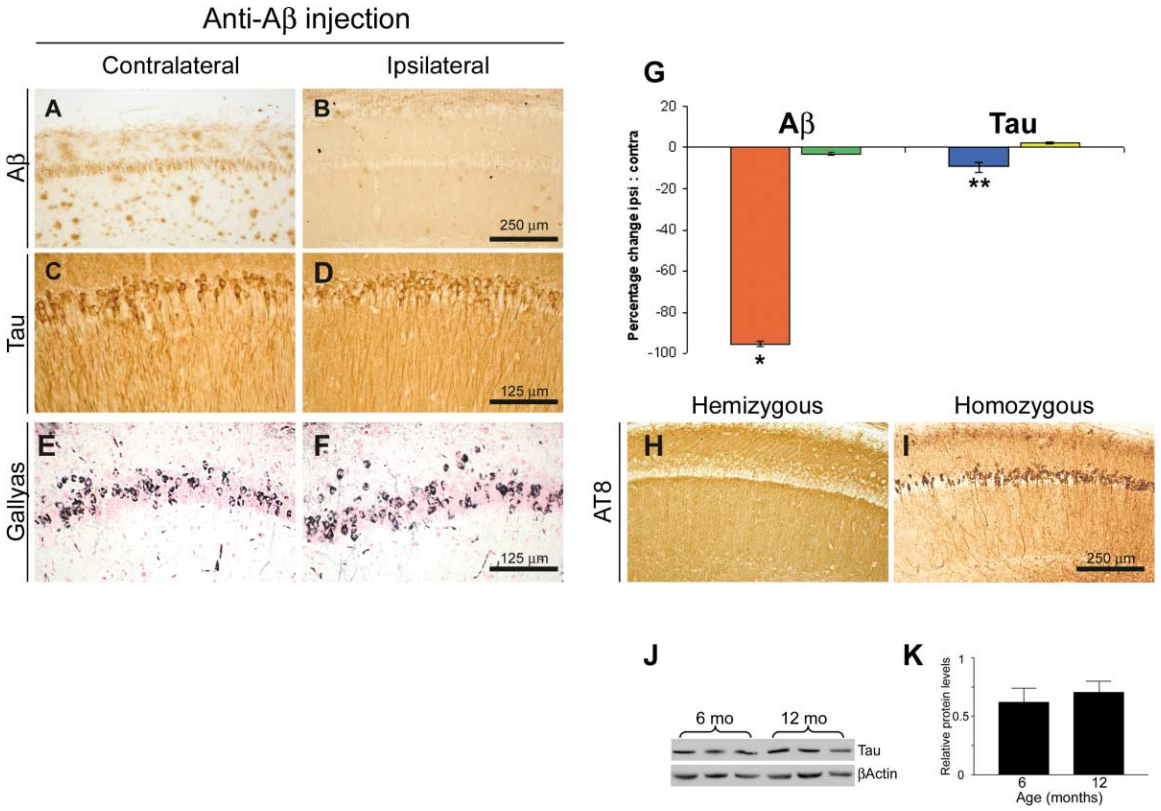

Figure 6. Hyperphosphorylated Tau Aggregates Are Resistant to Aβ-Mediated Clearance

(A and B) Both intracellular and extracellular Aβ deposits are cleared from the hippocampal regions of 12-month-old homozygous 3xTg-AD mice following administration of an anti-Aβ antibody ($n = 5$).

(C–F) Administration of an anti-Aβ antibody did not appear to clear tau aggregates from the 12-month-old homozygous 3xTg-AD mice as determined by HT7 immunostaining (C and D) and Gallyas silver staining (E and F).

(G) Quantitative assessment of the percentage change in the Aβ and tau load after intrahippocampal injection of the Aβ antibody 1560 into the brains of 12-month-old homozygous 3xTg-AD mice. The percentage of decrease of Aβ between the ipsilateral: contralateral site within the injection site and the percentage decrease of the ipsilateral:contralateral site 10 sections away from the injection site was statistically significant. Despite the prominent immunostaining of tau in the ipsilateral side (D), there was also a small but statistically significant reduction, presumably due to clearance of nonhyperphosphorylated tau. *$p < 0.0001$; **$p = 0.0011$. Red and blue bars denote the percentage change in Aβ and tau load, respectively, in areas surrounding the injection site compared to the same region in the contralateral site. Green and yellow bars denote the Aβ and tau load, respectively, in comparable areas about 10 sections away from the injection site.

(H and I) The disparity in tau clearance between the hemizygous and homozygous mice is likely due to the disparity in the hyperphosphorylation state of tau; AT8-positive tau staining is readily apparent in 12-month-old homozygous mice (I) but devoid in age-matched hemizygous mice (H) and in 6-month-old homozygous mice (not shown).

(J) Representative Western blot of tau steady-state levels in 6- and 12-month-old homozygous mice.

(K) Quantitative analysis reveals no significant differences in tau steady-state levels in 6- and 12-month-old homozygous mice.

pathology, as double transgenic mice ($PS1_{M146V/M146V}$ KI/tau_{P301L}) develop tau pathology much later than the 3xTg-AD mice, despite equivalent expression of the tau transgene in both models (Oddo et al., 2003b). Finally, other groups have also shown that tau is essential for Aβ-induced neurotoxicity (Rapoport et al., 2002; Rissman et al., 2004).

Concluding Remarks

There are several therapeutic implications of this study. The most clinically relevant finding is that using anti-Aβ-based therapeutic approaches can clear the tau burden. Because the clearance of the tau pathology is dependent on its phosphorylation state, it indicates that Aβ immunotherapy late during the disease course may still effectively clear amyloid plaques, although it will be insufficient to impact the neurofibrillary pathology. It is critical to note, however, that only one tau antibody was tested during these studies. Therefore, it is essential that other tau antibodies, including those that recognize hyperphosphorylated and conformation-specific epitopes, be evaluated for their efficacy in removing tau from the mammalian brain. These findings raise the intriguing possibility that a multiantibody-based approach (i.e., one targeted against Aβ and one against tau) may provide the most significant clinical benefit for the treatment of AD. Finally, we conclude that the Aβ-mediated clearance of tau from the 3xTg-AD brains suggests that tau aggregates are also in a dynamic state and that immune- or pharmacologic-based strategies may also be effective for treating tauopathies, such as corticobasal degeneration, frontotemporal dementia with Parkinsonism

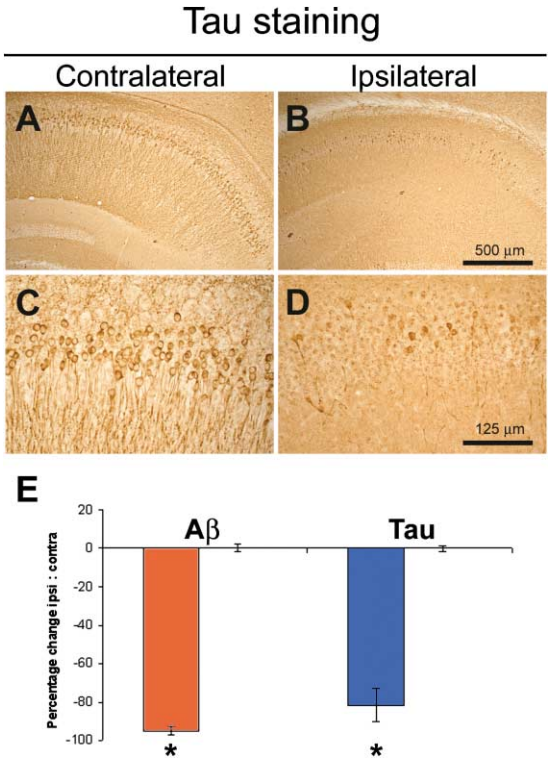

Figure 7. Clearance of the Tau Burden from Young Homozygous 3xTg-AD Mice by Aβ Immunotherapy

(A–D) Administration of the anti-Aβ antibody leads to a marked reduction in the tau burden in the hippocampus of 6-month-old homozygous 3xTg-AD mice. At this age, tau is not extensively hyperphosphorylated. These studies show that the increased steady-state levels of tau in the homozygous (versus hemizygous) mice do not represent a hindrance to clearance by Aβ-based immunotherapy. These findings support the hypothesis that the phosphorylation state of tau is a critical determinant in its ability to be cleared by anti-Aβ antibodies.

(E) Quantitative assessment of the percentage change in the Aβ and tau load after intrahippocampal injection of the Aβ antibody 1560 into the brains of 6-month-old homozygous 3xTg-AD mice. For both Aβ and tau, the percentage of decrease in the ipsilateral:contralateral site within the injection site differed significantly from the percentage decrease of the ipsilateral:contralateral site 10 sections away from the injection site (Aβ: $p < 0.001$; tau: $p = 0.0007$). Red and blue bars denote the Aβ and tau load, respectively, in areas surrounding the injection site compared to the same region on the contralateral side. Green and yellow bars denote the Aβ and tau load, respectively, in comparable areas about 10 sections from the injection site.

linked to chromosome 17, and progressive supranuclear palsy.

Experimental Procedures

Mice and Surgical Procedures

The 3xTg-AD mice used in this study have already been characterized (Oddo et al., 2003a, 2003b). Male and female 3xTg-AD mice weighing 25–49 g at the time of surgery were group housed and kept on a 12 hr light:12 hr dark schedule. All mice were given ad libitum access to food and water. Surgeries were carried out during the light cycle. Homozygous and hemizygous mice were anesthetized with avertin (1.3% tribromoethanol, 0.8% amylalcohol, given 0.6 ml/25 g body weight) and placed in a stereotactic apparatus (MyNeuroLab, St. Louis, MO) with a mouse adaptor. Antibodies (2 μl) or PBS vehicle were injected into the left hippocampus through a 33-gauge injector attached to a 5 μl Hamilton syringe (Hamilton Company, Reno, NV). The coordinates, with respect to bregma, were −2.7 mm posterior, +2.5 mm lateral, and −3.0 ventral to the skull. Injections occurred over the span of 5 min, after which the cannula was left in place for an additional 5 min to allow for diffusion. Animals were kept on a warming pad until they had fully recovered from anesthesia and were kept in individual cages until they were sacrificed for tissue processing to prevent damage to the scalp sutures. All animal procedures were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and all appropriate measures were taken to minimize pain and discomfort in experimental animals.

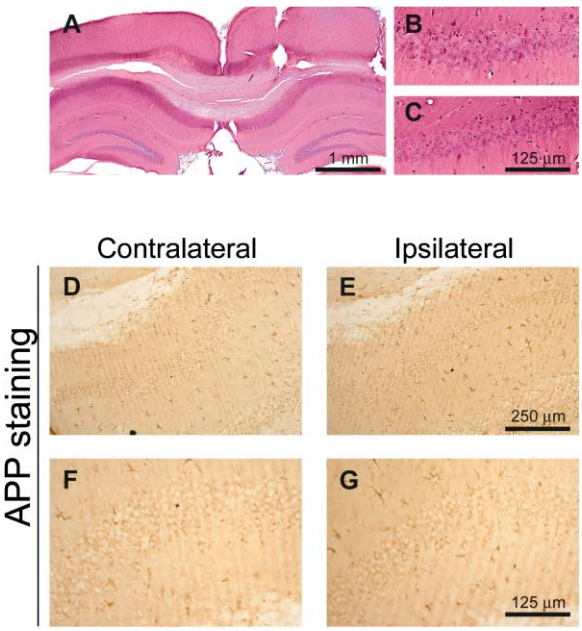

Figure 8. Clearance of Aβ/Tau Is Not Due to Neuronal Damage, nor to Changes in APP Steady-State Levels

(A–C) Hematoxylin-eosin staining shows that the CA1 pyramidal neurons appear morphologically unaltered in both the ipsilateral and contralateral hemispheres, indicating that clearance was specific for the anti-Aβ antibody treatment and not due to unintentional damage by the injection procedure. (B) and (C) show high-magnification views of the contra- and ipsilateral subiculum/CA1 region, respectively.

(D–G) Steady-state levels of APP were comparable between the ipsi- and contralateral hippocampi after a single injection of an anti-Aβ antibody, indicating that the clearance of Aβ is not likely due to diminished formation from APP. (F) and (G) show higher-magnification views of (D) and (E).

Antibodies and Inhibitors

The following antibodies were injected during the surgeries: anti-Aβ antibodies 1560 (1 μg/μl) (Chemicon) or 4G8 (1 μg/μl) and anti-tau HT7 (1 μg/μl) (Innogenetics). The following antibodies were used for immunohistochemistry and Western blotting: anti-Aβ 6E10 and 4G8 (Signet Laboratories, Dedham, MA), anti-Aβ 1560, anti-Tau HT7, AT8 (Innogenetics), Tau 5 (Calbiochem), and anti-actin (Sigma). Primary antibodies were applied at dilutions of 1:1000 for 6E10, 1:3000 for 1560, 1:500 for AT8 and AT180, 1:1000 for HT7, and 1:200 for 22C11. The proteasome inhibitor epoxomicin (Sigma) was intrahippocampally injected (15 μg) in combination with anti-Aβ antibody 1560. The γ-secretase inhibitor, DAPT (Calbiochem), was also injected into the hippocampus.

Immunohistochemistry

Mice were sacrificed by CO_2 asphyxiation and the brains were fixed for 48 hr in 4% paraformaldehyde. 50 μm thick free-floating sections

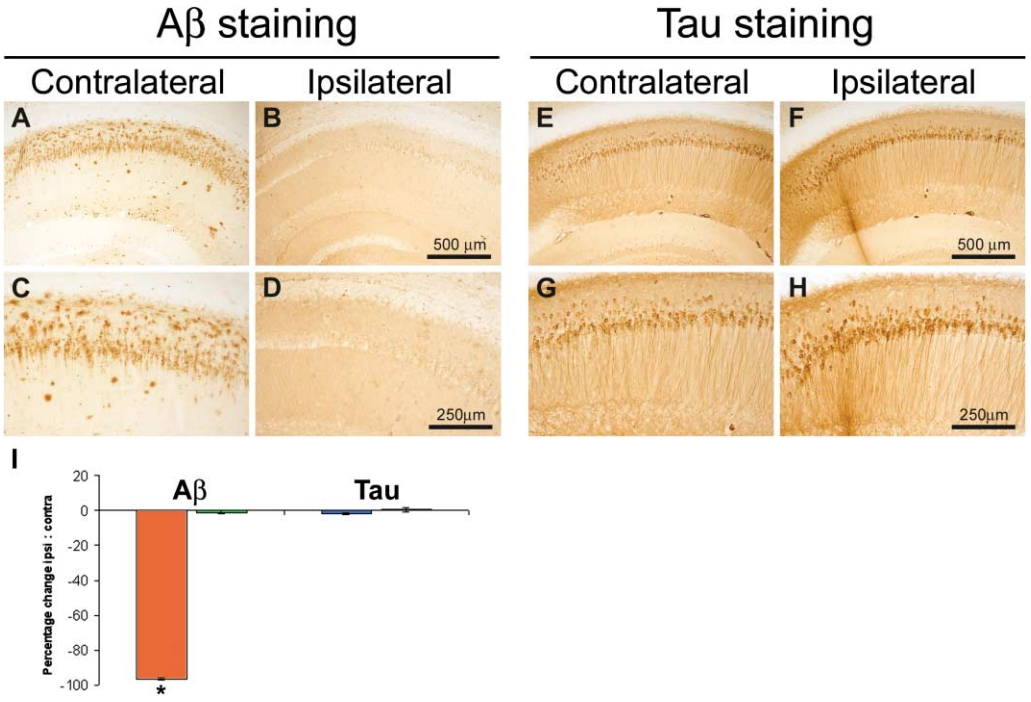

Figure 9. Clearance of Tau Is Dependent on Proteasome Activity

(A–D) Immunotherapy-mediated clearance of Aβ is not dependent on the proteasome. The proteasome inhibitor, epoxomicin, and the monoclonal antibody 1560 were concomitantly injected into the hippocampus of 12-month-old hemizygous mice. Even in the presence of the proteasome inhibitor, Aβ levels were significantly reduced.

(E–H) Inhibiting the proteasome prevents Aβ-mediated clearance of tau. Unlike the case for Aβ, there was no significant clearance of tau in the presence of the proteasome inhibitor.

(I) Quantitative assessment of the percentage change in the Aβ and tau load following concomitant intrahippocampal injection of the Aβ antibody 1560 and the proteasome inhibitor epoxomicin, into the brains of 12-month-old hemizygous 3xTg-AD mice. For Aβ, the percentage of decrease in the ipsilateral:contralateral site within the injection site differed significantly from the percentage decrease of the ipsilateral:contralateral site 10 sections away from the injection site ($p < 0.0001$). For tau, however, no significant reduction was apparent ($p = 0.1351$). These findings indicate that clearance of tau pathology is dependent on the proteasome, whereas Aβ clearance is not. Red and blue bars denote the Aβ and tau load, respectively, in areas surrounding the injection site compared to the same region on the contralateral side. Green and yellow bars denote the Aβ and tau load, respectively, in comparable areas about 10 sections away from the injection site.

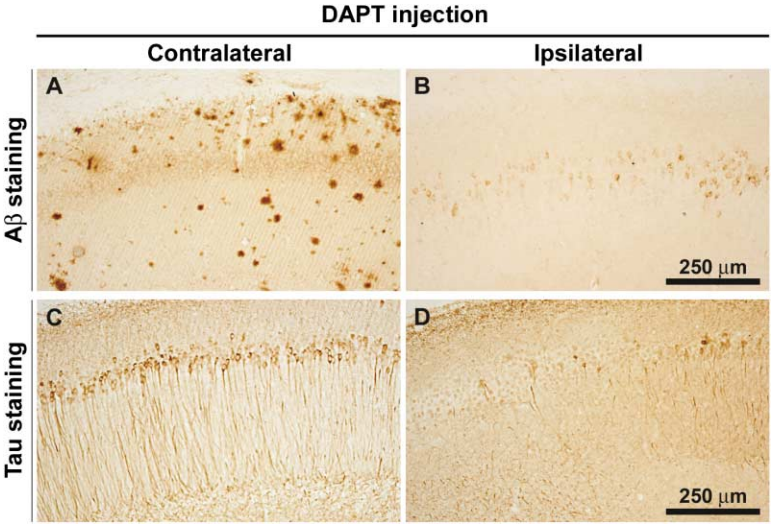

Figure 10. Inhibiting Aβ Formation also Leads to the Clearance of Tau Pathology

The γ-secretase inhibitor, DAPT, was administered via intrahippocampal injection into 12-month-old hemizygous 3xTg-AD mice ($n = 3$). Representative microphotographs of the hippocampus/CA1 pyramidal cell layer show that a single administration of DAPT is sufficient to markedly reduce both Aβ and tau pathology.

were obtained using a vibratome slicing system (Pelco) and stored in PBS. The endogenous peroxidase activity was quenched for 30 min in 0.3% H_2O_2. Sections were then incubated in 90% formic acid for 7 min to expose the epitope. The appropriate primary antibody was applied overnight at 4°C. Sections were developed with diaminobenzidine (DAB) substrate using the avidin-biotin horseradish peroxidase system (Vector Labs).

Immunoblot

For immunoblot, brains from transgenic and control mice were dounce homogenized in a solution of 2% SDS in H_20 containing 0.7 mg/ml Pepstatin A supplemented with complete Mini protease inhibitor tablet (Roche 1836153) and phosphatases inhibitors (Invitrogen). The homogenized mixes were briefly sonicated to sheer the DNA and then centrifuged at 4°C for 1 hr at 100,000 × g. The supernatant was used for immunoblot analysis. Proteins were resolved by SDS/PAGE (10% Bis-Tris from Invitrogen) under reducing conditions and transferred to nitrocellulose membrane. The membrane was incubated in a 5% solution of nonfat milk for 1 hr at 20°C. After overnight incubation at 4°C with the primary antibody, the blots were washed in tween-TBS for 20 min and incubated at 20°C with the secondary antibody. The blots were washed in T-TBS for 20 min and incubated for 5 min with Super Signal (Pierce).

Aβ and Tau Quantification

Using a Zeiss digital camera, microphotographs were taken of the hippocampal area surrounding the injection site and similar areas 10 sections away from the injection site. The contralateral side was processed in parallel. Photomicrographs were imported into the Scion Image system (NIH) and converted to black and white images. Threshold intensity was manually set and kept constant, and the number of pixels were determined for both Aβ- and tau-immunostained sections. To obtain the percentage difference between the ipsilateral and the contralateral hippocampi, we applied the following formula: (number of pixels in the ipsilateral hippocampus − number of pixels in the contralateral hippocampus)/number of pixels in the contralateral hippocampus.

The data were subsequently analyzed by ANOVA or t test comparison, using Graphpad Prism software.

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