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Neurobiology of Aging 34 (2013) 137–145

NEUROBIOLOGY OF AGING

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Immunization targeting a minor plaque constituent clears β-amyloid and rescues behavioral deficits in an Alzheimer's disease mouse model

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Received 10 January 2012; received in revised form 30 March 2012; accepted 15 April 2012

Abstract

Although anti-human β-amyloid (Aβ) immunotherapy clears brain β-amyloid plaques in Alzheimer's disease (AD), targeting additional brain plaque constituents to promote clearance has not been attempted. Endogenous murine Aβ is a minor Aβ plaque component in amyloid precursor protein (APP) transgenic AD models, which we show is ~3%–8% of the total accumulated Aβ in various human APP transgenic mice. Murine Aβ codeposits and colocalizes with human Aβ in amyloid plaques, and the two Aβ species coimmunoprecipitate together from brain extracts. In the human APP transgenic mouse model Tg2576, passive immunization for 8 weeks with a murine-Aβ-specific antibody reduced β-amyloid plaque pathology, robustly decreasing both murine and human Aβ levels. The immunized mice additionally showed improvements in two behavioral assays, odor habituation and nesting behavior. We conclude that passive anti-murine Aβ immunization clears Aβ plaque pathology—including the major human Aβ component—and decreases behavioral deficits, arguing that targeting minor endogenous brain plaque constituents can be beneficial, broadening the range of plaque-associated targets for AD therapeutics.

Keywords: Alzheimer's disease; Aβ; Codeposition; Immunization; Immunotherapy

1. Introduction

The β-amyloid (Aβ) plaque, a pathological hallmark of Alzheimer's disease (AD), consists primarily of aggregated Aβ peptide and is central to the pathobiology of the disease. Aβ-depositing amyloid precursor protein (APP) transgenic (tg) mice are an important experimental system in which to evaluate AD therapies, such as inhibitors of Aβ generation (Citron, 2010) or Aβ immunotherapy (Lemere and Masliah, 2010; Schenk et al., 1999). Human Aβ-directed active and passive immunization clears Aβ plaques in both mouse models (Schenk et al., 1999) and human patients (Bayer et al., 2005; Nicoll et al., 2003), although cognitive improve-

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0197-4580/$ – see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.neurobiolaging.2012.04.007

ment has not been seen in clinical trials (Holmes et al., 2008). This is in contrast with immunotherapy targeting constituents of peripheral amyloids such as liver and spleen amyloidoses, where substantial clearance of the amyloid leads to reduced disease pathology (Bodin et al., 2010). In our study, we use an analogous therapeutic approach for brain Aβ by targeting a minor endogenous brain component of plaques, murine Aβ, in tg mouse models to reduce Aβ plaque pathology and rescue behavioral deficits.

2. Methods

2.1. Transgenic and knockout mice

We used the following APP- and/or presenilin 1 (PS1)-overexpressing tg lines: TgPS1 ($PS1_{M146L}$ line 6.2) (Duff et al., 1996), Tg2576 (APP with the "Swedish" mutation or $APP_{K670N,\ M671L}$Hsiao et al., 1996), Tg2576/PS1 (APP "Swedish" + $PS1_{M146L}$) (Holcomb et al., 1998), TgCRND8 (APP "Swedish" + the "Indiana" mutation or $APP_{K670N,\ M671L\ +\ V717F}$) (Chishti et al., 2001), APPDutch (APP3 or APP_{E693Q}) (Herzig et al., 2004), APPDutch/PS45 ($APP_{E693Q} \times PS1_{G384A}$) (Herzig et al., 2004), and APPwt (APP51/16) (Herzig et al., 2004). The age at which Aβ deposition begins in each tg line varies from ~3 months (TgCRND8, Tg2576/PS1, and APPDutch/PS45) to ~8 months (Tg2576), to as long as ~18 or 23 months (APPwt and APPDutch lines, respectively). We also used an APP-knockout (APP-ko) model (Zheng et al., 1995). Experiments were conducted according to Nathan S. Kline Institute Animal Care and Use Committee guidelines. Ages of the mice are given in Supplemental Fig. 1B.

2.2. Brain processing and Western blot analysis

Ten percent (w/v) homogenates were prepared from mouse hemibrains lacking the olfactory bulb and cerebellum, and from cortical tissue from human control and AD brains, and used for biochemical analyses as previously described (Schmidt et al., 2005a). Contralateral hemibrains were sectioned by vibratome for histological examinations. Using an aliquot of the homogenates, soluble APP (sAPP) was isolated from membrane-associated APP by centrifugation at 100,000 × *g* (Schmidt et al., 2005a); using separate homogenate aliquots, formic acid or diethylamine (DEA) was used to extract plaque-associated Aβ or soluble endogenous murine Aβ in non-tg mice, respectively (Schmidt et al., 2005b). For Western blot analysis, polyvinylidene difluoride membranes were incubated with antibodies against APP metabolites, as previously described (Morales-Corraliza et al., 2009). A rabbit polyclonal anti-GFAP antibody (Sigma-Aldrich, St. Louis, MO, USA) was also used for Western blotting. Independently run and probed Western blots are shown in Fig. 2 and Supplemental Fig. 1; multiple mice were analyzed by Western blot in Figs. 1A, 2A and Supplemental Fig. 1.

2.3. ELISA

After formic acid or diethylamine extraction, human and murine Aβ quantification was performed by enzyme-linked immunosorbent assay (ELISA), as previously described (Schmidt et al., 2005b). Briefly, monoclonal antibodies JRF/cAβ40/10 or JRF/cAβ42/26 were used to capture Aβ ending at residue 40 or 42, respectively. Monoclonal antibody JRF/Aβtot/17 was used to detect human Aβ, and monoclonal antibody JRF/rAβ1–15/2 was used to detect murine Aβ (ELISA antibodies are a gift from Marc Mercken, Janssen Pharmaceutica, Beerse, Belgium) (Rozmahel et al., 2002a, 2002b). In Supplemental Fig. 1B, "human Aβ" was calculated as human Aβ40 + Aβ42, and "murine Aβ" as murine Aβ40 + Aβ42.

2.4. Immunohistochemistry

Formalin-fixed hemibrains were cut by vibratome into 40-μm-thick free-floating sections (Cataldo and Nixon, 1990). Aβ plaque was visualized with JRF/Aβtot/17, JRF/rAβ1–15/2, and Thioflavin S staining and quantified using the program AxioVision 4.6 (Carl Zeiss, Jena, Germany) (Mi et al., 2007). For antibody binding competition, a 10-fold molar excess of murine Aβ40 was preincubated with the antibody (Mathews et al., 2000). The rabbit polyclonal anti-GFAP (Sigma-Aldrich, St. Louis, MO, USA) and an anti-ubiquitin antibody (Dako, Carpinteria, CA, USA) were used for the immunolabeling shown in Fig. 2.

2.5. Immunization

In-house murine-Aβ-specific monoclonal antibody m3.2 (Morales-Corraliza et al., 2009) or, as control, NT1 monoclonal antibody, also an immunoglobulin G1 (IgG1) that does not recognize any murine protein (Mathews et al., 2000), were administered by weekly intraperitoneal injection (400 μg in saline) for 8 weeks in 20-month-old Tg2576 and non-tg mice. Brain-deposited human and murine Aβ levels after m3.2 immunotherapy were determined by ELISA using formic acid-extracted Aβ as described above. Amyloid plaque burden was visualized by Thioflavin S staining as described (Mi et al., 2007).

2.6. Behavioral studies

(a) Odor habituation test—Mice were individually housed in clean plastic cages and were screened for olfactory deficits (Sundberg et al., 1982; Wesson et al., 2010; Wilson and Linster, 2008). Four odors (2-heptanone, isoamyl acetate, limonene, and ethyl valerate; Sigma Aldrich, St. Louis, MO, USA) were diluted in mineral oil (dilution of 1:100, 1:100, 1:50, 1:100, respectively) and applied to a cotton-applicator stick enclosed in a piece of odorless plastic tubing. Odors were delivered for four consecutive trials of 20 seconds each, with 30-second intertrial intervals, by inserting the odor stick into a port on the side of the animal's home cage. (b) Nesting behavioral test—Based on

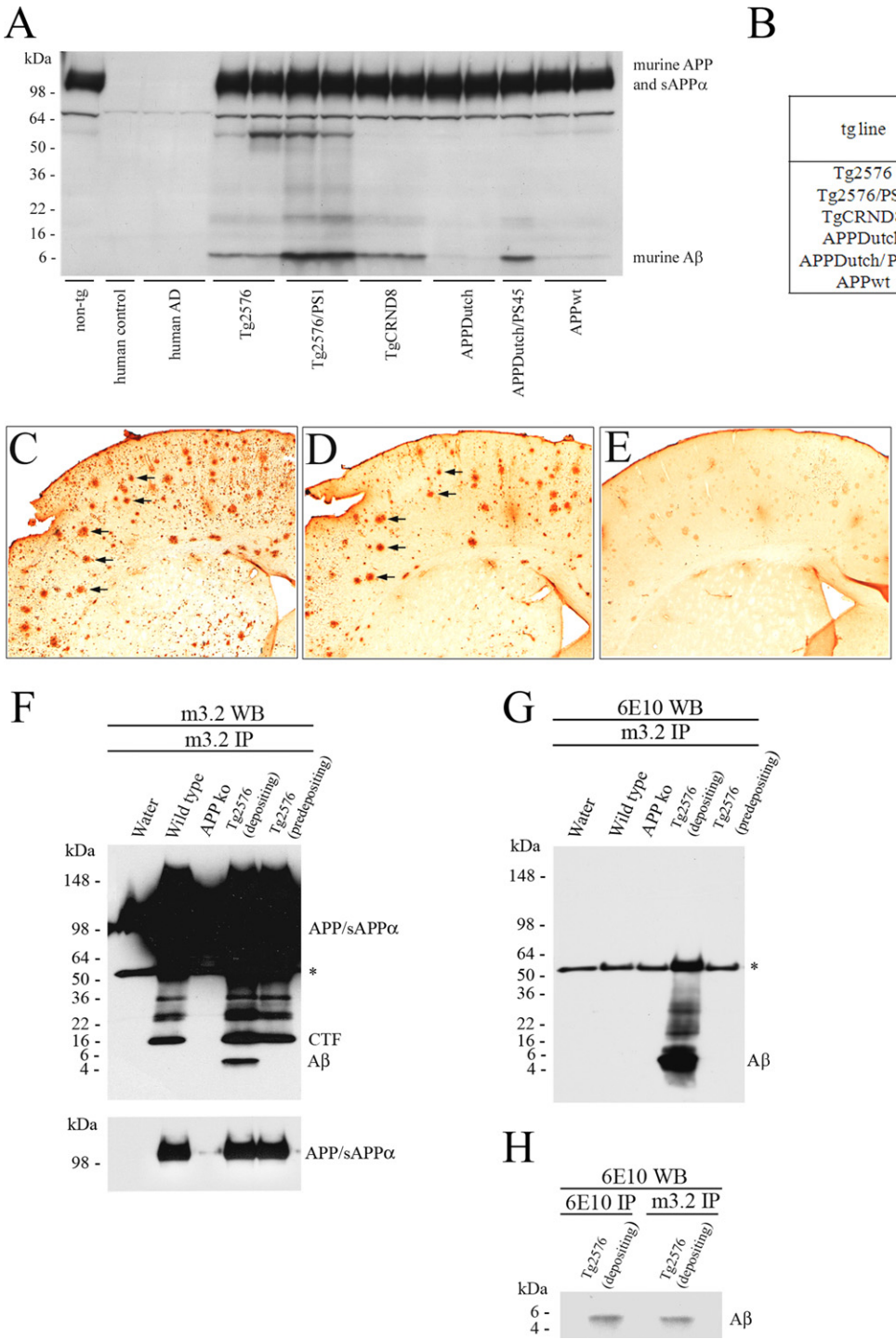

Fig. 1. Codeposition of murine and human Aβ in Aβ-depositing mice. (A) Endogenous murine APP, sAPPα, and Aβ levels in various APP- and/or PS1-overexpressing tg lines and non-tg mice (see Methods for details) as detected by Western blot analysis of brain homogenates with antibody m3.2. Antibody m3.2 showed no reactivity with human control and AD brain tissue, in agreement with the specificity of this antibody for murine APP metabolites. (B) Percentage of murine Aβ of total Aβ (human + murine Aβ) accumulating in the brains of these tg lines as determined by ELISA. (C–E) Serial brain coronal sections were immunolabeled either with human-Aβ-specific antibody (C) or murine-Aβ-specific antibody (D) (with arrows marking colabeled plaques) or with an addition of a 10-fold molar excess of murine Aβ to the murine-Aβ-specific antibody-binding solution (E). (F–H) The murine-Aβ-specific antibody m3.2 was used to immunoprecipitate murine Aβ from brain homogenates of wild-type, APP ko, 16-month-old Tg2576 (depositing), and 4-month-old Tg2576 (predepositing) mice. m3.2-immunoprecipitation products were analyzed by Western blots probed with m3.2 for murine Aβ (F) or with 6E10 for human Aβ (G). The relative amount of human Aβ immunoprecipitated with 6E10 or coimmunoprecipitated with m3.2 is shown by 6E10 Western blot analysis (H). The asterisk (*) indicates nonspecific reactivity. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

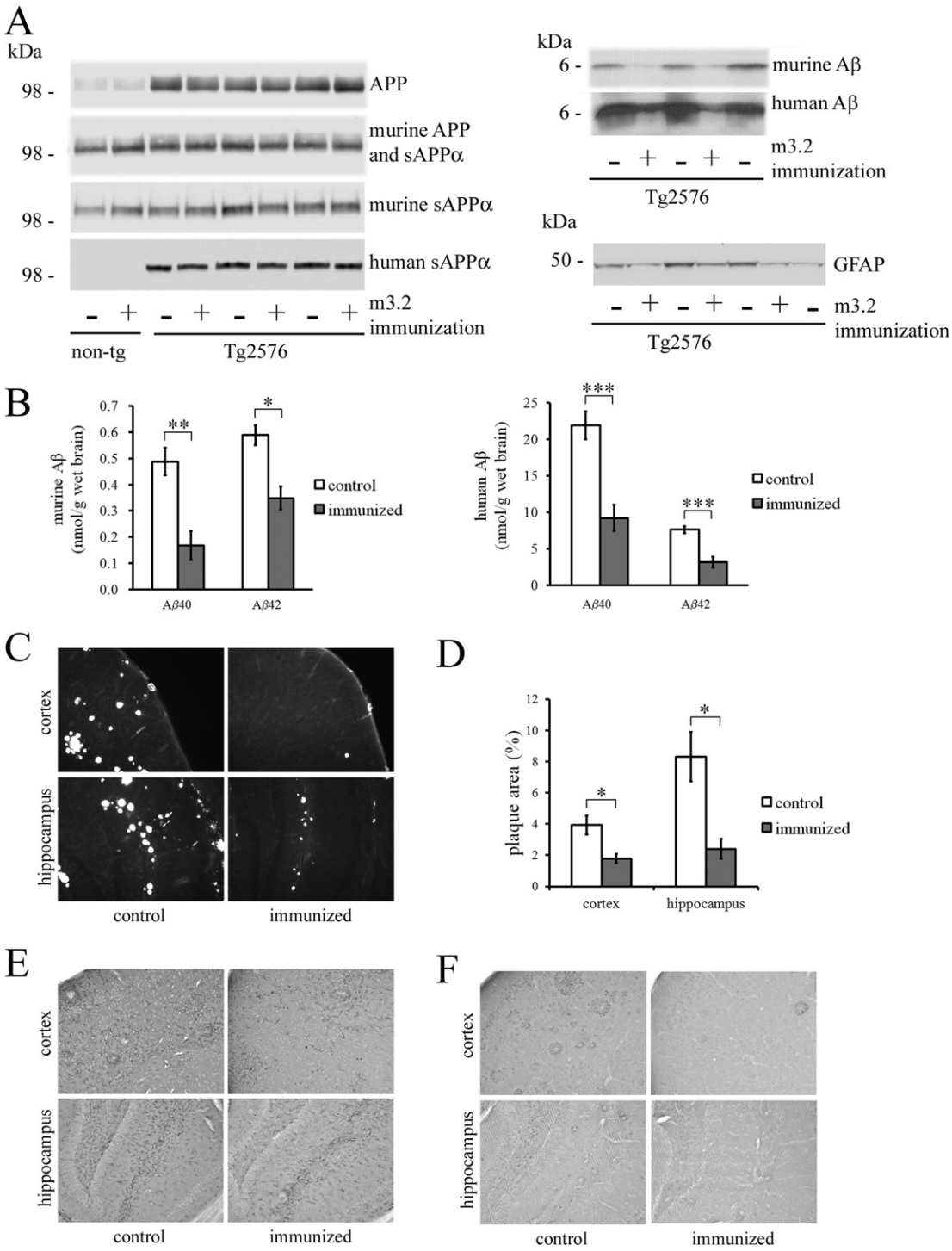

Fig. 2. Human and murine APP metabolite levels and Aβ plaque burden in the brains of Tg2576 mice after m3.2 immunotherapy. (A, left) Western blot analysis of total APP (both human and murine; detected by C1/6.1 from homogenate samples), both murine APP and sAPPα (detected by m3.2 from homogenate samples), murine sAPPα alone (detected by m3.2 from diethylamine (DEA)-extracted samples), and human sAPPα levels (detected by 6E10 from DEA-extracted samples) in brains of Tg2576 and non-tg mice injected with either m3.2 or control antibody. (A, right) Western blots of brain homogenates probed with m3.2 or 6E10 showing murine and human Aβ levels, respectively. Brain GFAP expression is shown by Western blot analysis in the bottom right. (B) ELISA measurement of formic acid-extracted murine Aβ40 and murine Aβ42 (left graph) and human Aβ40 and human Aβ42 (right graph) in Tg2576 mouse brains in m3.2-injected mice (n = 7) compared with controls (n = 6). (C) Thioflavin S staining of Aβ plaques in the cortex (top) and hippocampus (bottom) of Tg2576 mouse brains, comparing m3.2-injected (right) with control antibody-injected mice (left). (D) Quantification of Thioflavin S-positive plaque area in these mice (n = 7, m3.2-injected mice and n = 6, controls). Immunolabeling with anti-GFAP (E) and anti-ubiquitin (F) antibodies of representative brain coronal sections showing the cortex and hippocampus of m3.2-injected Tg2576 mice compared with control antibody-injected mice. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

the failure to construct nests of Tg2576 compared with non-tg mice (Wesson and Wilson, 2011), nest construction abilities were analyzed weekly for 8 weeks immediately after immunization. Mice were individually housed in clean plastic cages with ten pieces of paper towels overnight. Paper towel nest construction was scored the following morning along a 4-point system: (1) no biting/tearing with random dispersion of the paper, (2) no biting/tearing of paper with gathering in a corner/side of the cage, (3) moderate biting/tearing on paper with gathering in a corner/side of the cage, and (4) extensive biting/tearing on paper with gathering in a corner/side of the cage (see Supplemental Fig. 1I)

2.7. Statistical analysis

Western blots were quantitated using ImageJ (rsb.info.nih.gov). ELISA measurements were assessed using the nonparametric Mann–Whitney *U* test. All data were plotted with GraphPad Prism v.5 (GraphPad Software, San Diego, CA, USA) for statistical analysis. The method of estimation of the sampling distribution throughout this study was the standard error of the mean (mean ± SEM).

3. Results and discussion

The concurrence of human and murine Aβ in amyloid plaques in APP tg mice has been reported (Jankowsky et al., 2007; Pype et al., 2003), but the extent to which the peptides from the two species are integrated remains unclear. *In vitro*, synthetic murine Aβ has been shown to aggregate and form fibrils, although whether murine Aβ aggregates *in vivo* and initiates plaque deposition independent of human Aβ is not known (Fung et al., 2004). Here, by Western blot analysis with our in-house murine-Aβ-specific antibody m3.2 (Morales-Corraliza et al., 2009), we assess murine Aβ, APP, and sAPPα levels in the brains of different APP- and/or PS1-overexpressing tg lines (Fig. 1A) (see Methods for details). Although murine APP and sAPPα levels showed no differences between tg and non-tg mice, murine Aβ accumulation in amyloid plaques in these tg lines is evidenced by the presence of an ~4-kDa Aβ band (Fig. 1A; see also Supplemental Fig. 1A). We analyzed human and murine Aβ levels by ELISA in these tg lines (see Supplemental Fig. 1B), and, although the absolute amount of both human and murine Aβ varied considerably among the various tg models, murine Aβ levels comprised approximately 5% (range: 2.5–7.7%) of the total Aβ (murine + human Aβ) (Fig. 1B). Thus, our data argue that, within a broad range of Aβ accumulation in tg mice expressing various pro-amyloidogenic human APP mutations, the ratio of codeposited murine Aβ remains at approximately 1/20th of the total Aβ that accumulates within the brain. No brain accumulation of murine Aβ was seen with aging in wild-type mice (Supplemental Fig. 1B). Immunolabeling of serial brain sections from Tg2576 mice with either human-Aβ-specific antibody (Fig. 1C) or murine-Aβ-specific antibody (Fig. 1D) showed colabeling of plaques; murine Aβ labeling was blocked by preincubation of the antibody with murine Aβ peptide (Fig. 1E). Immunolabeling showing plaque-associated murine Aβ in additional tg mouse models is shown in Supplemental Fig. 1C–H. To further assess the interaction between human and murine Aβ in the brains of tg mice, we performed coimmunoprecipitation (IP)-Western blot analysis (Fig. 1F–H). Antibody m3.2 was used to immunoprecipitate murine Aβ from brain homogenates of wild-type, APP ko, and Tg2576 (depositing and predepositing) mice. Antibody m3.2 Western blot analysis of the m3.2 IP products revealed abundant murine Aβ in the depositing Tg2576 mouse brain but not in any of the other samples (Fig. 1F). Human-Aβ-specific Western blot analysis using antibody 6E10 of the same IP products detected coimmunoprecipitated human Aβ in depositing Tg2576 mice (Fig. 1G). The human-APP-specific 6E10 did not detect murine APP (as seen in Fig. 1G probed with 6E10), nor was human APP coimmunoprecipitated using the m3.2 antibody, arguing that the human/murine Aβ interaction detected by this technique is specific and unique to Aβ. Additionally, the relative amount of human Aβ directly immunoprecipitated by 6E10 or through its interaction with murine Aβ by coimmunoprecipitation with m3.2 was found to be similar (Fig. 1H). Our findings demonstrate an extensive and integral association between human and murine Aβ in the brain that is maintained through the coimmunoprecipitation.

Given this close association of murine and human Aβ in the APP tg mouse brain, we tested whether passive immunization with the murine-APP/Aβ-specific antibody m3.2 could reduce Aβ pathology in Aβ-depositing mice (Fig. 2). We administered weekly intraperitoneal injections of the antibody for 8 weeks to 20-month-old Tg2576 and non-tg mice. Western blots of brain homogenates probed with m3.2 or 6E10 antibodies (Fig. 2A, right) showed that both murine and human Aβ were significantly decreased after passive immunization, whereas murine APP and sAPPα were unaltered, suggesting a specificity of the m3.2 immunization for plaque-associated Aβ (Fig. 2A, left). Additionally, ELISA measurements of formic acid-extracted murine Aβ40, murine Aβ42, human Aβ40, and human Aβ42 showed significantly decreased levels of both human and murine Aβ in the m3.2-injected Tg2576 mice compared with control antibody-injected mice: murine Aβ40 levels decreased 65.3% ± 10.2%, murine Aβ42 levels decreased 40.7% ± 6.8%, human Aβ40 levels decreased 58.0% ± 8.7%, and human Aβ42 levels decreased 57.9% ± 9.2% (Fig. 2B). Thioflavin S staining of brain tissue sections showed significant clearance of amyloid plaques in both cortex (Fig. 2C, top) and hippocampus (Fig. 2C, bottom) when comparing m3.2-injected mice (right) with controls (left). Quantification of amyloid area of brain coronal sections is shown in Fig. 2D: amyloid plaque area decreased 54.4% ± 15.0% in cortex and decreased

71.0% ± 19.0% in hippocampus in m3.2-injected mice compared with controls. Thus, these results following passive immunization with m3.2 in aged plaque-containing Tg2576 mice are consistent with clearance via an integral association in plaques of the murine Aβ with human Aβ. Western blot and immunolabeling analyses with an anti-glial fibrillary acidic protein (GFAP) antibody showed a decrease in GFAP levels in brain homogenates (Fig. 2A and 2E), consistent with a reduction in reactive glia in the immunized mice (Simpson et al., 2010). Similarly, an anti-ubiquitin antibody (Fig. 2F), which labeled the neuropil adjacent to Aβ plaques in both cortex and hippocampus in the Tg2576 mouse brains, showed reduced labeling in the m3.2-injected mice. Thus, the mice immunized with the anti-murine Aβ antibody show a decrease in astrogliosis and dystrophic neurites (Perry et al., 1987), consistent with a reduction in Aβ.

To determine whether m3.2 immunization affects behavioral deficits that have been previously described in Tg2576 mice (Hsiao et al., 1996; Wesson et al., 2010; Wesson and Wilson, 2011), two behavioral assays were performed: odor habituation and nesting behavior tests. AD pathology results in impaired olfactory perceptual acuity in both humans (Doty, 1991; Murphy, 1999) and Tg2576 mice (Wesson et al., 2010). We determined that after 8 weeks, m3.2-injected Tg2576 mice show odor habituation behavior similar to that of non-tg mice, with an increase of 2.3-fold of the percentage odor habituation compared with control IgG-injected Tg2576 mice (comparison of odor habituation percentage: 61.0% ± 9.6% in m3.2-injected Tg2576 mice, 26.3% ± 6.3% in control IgG-injected Tg2576 mice, 66.2% ± 13.3% in m3.2-injected non-tg mice, and 72.8% ± 4.9% in control IgG-injected non-tg mice) (Fig. 3A). Tg2576 mice fail to construct nests, a complex goal-directed behavior requiring multiple brain regions (Wesson and Wilson, 2011) that can be assessed repeatedly during a treatment period. Consistent with the odor habituation findings, m3.2-injected Tg2576 mice show a significant improvement in nesting behavior when compared with control-injected Tg2576 mice (comparison of nesting behavior at week 8: 2.3 ± 0.2 in m3.2-injected Tg2576 mice, 1.5 ± 0.2 in control-injected Tg2576 mice, 2.8 ± 0.6 in m3.2-injected non-tg mice, and 3.2 ± 0.3 in control-injected non-tg mice) (Fig. 3B) based upon a 4-point scoring system of nest construction (see Methods and Supplemental Fig. 1I). Therefore, in addition to reducing Aβ pathology, passive anti-murine/endogenous-Aβ antibody therapy rescued two highly distinct behavioral deficits in Tg2576 mice after only 2 months of intraperitoneal injections.

Given that only ~5% of the total Aβ is murine Aβ, it is striking that an antibody directed at this small pool is adequate to robustly clear the total Aβ burden without apparent preferential clearance of the murine peptide, arguing that m3.2-mediated clearance of Aβ from the brain involves the co-removal of associated murine and human Aβ. The models of Aβ clearance by immunization that have been proposed include the direct disruption of plaques, microglial phagocytosis and clearance of antibody-bound Aβ (DeMattos et al., 2001; Schenk et al., 1999; Wilcock et al., 2003), and the "peripheral sink" concept in which antibody outside the CNS binds Aβ, promoting its redistribution from the brain to the periphery (DeMattos et al., 2001; Zotova et al., 2010). Presumably the strong association of the human and murine peptides allows a robust clearance of both Aβs by an anti-murine-Aβ antibody, demonstrating that clearance of an endogenous peptide that comprises only a small fraction of the total heterogeneous Aβ plaque is sufficient to lead to the clearance of the bulk Aβ within the plaque, leading to substantial cognitive improvements. This suggests that, a priori, anti-Aβ immunotherapies need not be necessarily directed at Aβ, but that effective plaque clearance can follow immunotherapies directed at a limited subset of Aβ and/or non-Aβ amyloid plaque components. Such a strategy has been shown to work in animal models of peripheral amyloidosis, where immunotherapies targeting serum amyloid P component—a minor constituent in peripheral amy-

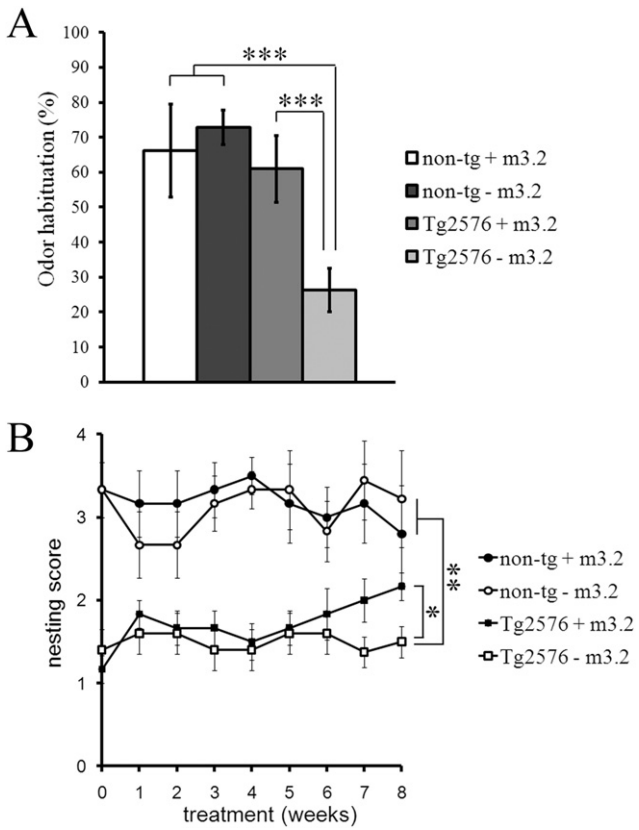

Fig. 3. Rescue of behavioral deficits in Tg2576 mice after m3.2 immunotherapy. (A) Odor habituation measurements of m3.2-injected Tg2576 mice (n = 7) compared with control-injected Tg2576 mice (n = 9). m3.2-injected (n = 6) and control-injected (n = 9) non-tg mice were also analyzed for comparison. (B) Nesting behavioral test analysis of the same groups of mice (see Supplemental Fig. 1). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

loidoses that does not independently form amyloid—is sufficient to clear the major plaque peptide species (Bodin et al., 2010). Potential human-AD targets include proteins that have been shown to occur in plaques such as pyroglutamate Aβ (Saido et al., 1995), cystatin C (Levy et al., 2006), cathepsin B and cathepsin D (Nixon and Cataldo, 2006), Apo E (Namba et al., 1991; Richey et al., 1995; Wisniewski and Frangione, 1992), Apo J (Choi-Miura et al., 1992; Giannakopoulos et al., 1998; McGeer et al., 1992), or specific heparin sulfate glycosaminoglycan and proteoglycans (Leveugle et al., 1994). For example, cystatin C is a minor Aβ plaque constituent found codeposited in human AD and APP tg mouse models, and, like murine Aβ in the tg models, is broadly expressed in peripheral tissues as well as the brain (Gauthier et al., 2011; Levy et al., 2006). We and our colleagues have shown that cystatin C interacts with soluble Aβ (Mi et al., 2009) and that the brain expression of cystatin C can modulate Aβ deposition *in vivo* (Kaeser et al., 2007; Mi et al., 2007), making cystatin C an appealing target for future studies of immunization directed against a minor Aβ-binding protein. A recent study showing that modulation of Apo E expression levels in the brain is sufficient to reduce Aβ pathology is further evidence that manipulating Aβ-binding partners can be an effective strategy for anti-amyloid therapeutics (Cramer et al., 2012). Our study, in which an antibody against an endogenous and minor brain plaque component clears both the direct target (i.e., the murine peptide) as well as the closely associated bulk component (i.e., the human peptide) and at the same time rescues cognitive impairment, suggests that the range of anti-amyloid immunotherapeutic targets that may be available for Alzheimer's disease treatment strategies is more extensive than previously appreciated.

Disclosure statement

All authors disclose: (a) No actual or potential conflicts of interest including any financial, personal, or other relationships with other people or organizations within 3 years of beginning the work submitted that could inappropriately influence (bias) our work. (b) None of the authors' institutions have contracts relating to this research through which they or any other organization may stand to gain financially now or in the future. (c) No other agreements of authors or their institutions that could be seen as involving a financial interest in this work.

Data contained in the manuscript being submitted have not been previously published, have not been submitted elsewhere, and will not be submitted elsewhere while under consideration at *Neurobiology of Aging*.

Statements verifying that appropriate approval and procedures were used concerning human subjects and animals have been included. All experiments involving mice received prior approval from the Nathan Kline Institute Animal Care and Use committee (AP2010-362).

All authors have reviewed the contents of the manuscript being submitted, approve of its contents, and validate the accuracy of the data.

Acknowledgements

We thank Philip Stavrides for his help with the behavioral tests. This work was supported by the Alzheimer's Association (IIRG-07-60047; P.M.M.) and the National Institutes of Health (NS045205, P.M.M.; AG017617, P.M.M., R.A.N., E.L.; DC003906, D.A.W.; AG037693, D.A.W., R.A.N., E.L.).

Appendix. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neurobiolaging.2012.04.007.

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