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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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Abstract

While anti-human-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 β-amyloid plaque component in amyloid precursor protein transgenic AD models, which we show is ~2–8% of the total accumulated Aβ in various human APP transgenic mice. Murine Aβ co-deposits and co-localizes with human Aβ in amyloid plaques and the two Aβ species co-immunoprecipitate together from brain extracts. In the human APP transgenic mice Tg2576, passive immunization for eight weeks with a murine-Aβ-specific antibody reduced β-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 β-amyloid

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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; co-deposition; immunization; immunotherapy

Introduction

The β-amyloid plaque, a pathological hallmark of Alzheimer's disease (AD), consists primarily of aggregated Aβ peptide and is central to the pathobiology of the disease. β-amyloid-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 β-amyloid plaques in both mouse models (Schenk, et al., 1999) and human patients (Bayer, et al., 2005,Nicoll, et al., 2003), although cognitive improvement has not been seen in clinical trials (Holmes, et al., 2008). This is in contrast to 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 β-amyloid by targeting a minor endogenous brain component of plaques, murine Aβ, in tg mouse models in order to reduce β-amyloid plaque pathology and rescue behavioral deficits.

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 brain of different APP and/or PS1 overexpressing tg lines (Figure 1A) (see Materials and Methods for details). While 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 a ~4 kDa Aβ band (Figure 1A; see also Supplemental Figure 1A). We analyzed human and murine Aβ levels by ELISA in these tg lines (see Supplemental Figure 1B) and, while 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) (Figure 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 co-deposited murine Aβ remains at approximately $1/20^{th}$ of the total Aβ that accumulates within the brain. No brain accumulation of murine Aβ was seen with aging in wild-type mice (Supplemental Figure 1B). Immunolabeling of serial brain sections from Tg2576 mice with either human-Aβ-specific antibody (Figure 1C) or murine-Aβ-specific antibody (Figure 1D) showed co-labeling of plaques; murine Aβ labeling was blocked by pre-incubation of the antibody with murine Aβ peptide (Figure 1E). Immunolabeling showing plaque-associated murine Aβ in additional tg mouse models is shown in Supplemental Figure 1C–H. In order to further assess the interaction between human and murine Aβ in the brains of tg mice, we performed co-immunoprecipitation (IP)-

Western blot analysis (Figure 1F–H). Antibody m3.2 was used to immunoprecipitate murine Aβ from brain homogenates of wild-type, APP ko, and Tg2576 (depositing and pre-depositing) 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 (Figure 1F). Human-Aβ-specific Western blot analysis using antibody 6E10 of the same IP products detected co-immunoprecipated human Aβ in depositing Tg2576 mice (Figure 1G). The human-APP-specific 6E10 did not detect murine APP (as seen in Figure 1G probed with m3.2), nor was human APP co-immunoprecipitated 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 co-immunoprecipitation with m3.2 was found to be similar (Figure 1H). Our findings demonstrate an extensive and integral association between human and murine Aβ in the brain that is maintained through the co-immunoprecipitation.

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 β-amyloid pathology in β-amyloid depositing mice (Figure 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 (Figure 2A, right panels) showed that both murine and human Aβ were significantly decreased after passive immunization, while murine APP and sAPPα were unaltered, suggesting a specificity of the m3.2 immunization for plaque associated Aβ. 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 to 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% (Figure 2B). Thioflavin S staining of brain tissue sections showed significant clearance of amyloid plaques in both cortex (Figure 2C, upper panels) and hippocampus (Figure 2C, lower panels) when comparing m3.2-injected mice (right panels) with controls (left panels). Quantification of amyloid area of brain coronal sections is shown in Figure 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 to 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. Analyses with an anti-glial fibrillary acidic protein (GFAP) antibody showed a decrease in GFAP levels in brain homogenates (Figure 2A) and by immunolabeling (Figure 2E), consistent with a reduction in reactive glia in the immunized mice (Simpson, et al., 2010). Similarly, an anti-ubiquitin antibody (Figure 2F), which labeled the neuropil adjacent to β-amyloid 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 β-amyloid.

In order 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 to 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) (Figure 3A). Tg2576 mice fail to construct nests, a complex, goal-directed behaviour 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 of nesting behavior when compared to 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) (Figure 3B) based upon a 4-point scoring system of nest construction (see Materials and Methods, and Supplemental Figure 1I). Therefore, in addition to reducing Aβ pathology, passive anti-murine/endogenous-Aβ antibody rescued two highly distinct behavioral deficits in Tg2576 mice after only two months of intraperitoneal injections.

Given that only ~5% of the total β-amyloid 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 β-amyloid (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 β-amyloid 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-β-amyloid 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 as strategy has been shown to work in animal models of peripheral amyloidosis, where immunotherapies targeting serum amyloid P component – a minor constituent in peripheral amyloidoses 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 β-amyloid plaque constituent found co-deposited 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 β-amyloid 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 β-amyloid 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.

Materials and Methods

Transgenic and knockout mice

We used the following APP and/or PS1 overexpressing tg lines: TgPS1 (PS1M146L 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 β-amyloid 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 noted in Supplemental Figure 1B.

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 Aβ and 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 β-amyloid 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) was also used for Western blotting. Independently run and probed Western blots are shown in Figure 2 and Supplemental Figure 1; multiple mice were analyzed by Western blot in Figures 1A and 2A.

ELISA

Following formic acid or diethylamine extraction, human and murine Aβ quantification was performed by 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 (Rozmahel, et al., 2002a,Rozmahel, et al., 2002b). In Supplemental Figure 1B, "human Aβ" was calculated as human Aβ40 + Aβ42, and "murine Aβ" as murine Aβ40 + Aβ42.

Immunohistochemistry

Formalin-fixed hemibrains were cut by vibratome into 40-μm thick free-floating sections (Cataldo and Nixon, 1990). β-Amyloid plaque was visualized with JRF/Aβtot/17, JRF/rAβ1–15/2 and Thioflavin S staining and quantified using the program AxioVision 4.6 (Mi, et al., 2007). For antibody binding competition, a 10-fold molar excess of murine Aβ40 was pre-incubated with the antibody (Mathews, et al., 2000). The rabbit polyclonal anti-GFAP (Sigma-Aldrich, St. Louis, MO) and an anti-ubiquitin antibody (Dako, Carpinteria, CA) were used for the immunolabeling shown in Figure 1.

Immunization

In-house murine-Aβ-specific monoclonal antibody m3.2 (Morales-Corraliza, et al., 2009) or, as control, NT1 monoclonal antibody, also an 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 following 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).

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) 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 20s each, with 30s inter-trial intervals, by inserting the odor stick into a port on the side of the animal's home cage. (b) Nesting behavioural test. Based in the failure to construct nests of Tg2576 compared to 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 Figure 1I)

Statistical Analysis

Western blots were quantitated using ImageJ (http://rsb.info.nih.gov). ELISA measurements were assessed using the non-parametric 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).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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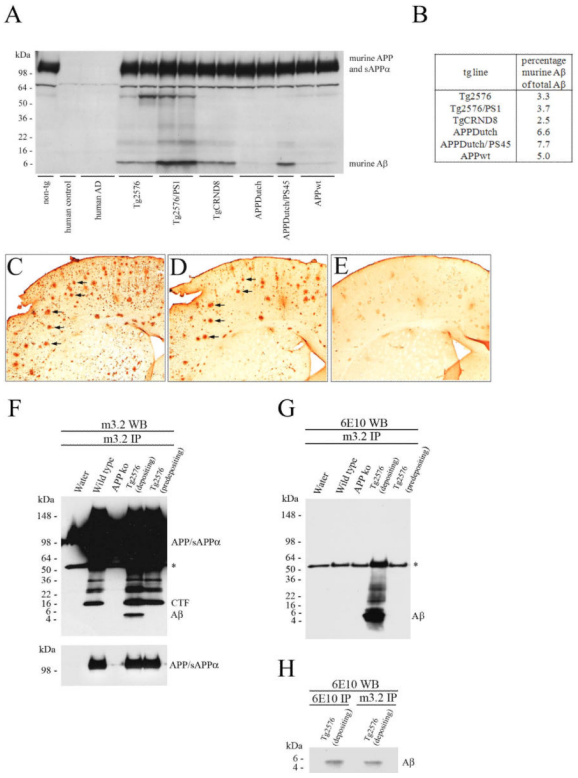

Figure 1. Co-deposition of murine and human Aβ in β-amyloid depositing mice

(A) Endogenous murine APP, sAPPα and Aβ levels in various APP and/or PS1 overexpressing tg lines and non-tg mice (see Materials and 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 co-labeled 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 co-immunoprecipitated with m3.2 is shown by 6E10 Western blot analysis (H). The asterisk (*) indicates non-specific reactivity.

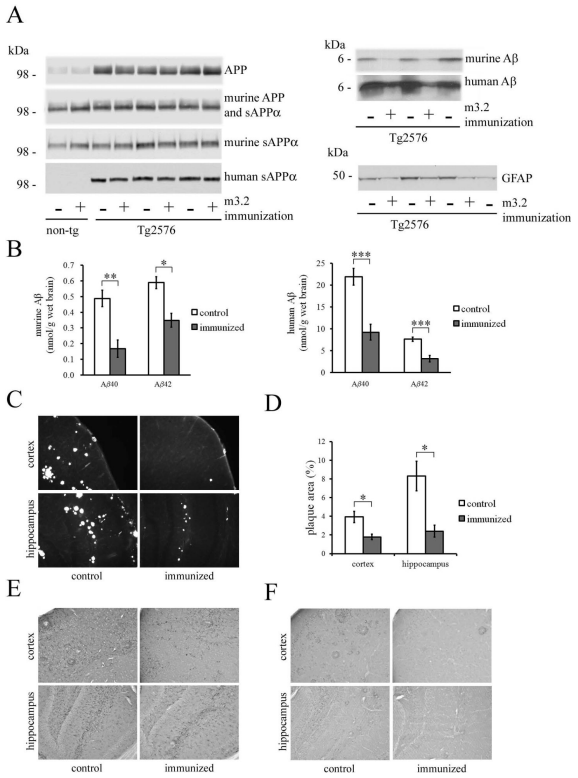

Figure 2. Human and murine APP metabolite levels and Aβ plaque burden in the brains of Tg2576 mice following m3.2 immunotherapy

(A, left panels) 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 panels) 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 panel. (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 to controls (n=6). (C) Thioflavin S staining of amyloid plaques in the cortex (upper panels) and hippocampus (lower panels) of Tg2576 mouse brains, comparing m3.2-injected (right panels) with control-antibody-injected mice (left panels). (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 to control-antibody injected mice. *p<0.05; ** p<0.01; ***p<0.001.

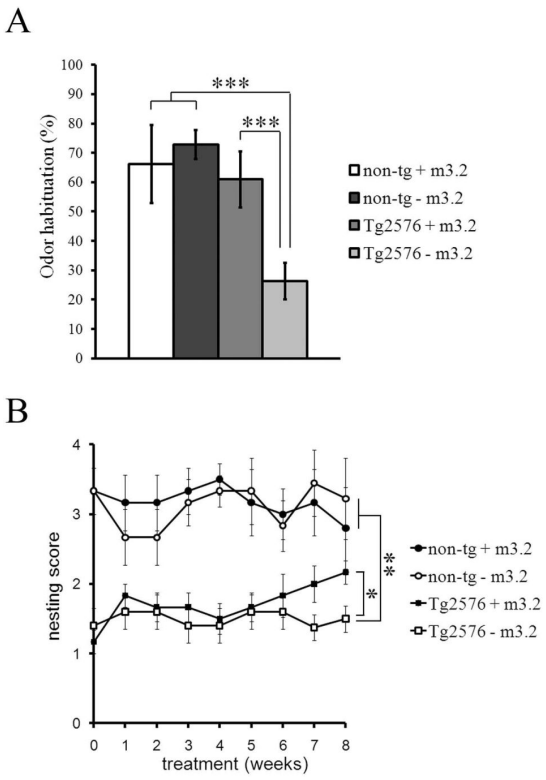

Figure 3. Rescue of behavioral deficits in Tg2576 mice following m3.2 immunotherapy

(A) Odor habituation measurements of m3.2-injected Tg2576 mice (n=7) compared to 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 behavioural test analysis of the same groups of mice (see Supplemental Figure 1). *p<0.05; ** p<0.01; ***p<0.001.