Chronic anti-murine Aβ immunization preserves odor guided behaviors in an Alzheimer's β-amyloidosis model

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Abstract

Olfaction is often impaired in Alzheimer''s disease (AD) and is also dysfunctional in mouse models of the disease. We recently demonstrated that short-term passive anti-murine-Aβ immunization can rescue olfactory behavior in the Tg2576 mouse model overexpressing a human mutation of the amyloid precursor protein (APP) after β-amyloid deposition. Here we tested the ability to preserve normal olfactory behaviors by means of long-term passive anti-murine-Aβ immunization. Seven-month-old Tg2576 and non-transgenic littermate (NTg) mice were IP-injected biweekly with the m3.2 murine-Aβ-specific antibody until 16 months of age when mice were tested in the odor habituation test. While Tg2576 mice treated with a control antibody showed elevations in odor investigation times and impaired odor habituation compared to NTg, olfactory behavior was preserved to NTg levels in m3.2-immunized Tg2576 mice. Immunized Tg2576 mice had significantly less β-amyloid immunolabeling in the olfactory bulb and entorhinal cortex, yet showed elevations in Thioflavin-S labeled plaques in the piriform cortex. No detectable changes in APP metabolite levels other than Aβ were found following m3.2 immunization. These results demonstrate efficacy of chronic, long-term anti-murine-Aβ m3.2 immunization in preserving normal odor-guided behaviors in a human APP Tg model. Further, these results provide mechanistic insights into olfactory dysfunction as a biomarker for AD by yielding evidence that focal reductions of Aβ may be sufficient to preserve olfaction.

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Keywords

Olfaction; Neurodegeneration; Alzheimer's disease; amyloid-beta; APP; immunization

1. Introduction

Olfactory perceptual impairments are commonly reported in Alzheimer's disease (AD). In particular, persons with AD often display reduced abilities to detect, discriminate, and identify odors (for review [1, 2]). These impairments in olfaction are even reported to precede significant cognitive dysfunction [3], highlighting the vulnerability of the olfactory system to the early events of AD and the possible clinical utility of olfactory dysfunction as a biomarker for the disease (e.g., [4, 5]). Understanding the mechanisms of olfactory perceptual loss in AD may help to elucidate general principles of disease pathogenesis and will be critical in treating olfactory dysfunction in the disease.

Olfactory perception requires that odor information originating with the binding of odorants to olfactory receptor neurons in the nose be transferred throughout multiple brain regions essential to odor processing. Following the initial events of odor processing within the olfactory bulb (OB) [6], odor information travels into olfactory cortices, including the piriform cortex (PCX) wherein processes critical for odor habituation and olfactory learning occur [7–12]. Odor information then enters the lateral entorhinal cortex (EC) [13–15] and ultimately the hippocampus (hipp) for odor memory storage and future retrieval [16]. The normal function of this network, which is well conserved through evolution and highly similar in rodent and human [17], is critical for olfactory perception, and indeed disrupting odor information flow throughout any of these regions can impair olfactory perception (e.g., [15, 18–22]).

While the neural basis for olfactory impairments in AD remain unclear, recent work from AD mouse models has suggested a role for amyloid-β (Aβ) in disrupting normal olfactory network function and olfactory behaviors [23–26]. Recent work from our group [26] in the Tg2576 mouse overexpressing human APP with the Swedish familial AD mutation demonstrated that behavioral dysfunction in the odor habituation task positively correlates with levels of fibrillar and non-fibrillar Aβ within olfactory structures, including the OB, PCX, EC, and hipp. Indeed, dysfunction in various olfactory behaviors has been reported in multiple AD model mouse lines [24, 27–30]. More recently, we reported that OB and PCX neural activity is highly aberrant in Tg2576 transgenic mice and that this is restored to near wild type levels following acute pharmacological intervention to lower Aβ levels [23, 25]. Thus, it is likely that Aβ and/or other factors related to APP processing are responsible for decline in olfactory system function. Exploring anti-Aβ strategies as potential therapies against olfactory perturbations in this model may provide insights into mechanisms of sensory decline in AD and its treatment.

We recently demonstrated that acute (short-term) passive anti-murine-Aβ immunization can rescue olfactory behavioral impairments in the Tg2576 mouse model [31]. In this study, 8 week treatment with the anti-murine Aβ antibody, m3.2, which is a monoclonal antibody with a selective affinity for murine Aβ (mAβ) [32], was found to have reduced both brain mAβ and human Aβ (hAβ) levels and also preserved normal odor habituation behaviors in Tg2576 mice when the immunization was begun after significant β-amyloid deposition. As summarized in Table 1, this 8 week treatment study showed that acute (short-term) passive anti-murine-Aβ immunization lowered brain Aβ levels in aged Tg2576 mice without altering other measured APP metabolite levels. However, whether these behavioral changes are accompanied by altered Aβ burden specifically in the olfactory system and whether

similar findings can be observed following chronic treatment beginning at the earliest stages of Aβ deposition remain to be determined. Therefore, here we tested the hypothesis that ongoing anti-mAβ immunization would prevent the deposition of Aβ within the brain, specifically within olfactory structures, and thereby within those very same animals, preserve normal odor habituation behavior.

2. Methods

2.1 Subjects

Male and female mice bred and maintained within the Nathan S. Kline Institute for Psychiatric Research animal facility were used. Tg2576 mice were generated previously [33] by overexpressing the 695-amino acid isoform of human APP containing the K670N-M671L Swedish mutation. Littermate, non-transgenic (NTg) mice were used as controls. Mice were maintained on a 12:12 (light:dark, 0600:1800hrs) day cycle in standard plastic cages with corn cob bedding. Mice were genotyped by PCR analysis of tail DNA using standard methods. Experiments were conducted in accordance with the guidelines of the National Institutes of Health and were approved by the Nathan S. Kline Institute's Institutional Animal Care and Use Committee.

2.2 Immunization

Mice were injected with the murine-Aβ-specific monoclonal antibody m3.2 (Tg2576+m3.2, NTg+m3.2) [32] or, as control, NT1 monoclonal antibody, also an IgG1a that does not recognize any murine protein (Tg2576+Ctl) [31, 34]. Injections were administered biweekly (400μg/mouse, I.P.) from 7–16mo of age. A separate group of Tg2576 mice were left untreated for comparison to both Tg2576+m3.2 and Tg2576+Ctl mice. Antibody m3.2 shows no reactivity with human Aβ or other human APP metabolites when examining synthetic peptides, human tissue, or APP transgenic mice [31, 32].

2.3 Odor habituation assay

Mice were screened for olfactory deficits using an odor habituation test [26]. Odors (n=7; limonene, ethyl valerate, isoamyl acetate, pentanol, heptanone, propyl butyrate, and nonane; Sigma Aldrich, St. Louis, MO) were diluted 1×10^{-3} in mineral oil and applied to a cotton-applicator stick which was then enclosed in a piece of odorless plastic tubing to prevent contact of the liquid odor with the testing chamber or animal yet still allowing for volatile odor delivery. Odors were delivered for 4 successive trials (1 block), 20sec each, separated by 30sec intertrial intervals, by inserting the odor stick into a port on the side of the animal's home cage. Testing took place during the light phase of the animals' (12:12) day:light cycle, over two daily sessions (3–4 odors/session) separated by 24–48 hrs. The duration of time spent investigating, defined as snout-oriented sniffing within 1cm of the odor presentation port, was recorded across all trials by a single observer blind to genotypes (D.W.W.). Home cages were cleaned with fresh corn cob bedding 24–48hrs prior to behavioral testing. The stainless steel food bin and water bottle were removed from cages immediately prior to testing.

2.4 Aβ quantification

Following behavioral testing, tissue was collected from all mice immediately after euthanasia (CO_2 overexposure and decapitation). On ice, one hemi-brain was dissected into OB, PCX, and `whole brain' including the remaining regions of the brain excluding the cerebellum and brain stem. These three samples were immediately flash frozen on dry ice and stored at −80°C. The other hemi-brain was placed in 10% formalin for fixation and stored at 4°C. Following fixation, brains were transferred into 30% sucrose solution in

formalin and, 48hrs later, coronally sectioned (40μm) on a microtome. A subset of these sections were slide-mounted and immersed in a solution of filtered 1% Thioflavin-S for 10min (Sigma Aldrich), rinsed (×3) for 1min in ddH$_2$0 and subsequently cover-slipped with Vectashield hardmount with DAPI (4′,6-diamidino-2-phenylindole; Vector Laboratories, Inc., Burlingame, CA) for nuclear counter-stain. The remaining sections were left floating in tris-buffered saline (TBS) for 4G8 (anti-Aβ, see Table 2) immunohistochemistry [35]. Sections were washed (3× @ 5min) in TBS after which they were treated with 85% formic acid for 5min to enhance amyloid staining. Sections were then washed in blocking buffer (0.05 M Tris-HCl pH 7.6, .9% NaCl, 0.25% Triton-X 100, 20% normal goat serum & 0.2% bovine serum albumin) 3× (10min each) before incubating for 12hrs in 4G8 primary antibody at 4°C (Signet Labs, Dedham, MA; 1:200 in blocking buffer). Sections were rinsed (3× @ 5min) in blocking buffer before incubating for 2hrs at room temperature in Alexa488 secondary antibody (Invitrogen, Carlsbad, CA). Finally sections were rinsed in TBS (3× @ 5min), placed on slides and cover-slipped with Vectashield hardmount with DAPI (Vector). Brains from all groups were stained during the same procedure. Optical scans of brain sections were collected within 7 days following staining by use of a Zeiss Axioscope microscope (model 200M) and a Zeiss digital camera (Carl Zeiss, Inc., Thornwood, NY).

2.5 Western blot analysis

Ten-percent (weight-to-volume) homogenates were prepared from the flash frozen hemibrain samples (OB, PCX, and `whole brain') and used for biochemical analyses [36]. Due to insufficient tissue weight, samples from 3 mice were used to construct each OB and PCX homogenate. An aliquot of the homogenates was extracted in diethylamine (DEA) and used for sAPP isolation prior to Western blot analyses [32]. Proteins were sorted by SDS-PAGE, transferred to polyvinylidene difluoride membrane [37], and incubated with antibodies as previously described [32] (see Table 2). Antibody C1/6.1 recognizes the carboxyl-terminal cytoplasmic domain of APP [37], and m3.2 recognizes residues 10–15 of murine Aβ, detecting murine APP, APPα and Aβ [32]. 22C11 was purchased from Millipore (Temecula, CA) and detects the APP N-terminus from both human and mouse. Monoclonal antibody 6E10 (Covance, Princeton, NJ), which recognizes residues 1–16 of human Aβ, was used to detect human APP, sAPPα, and Aβ. Monoclonal antibody 4G8 recognizes both human and mouse Aβ17–24 (Senentek, Napa, CA)

2.6 Data analysis

For analysis of behavior data (as described previously [26]), all raw investigatory values (sec) were organized within animals and according to odor presentation number (trial #). First, as a measure of novel odor investigation, the durations of all trial #1 odor investigations were averaged within each subject, pooled within each group, and analyzed using 2-way ANOVAs for independent groups followed by post-hoc group comparisons with Fisher's PLSD. As a measure of odor habituation, raw investigatory values (trials #1–4) were pooled within each group and either analyzed following normalization to the maximum investigation duration/animal for each odor (maximum during trials #1–4). Normalization of data was performed due to group differences in trial # 1 novel odor investigation behavior. For normalization, the maximum investigation duration was assigned a value of "1" and the lesser investigation times a proportion of 1.

Histological analysis of Aβ levels was performed with NIH ImageJ (http://rsbweb.nih.gov/ij). Four individual brain areas, OB, PCX (including both anterior- and posterior-areas), Hipp, and EC were analyzed for levels of Thioflavin-S and anti-Aβ staining in order to quantity Aβ deposition (% area) across olfactory structures. Primary somatosensory and motor cortices (S1 and M1) were used as `non-olfactory' brain regions for comparison to Aβ within the above listed olfactory structures. Fluorescence levels of anti-Aβ 4G8 and

Thioflavin-S were thresholded and regions of interest (ROIs) determined with the guidance of the DAPI counter stain and standard anatomical coordinates [38].

Aβ deposition area (%) was quantified within each ROI separately as previously described [26]. Within all structures, cumulative % area across all layers was used by outlining the entire region using DAPI labeling as a guide. To quantify Aβ deposition in the Hipp we manually outlined all Hipp regions (dentate gyrus, CA1, CA2 and CA3). Aβ deposition (% area) was defined as the cumulative area of fluorescent pixels above threshold within each ROI. At least 3 coronal brain sections (range 3–4) containing each ROI per mouse were used for analysis. Percent area values were analyzed using one-way ANOVAs for independent groups followed by post-hoc group comparisons using Fisher's PLSD. Percent area values for each ROI within each section were treated as independent measures for analysis.

All statistical analyses were performed in StatVIEW (SAS Institute Inc., Cary, NC). All values are reported as mean ± standard error of the mean (SEM) unless otherwise stated.

3. Results

Tg2576 mice display progressive impairments in olfactory behaviors, including abnormally long novel odor investigation times and deficient odor habituation which each positively correlate with the regional levels of Aβ throughout the olfactory system [26]. Here we explored the ability to preserve normal odor habituation behaviors in Tg2576 mice by means of chronic passive immunization against murine Aβ, which is codeposited along with hAβ [31]. Mice were treated biweekly with either the m3.2 antibody (Tg2576+m3.2) [31] or a control antibody (Tg2576+Ctl) starting at 7mo of age (see Materials and Methods). Importantly, this time point precedes major elevations in brain Aβ burden [33]. Indeed, in a separate group of untreated 7mo old mice (n=5) we found Aβ deposition to be minimal (Thioflavin-S: 0.3 ± 0.04 [OB], 0.02 ± 0.01 [PCX], 0.1 ± 0.05 [EC], 0.52 ± 0.03 [hipp]; 4G8: 0.88 ± 0.1 [OB], 0.52 ± 0.1 [PCX], 0.03 ± 0.02 [EC], 0.01 ± 0.01 [hipp], mean ± SEM). At 16mo, following 9mo of treatment, mice were single housed for measures of olfactory behavior using the odor habituation test [26] and following behavioral testing, brains removed for Aβ deposition and APP metabolite analysis.

3.1 Preservation of olfactory behaviors with long-term m3.2 immunization

The time spent investigating novel odors can be an indicator of arousal/motivation [39] or habituation [40]. To quantify effects of m3.2 immunotherapy on novel odor investigation behavior, we pooled all novel (trial #1) odor investigation durations across all odors (n=7) and animals within each genotype (Figure 1A). Similar to that reported previously [26], 16mo old Tg2576+Ctl mice displayed significantly prolonged novel odor investigation times compared to NTg counterparts (p<0.001, 2-tailed t-test). Strikingly, Tg2576+m3.2 mice spent significantly less time investigating novel odors than Tg2576+Ctl mice (p<0.001, 2-tailed t-test), maintaining levels statistically similar to NTg mice (p>0.05, 2-tailed t-test).

We next assessed odor habituation over repeated odor exposures in each of our groups of mice. To do this, odor investigation durations across all trials (4 total, including the data from trial #1 (Figure 1A)) of the odor cross-habituation task were analyzed for differences between groups. Due to the significant effects shown in Figure 1A in the groups' investigation levels within trial #1, all data were normalized before statistical analysis to the maximum odor investigation duration within each individual odor presentation block (trials #1 – 4, see Methods).

We found, similar to before [26], that Tg2576 mice (here Tg2576+Ctl) display a failure to normally habituate across odor presentations in comparison to age-matched NTg controls

(Figure 1B). In particular, enhanced investigation times were displayed upon trial #2 (p<0.01), #3 (p<0.001) and #4 (p<0.001) (all 2-tailed t-tests) in Tg2576+Ctl mice compared to NTg. Strikingly, Tg2576+m3.2 mice showed no significant differences to their NTg counterparts (Figure 2B), reflecting their ability to normally habituate to odors and suggesting a preservation of olfactory circuits supporting this behavior with chronic m3.2 treatment.

3.2 Differential regulation of regional Aβ deposition in m3.2-immunized mice

Within 1 week following behavioral testing, brain tissue was collected from all groups of mice. One hemibrain was drop-fixed in 10% formalin and later sectioned and stained with an anti-Aβ antibody (4G8) (4G8 reacts with human and mouse Aβ, see Table 2) and separately, Thioflavin-S as a measure of fibrillar Aβ plaques. We used these stained sections to quantify regional Aβ deposition throughout 4 principle structures essential to olfactory perception and odor learning and memory, namely the OB, PCX, EC, and hipp.

Interestingly, we found that Tg2576+m3.2 mice had differential levels of Aβ deposited across olfactory and other brain structures in manners different from Tg2576+Ctl or Tg2576 (untreated) mice. For example, greater levels of Thioflavin-S staining was found within the PCX in Tg2576+m3.2 mice compared to Tg2576+Ctl or Tg2576 mice (p<0.001, 2-tailed t-test). No effects of m3.2 immunization on Thioflavin-S staining in the other 3 structures analyzed were found (p>0.05, Tg2576+m3.2 vs. Tg2576+Ctl mice). In contrast, significant decreases in 4G8 staining within the OB (p<0.01) and EC (p<0.01) were observed in Tg2576+m3.2 mice compared to Tg2576+Ctl and Tg2576 mice. As a `non-olfactory' comparison, we additionally measured 4G8 and separately Thioflavin-S staining within the primary somatosensory and motor cortices (S1 and M1). Compared to Tg2576+Ctl mice, we found a significant decrease in 4G8 (p<0.05, 0.36 ± 0.15 [Ctl] vs 0.04 ± 0.01 [m3.2], mean ± SEM) and Thioflavin-S staining (p<0.01, 0.7 ± 0.09 [Ctl] vs 0.36 ± 0.07 [m3.2], mean ± SEM) in S1 and M1, consistent with our prior finding of a reduction in cortical β-amyloid following acute m3.2 immunization [31] (see Table 1). These data show that chronic anti-murine-Aβ immunization in Tg2576 mice alters the pattern of fibrillar and/or nonfibrillar Aβ accumulation throughout both olfactory and non-olfactory brain structures.

3.3 APP metabolite levels other than Aβ are unchanged by m3.2 treatment

The finding that 4G8 positive Aβ deposits were significantly reduced in the OB and EC of Tg2576+m3.2 mice suggests that changes in the levels of these deposits may be sufficient and necessary to remediate olfactory behavioral impairments in Tg2576 mice. However, given the reactivity of m3.2 with other APP metabolites [32] levels of other APP metabolites and even full-length APP itself may also be altered by m3.2 immunotherapy [31], thus confound the interpretation of the behavioral improvements in Tg2576+m3.2 mice. Therefore, we explored whether levels of APP and its metabolites were altered in Tg2576+m3.2 mice. Western blot analysis, however, did not reveal any changes in APP, APP+human sAPPα, sAPP total, or murine sAPPα in either whole brain, OB, or PCX homogenates from Tg2576+m3.2 mice compared to Tg2576+Ctl (Figures 3A & B). No significant changes (p>0.05, 2-tailed t-tests) in APP metabolite levels were found between whole brain, OB, or PCX samples in Tg2576 mice treated with m3.2 compared to control antibody. Total levels of APP and sAPP total were approximately 4.9-fold and 5.8-fold, respectively, in Tg2576 compared to NTg mice, consistent with our prior work [32]. These results suggest that remediation of olfactory behaviors in Tg2576+m3.2 mice is independent of APP metabolite levels other than Aβ.

4. Discussion

A better understanding of the mechanisms of olfactory perceptual loss in AD may help to elucidate general principles of disease pathogenesis and will provide further support for the use of olfactory dysfunction as a biomarker of the disease [5]. Here our primary goal was to test whether chronic anti-murine Aβ (mAβ) immunotherapy with the m3.2 antibody [32] – beginning prior to prominent β-amyloid accumulation – can preserve olfactory behavior in Tg2576 mice. To address this we examined odor-guided behaviors in an odor habituation task [26] which allows for measurements of novel odor investigation and odor learning and memory (habituation) – both within a single behavioral test. Using this same task, we recently established novel odor investigation and odor habituation deficits in Tg2576 mice, which appear starting at 6mo of age, and correlate highly with the levels of Aβ burden throughout olfactory regions [26]. Thus, one advantage of using this task is that the level of olfactory impairments in Tg2576 mice is known, and indeed, the present data closely replicates our previous finding. Another advantage of this assay in the present studies is that the synaptic mechanisms underlying normal behavioral function in this task are mostly known [40, 41]. Olfactory circuit disruption has been implicated as a cause of olfactory dysfunction in AD in both rodent models [23, 25, 26] and in humans [42].

Here we found that chronic m3.2 immunization, which only directly targets the relatively small pool of the mAβ present in Tg2576 mice (~5% of the total, see [31]), was sufficient to preserve the olfactory behaviors of novel odor investigation and habituation. In our prior study [31] (see Table 1), we found that anti-mAβ immunization was sufficient to clear both hAβ and mAβ in the cortex and hipp when initiated after β-amyloid deposition. Somewhat surprisingly, here we did not find that the remediation of olfactory habituation deficits in Tg2576 mice matched a reduction in Aβ deposition within the PCX (Figure 2), a region highly implicated in odor habituation [40]. Instead, Thioflavin-S positive Aβ actually increased in the PCX with chronic m3.2 immunization, despite the improvements in habituation behavior. This finding may reflect the complex dynamics of the interaction between the exogenously expressed hAβ and the endogenous mAβ within the mouse brain during β-amyloid accumulation [31, 43, 44]. However, this is consistent with a more neurotoxic role for non-fibrillar Aβ (as partly measured by 4G8 staining) compared to β-amyloid plaque-associated Aβ in the AD brain [45], suggesting the possibility that reductions in non-fibrillar Aβ in the OB and EC are sufficient to rescue odor habituation behavior, despite persistence of fibrillar Aβ (Thioflavin-S positive) plaques in the PCX. In contrast, we did find that 4G8 positive Aβ deposits were reduced within the OB and EC of Tg2576+m3.2 mice. Importantly, here we were unable to quantify soluble Aβ, specifically within the OBs of the treated mice to explore m3.2-induced changes in soluble Aβ directly, if any. Future studies using methods to precisely regulate and measure soluble Aβ levels within local circuits will be needed to explore the possibility that reductions in Aβ in the OB and EC is sufficient to rescue odor habituation behavior, despite persistence of fibrillar Aβ (Thioflavin-S positive) plaques in the PCX.

Immunization-induced Aβ clearance may operate by several methods, including microglial phagocytosis, the direct disruption of plaques, the clearance of antibody bound Aβ, and the redistribution of brain Aβ to the periphery [43, 44, 46, 47]. In transgenic mouse models of human amyloidosis, the overexpression of hAPP results in the accumulation of hAβ along with the endogenously expressed mAβ [31, 48, 49]. Targeting mAβ with the m3.2 antibody is an immunotherapeutic approach to manipulating Aβ levels in the brains of AD transgenic models that relies upon targeting this relatively small pool (~5%) of the total Aβ [31]. This method is in contrast to strategies that directly target the abundant, overexpressed and non-endogenous human APP-derived hAβ (e.g., [50]). Importantly, m3.2 treatment altered hAβ levels but not the levels of APP or levels of other APP metabolites (Figure 3), suggesting

that long-term treatment with an antibody that reacts with the endogenous Aβ as well as potentially with other endogenous APP metabolites can be well tolerated.

In conclusion, this study presents evidence for the remediation of olfactory impairments in Alzheimer's mouse models overexpressing human mutations of the APP gene and therefore developing progressive elevations in Aβ deposition. This behavioral remediation did not require a global decrease in fibrillar Aβ deposition but is consistent with a decrease in non-fibrillar Aβ as a critical target for restoring circuit function in AD [45]. Further, this data suggests that chronic passive immunotherapy targeting endogenous Aβ can preserve normal olfactory function in a manner potentially relevant to those with olfactory loss due to AD and even other disorders related to APP gene dysregulation and Aβ deposition (e.g., Down's syndrome) [2]. Whether anti-hAβ immunotherapy also may serve to preserve and/or rescue olfactory dysfunction in similar manners as we found herein using anti-mAβ immunotherapy is yet to be determined. We predict that future clinical studies incorporating olfactory perceptual screens along with other standardized biomarker measures throughout course of anti-hAβ immunotherapy may reveal enhanced diagnostic and staging sensitivity.

Acknowledgments

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Highlights

- Olfaction is dysfunctional in mouse models of the Alzheimer's disease (AD).

- Chronic anti-murine amyloid-β immunization preserved odor habituation behavior.

- Less amyloid-β deposits were found in the olfactory bulb and entorhinal cortex of immunized mice.

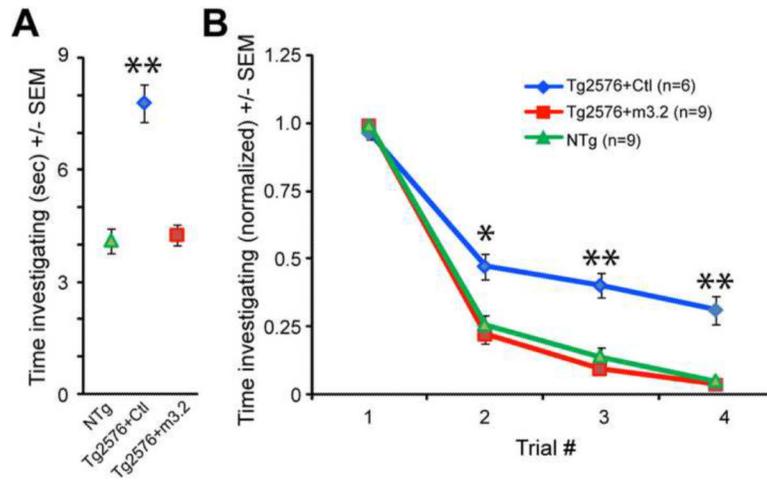- No detectable changes in APP metabolite levels other than amyloid-β were found.

Figure 1. Preservation of normal odor habituation behaviors in Tg2576 mice treated with m3.2

Results from an odor habituation test from 16mo old Tg2576+m3.2 and Tg2576+Ctl (control treated, see Methods) and age-matched non-transgenic control mice (NTg). **(A)** Investigation times for all trial #1 novel odor presentations. **p<.001 vs. both Tg2576+m3.2 and NTg. **(B)** Odor habituation (normalized, see Methods) across 4 successive odor presentation trials. *p<0.01, **p<0.001, 2-tailed *t*-tests, Tg2576+Ctl vs. both Tg2576+m3.2 and NTg within each odor presentation #.
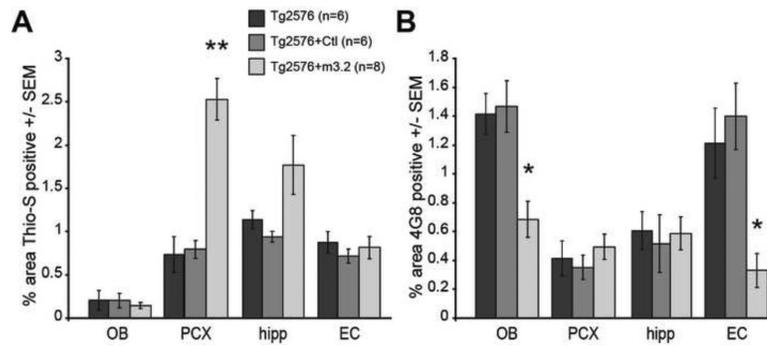
Figure 2. Regional variations in Aβ deposition in Tg2576 mice treated with m3.2
Quantification of Aβ deposition (% area, Thioflavin-S (Thio-S, (**A**)) and anti-Aβ (4G8, (**B**)))
in the olfactory bulb (OB), piriform cortex (PCX), dorsal hippocampus (hipp) and lateral
entorhinal cortex (EC) from 16mo old Tg2576 mice. *$p<0.01$, **$p<0.001$ Tg2576+m3.2 vs.
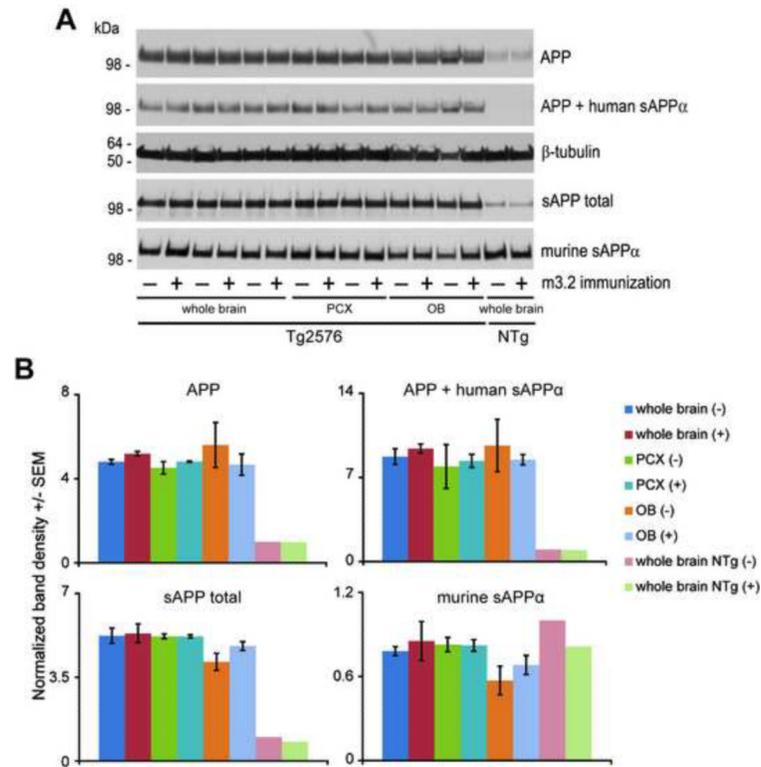Tg2576+Ctl or Tg2576, 2-tailed *t*-tests.

Figure 3. APP metabolite levels are independent of m3.2 immunization in the Tg2576 mouse brain

Western blot (A) and semi-quantitative analysis of western blot data (B) from three different brain regions (whole brain, PCX and OB) showing APP metabolite levels including APP (antibody C1/6.1), APP + human sAPPα (antibody 6E10), sAPP total (antibody 22C11) and murine sAPPα (antibody m3.2). Data in B = optical density normalized to NTg levels. β-tubulin was used as a loading control. OB and PCX homogenates were created by homogenizing tissue from 3 mice/sample. All data from 16mo old mice, treated with either control antibody (−) or m3.2 (+) and used in olfactory behavior testing (Fig 1).

Table 1

Summary of previous passive immunization study [31] with the murine-Aβ-specific antibody m3.2 in older (β-amyloid depositing Tg2576 mice (immunization from 20 to 22 months of age).

Tests	Tg2576 (compared to control injected)	NTg (compared to control injected)
Human Aβ40 ELISA	Decrease of 58.0% +/− 8.7%	N/A
Human Aβ42 ELISA	Decrease of 57.9% +/− 9.2	N/A
Murine Aβ40 ELISA	N/A	Decrease of 65.3% +/− 10.2%
Murine Aβ42 ELISA	N/A	Decrease of 40.7% +/− 6.8%
APP and sAPPα Western blotting	No significant changes	No significant changes
Thioflavin S staining (hippocampus)	Decrease of 71.0% +/− 19.0%	N/A
Thioflavin S staining (cortex)	Decrease of 54.4% +/− 15.0%	N/A
Odor habituation	Rescue in behavioral deficits	No significant changes
Nesting	Rescue in behavioral deficits	No significant changes

N/A: not applicable.

Table 2

Summary of antibodies used.

Antibody	Specificity	Reactivity	Source
m3.2	Recognizes residues 10–15 of murine Aβ, detecting murine APP, APPα and Aβ	Murine	[32]
6E10	Recognizes residues 1–16 of human Aβ, detecting human APP, sAPPα, and Aβ	Human	Covance, (Princeton, NJ)
4G8	Recognizes residues 17–24 of Aβ, a species-conserved epitope	Murine and human	Senentek, (Napa, CA)
C1/6.1	Recognizes the conserved C-terminal cytoplasmic domain of APP	Murine and human	[37]
22C11	Recognizes a species-conserved N-terminus extracellular domain of APP	Murine and human	Millipore (Temecula, CA)