Published in final edited form as:

Eur J Neurosci. 2010 February ; 31(4): 710–721. doi:10.1111/j.1460-9568.2010.07103.x.

Functional deprivation promotes amyloid plaque pathogenesis in Tg2576 mouse olfactory bulb and piriform cortex

Xue-Mei Zhang^{1,2,#}, Kun Xiong^{3,#}, Yan Cai^{3,4}, Huaibin Cai⁵, Xue-Gang Luo³, Jia-Chun Feng², Richard W. Clough¹, Peter R. Patrylo^{1,4}, Robert G. Struble⁶, and Xiao-Xin Yan^{1,*}

¹Department of Anatomy, Southern Illinois University School of Medicine, Carbondale, IL 62901, USA

²Department of Neurology, The First Hospital of Jilin University, Changchun, Jilin 130021, China

³Department of Anatomy and Neurobiology, Central South University Xiangya Medical School, Changsha, Hunan 410013, China

⁴Department of Physiology, Southern Illinois University School of Medicine, Carbondale, IL 62901, USA

⁵Laboratory of Neurogenetics, National Institute on Aging, National Institutes of Health, Bethesda, MD 20892, USA

⁶Department of Neurology & Center for Alzheimer's disease, Southern Illinois University School of Medicine, Springfield, IL 62794, USA

Abstract

Cerebral hypometabolism and amyloid accumulation are principal neuropathological manifestations of Alzheimer's disease (AD). Whether and how brain/neuronal activity might modulate certain pathological process of AD are interesting topics of recent clinical and basic research in the field, and may be of potential medical relevance in regard to both the disease etiology and intervention. Using the Tg2576 transgenic mouse model of AD, this study characterized a promotive effect of neuronal hypoactivity associated with functional deprivation on amyloid plaque pathogenesis in the olfactory pathway. Unilateral naris-occlusion caused BACE1 elevation in neuronal terminals in the deprived relative to the non-deprived bulb and piriform cortex in young adult mice. In parallel with the overall age-related plaque development in the forebrain, locally-increased BACE1 immunoreactivity co-occurred with amyloid deposition first in the piriform cortex then within the bulb, more prominent on the deprived relative to the non-deprived side. Biochemical analyses confirmed elevated BACE1 protein levels, enzymatic activity and products in the deprived relative to non-deprived bulbs. Plaque-associated BACE1 immunoreactivity in the bulb and piriform cortex was localized preferentially to swollen/sprouting glutamatergic axonal terminals, with A β immunoreactivity occurred inside as well as around these terminals. Together, these findings suggest that functional deprivation or neuronal hypoactivity facilitates amyloid plaque formation in the forebrain in a transgenic model of AD, which operates synergistically with age effect. The data also implicate an intrinsic association of amyloid accumulation and plaque formation with progressive axonal pathology.

*Correspondence to: Xiao-Xin Yan, Department of Anatomy, Southern Illinois University School of Medicine, Carbondale, IL 62901. Tel: 618-453-1577; xyan@siu.edu.

Authors equally contributed to this work

Keywords

cerebral hypometabolism; secretase; amyloidogenesis; axonal pathology; neuroplasticity

Introduction

Alzheimer's disease (AD) is characterized pathologically by extracellular amyloid deposition, neurofibrillary tangles, aberrant neuritic sprouting, and loss of neurons and synapses in vulnerable brain regions especially the limbic and neocortical areas. These pathological alterations appear to progress along anatomically definable brain pathways (Braak and Braak, 1996), implicating certain type of chain reaction in the spread of neuropathology presumably via synaptic connections. Accumulation of β -amyloid peptides ($A\beta$) is a principal pathogenic event in AD (Selkoe, 2000). A large body of evidence indicates that β -secretase-1 (BACE1), the enzyme obligatory for amyloidogenic processing of the amyloid precursor protein (APP), is elevated in the brain in prodromal and clinical AD, implicating a potential initiative role of this enzyme in $A\beta$ accumulation (Fukumoto et al., 2002; Holsinger et al., 2002; Tyler et al., 2002; Yang et al., 2003; Li et al., 2004). Therefore, identifying factors that elevates BACE1 *in vivo* might help understand the site-specific plaque pathogenesis in AD brain.

Cerebral hypometabolism is a prominent premortem imaging finding in prodromal and clinical AD cases (Reiman et al., 2001; Nestor et al., 2003; Perneczky et al., 2007). Epidemiological studies suggest that brain activity might play a role in AD etiology as cognitive and physical activities appear to delay the onset of the disease (Laurin et al., 2001; Yu et al., 2006; Fratiglioni and Wang, 2007; Kemppainen et al., 2008; Roe et al., 2008). In transgenic mouse models of AD, certain stimulative experimental paradigms, such as physical, cognitive or environment enrichments, appear to lower central $A\beta$ levels, ameliorate plaque development and improve cognitive performance (Adlard et al., 2005; Jankowsky et al., 2005; Lazarov et al., 2005; Billings et al., 2007). Therefore, it is of particular interest to investigate if and how physiological neuronal activity might affect plaque development in anatomically defined brain region or pathway.

We recently identified an inverse correlation between endogenous neuronal activity or metabolism and BACE1 expression at the olfactory glomeruli in rats (Yan et al., 2007). That study raised a compelling question as to whether functional deprivation would eventually promote plaque pathogenesis in the olfactory system. Using Tg2576 mice as an experimental model, the present study demonstrates that functional deprivation causes BACE1 upregulation trans-synaptically in the olfactory bulb and primary cortex, and may exacerbates plaque pathogenesis in these olfactory centers.

Materials and Methods

Animal and surgery

Adult male Tg2576 mice (APP^{sw}, K670N/M671L) were purchased from Taconic (Hudson, NY, USA). Plaque onset in Tg2576 mice occurs around 9 month of age in the cortex and hippocampus (Hsiao et al., 1996; Sarsoza et al., 2009). Therefore, unilateral naris-occlusion was performed on 6 month-old animals, which would allow investigations on whether or not deprivation might affect the timing of plaque onset as well as the progress of age-dependent plaque development. The left or right nostril was cauterized under anesthesia with sodium pentobarbital (50 mg/kg, i.p.). Occluded animals were allowed to survive until they were 7 (n=3), 8 (n=3), 9 (n=4), 12 (n=4), 18 (n=7, including n=3 for assessing β -site APP cleavage activity and ELISA) and 24 (n=7, including n=3 for western blots) month-old.

Animal use was in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. All experimental procedures were approved by the Animal Care and Use Committee of Southern Illinois University at Carbondale.

Tissue preparation

Mice were perfused transcardially with 4% paraformaldehyde in 0.01M phosphate-buffered saline (pH 7.4, PBS) under overdose anesthesia (sodium pentobarbital, 100 mg/kg, i.p.). The brains were carefully dissected out, postfixed in the perfusion fixative overnight at 4 °C, and then cryoprotected with 30% sucrose. The forebrains were cut either perpendicular to the long axis of the bulbs (7-8 month-old mice), or in parallel to the ventral surface of the bulbs (9-24 month-old mice). The latter preparation was especially suitable for concurrent analyses of labelings in the bulb and piriform cortex. Twelve sets of 30 µm sections across the entire bulb (together with the cortex) were collected in order in PBS in cell culture plates. For double immunofluorescence, 4 sets of 8 µm sections around the middle bulb levels in each brain were also collected by thaw-mounting on Superfrost Plus slides (VWR, West Chester, PA, USA). Before section collection, small needle punches were made around the center of the bulb, piriform cortical white matter or the striatum contralateral to the occluded nostril. These fiducial markers were used to determine the orientation of the sections (i.e., the side of the bulb or cerebral cortex). For biochemical studies, animals were briefly perfused with cold PBS to remove blood, and the forebrains were dissected out. The left and right bulbs were separated, weighed, snap-frozen with liquid nitrogen and stored at -70 °C. Bulbs from 18 month-old mice were used for measuring enzymatic activity of β -site APP cleavage and soluble A β concentration, and bulbs from 24 month-old mice were used for western analyses.

Immunohistochemistry

Two out of the 12 sets of sections from each brain were immunostained for BACE1 (#1, #7) and A β (#2, #8, using the rabbit anti-A β antibody, Ter-40) with the DAB-peroxidase method for systematic quantitative analyses. The remaining sets were used for Nissl stain (#3, #9) and immunolabelings for some other A β antibodies (see Table 1). Sections were first treated with 1% H₂O₂ in PBS for 30 minutes, and pre-incubated in 5% normal goat or horse serum with 0.3% Triton X-100 for 1 hour. Antigen retrieval techniques were used for BACE1 (50% formamide and 50% 2XSSC at 65 °C for 1 hour) and A β antibody (50% formic acid in PBS for 30 minutes at room temperature) labelings before H₂O₂ treatment. Sections were incubated overnight at 4 °C with BACE1 and A β antibodies diluted in PBS containing appropriate blocking sera (see Table 1 for antibody sources and dilutions). The sections were then reacted with biotinylated goat anti-rabbit or horse anti-mouse IgGs at 1:400 for 2 hours, and subsequently with the ABC reagents (1:400) (Vector Laboratories, Burlingame, CA, USA) for an additional hour. Immunoreactivity was visualized using 0.003% H₂O₂ and 0.05% diaminobenzidine (DAB, Sigma-Aldrich, St. Louis, MO, USA). Immunostained sections were mounted on slides, allowed to air-dry, and coverslipped. Some sections were lightly counterstained with cresyl violet to verify the laminar distribution of the immunolabeling.

Double immunofluorescence was carried out by incubating sections in PBS containing 5% donkey serum and a pair of primary antibodies from different animal species (Table 1), followed by a 2 hour reaction with Alexa Fluor® 488 and Alexa Fluor® 594 conjugated donkey anti-mouse, rabbit and goat IgGs (1:200, Invitrogen, Carlsbad, CA, USA). After fluorescent immunolabeling, sections were counter-stained with bisbenzimidazole (Hoechst 33342, 1:50000), washed 3 times in PBS and mounted with anti-fading medium. Initial antibody specificity tests included preabsorption of primary antibody with neutralizing peptide or omission of primary antibody in the incubation buffer. These controls yielded no specific labeling in brain sections.

NADPH-diaphorase and CO histochemistry—Sections were incubated in 0.05 M Tris-HCl buffered saline (pH 8.0, TBS) containing 0.3% Triton X-100, 1 mM nicotinamide adenine dinucleotide phosphate diaphorase (β -NADPH-d), 0.8 mM nitroblue tetrazolium and 5% dimethyl sulfoxide for 45 minutes at 37 °C. Selected sections were further immunostained for BACE1 using the DAB-peroxidase method to assess colocalization. Cytochrome c oxidase (CO) histochemical stain was processed by incubating the sections in 0.05% DAB, 0.02% cytochrome c oxidase, and 4% sucrose in 0.1 M phosphate buffer (pH 7.4) at 37°C in the dark for 3-5 hours, followed by several rinses with PBS at room temperature. All histochemical reagents were from Sigma-Aldrich (St. Louis, MO, USA).

Western blotting

Left and right bulbs were homogenized with a sonication device in T-PER buffer (10x w/v) (Pierce, Rockford, IL, USA) containing a cocktail of protease inhibitor (Roche Products, Welwyn Garden City, UK) at 4°C. Tissue extracts were centrifuged at 100,000 X g. Supernatants were collected and protein concentrations determined by DC protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Equal amount of proteins (25-50 μ g) were run on 20% (for A β 40 and A β 42) and 12% (for other protein products) SDS-PAGE gels (Hoefer Scientific Instruments, San Francisco, CA, USA). The polypeptides were electrotransferred to Tans-Blot® pure nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA, USA). Non-specific binding was blocked using 5% nonfat milk in PBST (9.1 mM dibasic sodium phosphate, 1.7 mM monobasic sodium phosphate, 150 mM NaCl, and 0.1% Tween-20). Full-length and proteolytic fragments of APP, BACE1 and reference protein β -tubulin-III were blotted with primary antibodies as listed in Table 1. Nitrocellulose membranes were incubated with HRP-conjugated second antibodies for 1 hour (1:20000, Bio-Rad), and protein bands visualized with an ECL Plus™ western blotting detection kit (GE healthcare, Pittsburgh, PA, USA), and images captured in a UVP Biodoc-it™ system (UVP, Inc, CA, USA).

β -Site APP cleavage activity and ELISA for A β 40 and A β 42

Frozen bulbs were homogenized in T-PER buffer (5x v/w) containing protease inhibitors (Roche Products) and 1% Triton X-100. Samples were then centrifuged and supernatants collected, followed by determination and normalization of protein concentrations. Beta-site APP cleavage activities were measured in 96-well transparent flat-bottom plates using a commercial kit (#565785, Calbiochem, La Jolla, CA, USA) following manufacturer's instruction. Triplicated loadings (20 μ g protein/well) were applied for each sample in a given experiment, and assay was repeated at least once. Levels of soluble A β 40 and A β 42 were assayed using commercial kits according to manufacturer's instruction (Catalog #88-348 and KHB3441, Invitrogen, Carlsbad, CA, USA). Triplicated loadings of 50 μ g protein (normalized to 50 μ l sample solution) were assayed for each bulb sample. Enzyme activity and ELISA signals were captured in a Bio-Rad microplate reader (Model 3550).

Imaging, densitometry, statistical testing and figure preparation

Sections were examined on an Olympus (BX60) fluorescent microscope equipped with a digital camera and image analysis system (Optronics, Goleta, CA, USA). Double immunofluorescent images were superimposed using the Optronics software. For densitometry, digital images were captured using a 4X objective lens with a numerical aperture of 0.1, such that both sides of the forebrain structures were included within the same image. Higher magnification images were taken using 10X, 20X and 40X objectives with numerical apertures of 0.3, 0.7 and 0.85, respectively. All original images contained 1200×1600 pixels. An identical photo-exposure setting was used for all sections. Optical densities (o.d.), expressed as digital light units (DLU)/mm², over areas of interest were obtained in equally-spaced (~180 μ m apart) and level-matched (5-7 levels/brain, i.e., with the right and left bulb sections attached together) sections using an

irregular interconnecting selection tool (OptiQuant 4.1, Parkard Instruments, Meriden, CT, USA), by an experimenter who was blinded to the treatment.

BACE1, CO or tyrosine hydroxylase (TH) labelings were measured over the glomerular layer (GL) and/or external plexiform layer (EPL) of the bulb, or layer I and II/III of the piriform cortex. Data were normalized to the averaged mean from the non-deprived side (defined as 100%). Plaque-related BACE1 and A β labelings were measured over the granule cell layer (GCL) and the subependymal zone (SEZ) of the bulb, layers I-III of the piriform cortex together with the lateral olfactory tract (LOT), and layers I-VI in the frontal cortex (see Supplemental-Fig. 1A-F). To calculate specific (or plaque-related) BACE1 and A β immunoreactivities, optic densities over the plaque-free striatum (both sides) were also measured (Supplemental-Fig. 1A-D). Measured optic densities (row data) were exported into Excel spreadsheets, and the averaged striatal density was used as the cut-off threshold to define specific densities [i.e., specific BACE1 or A β density (in the bulb or cortex) = measured BACE1/A β o.d. — average of the measured BACE1/A β o.d. in the striatum]. Means of specific densities were calculated for each brain and each surviving group, and data were statistically analyzed using student-*t* test or ANOVA together with Bonferroni posttests between paired means (Prism GraphPad, San Diego, CA, USA). The minimal significant level of difference between comparing means was set at $p < 0.05$.

Specific β -site APP cleavage activity was calculated by subtracting non-specific signal from the total signals in individual samples. The non-specific signal level was defined in the same experiment by including excessive enzyme inhibitor provided by the manufacturer in the assay buffer. Specific enzyme activities in individual bulb samples were normalized to the averaged mean (defined as 100%) from the non-deprived bulbs. For ELISA data, A β 40 and A β 42 concentrations were calculated according to a standard curve generated using serially-diluted synthetic peptides provided by the manufacturer. The maximal signal in the samples was in the linear range of the standard curve. Figure panels were assembled using Photoshop 7.1 and converted to a final TIFF file, with contrast/brightness adjusted as needed.

Results

Trans-neuronal BACE1 elevation in the deprived olfactory pathway

In the present study we used a rabbit antibody generated against human BACE1 protein sequence (anti-BACE1 α) to assess BACE1 alteration in Tg2576 mouse forebrain (Cai et al., 2001). This antibody detects mature BACE1 protein in cell lysate and brain homogenate migrating at ~70 kd in western blot (Xiong et al., 2007; Wang et al., 2008). The utility of this antibody in immunohistochemistry has been evaluated using BACE1 knockout (BACE1 $^{-/-}$) and wild-type (BACE1 $^{+/+}$) mouse brains as negative and positive controls (Zhang et al., 2009).

Our previous study showed significant BACE1 elevation and CO reduction in the GL in adult rat olfactory bulb after naris-occlusion (Yan et al., 2007). Sensory deprivation has been shown to induce trans-synaptic changes in the primary olfactory cortex (Kim et al., 2006). Therefore, sections from 7 and 8 month-old transgenics were used to determine potential trans-neuronal BACE1 and CO modulations by sensory activity (no plaque-like BACE1 and A β reactivities were visible in the forebrain in these animals) (Fig. 1). Densitometric analyses revealed significant increase of BACE1 immunolabeling and decrease of CO activity in piriform cortical layer I on the deprived relative to the non-deprived sides ($p < 0.01$ for BACE1 and $p < 0.001$ for CO, student-*t* test, $n = 6$ including both age groups) (Fig. 1C-H). Similar changes in BACE1 and CO reactivities were visible over layers II/III (Fig. 1E, F), and the difference of labeling density over these layers between the two sides was approaching statistical significance ($p = 0.086$ for BACE1 and $p = 0.091$ for CO, one-way ANOVA analysis, same below). As

expected, BACE1 elevation and CO reduction occurred in the GL and EPL in the deprived bulbs in these animals (Fig. 1A, B, G, H).

Increased plaque-like BACE1 and A β reactivity in the deprived olfactory centers

Ter-40 and 3D6 were used as the primary A β antibodies to assess amyloid deposition in Tg2576 mouse forebrain, both clearly labeled amyloid plaques in cerebral cortical sections from diagnosed AD cases (data not shown). In all long-term surviving animals, densitometry confirmed a 2-3 fold increase of BACE1 labeling in the GL in the deprived relative to the non-deprived bulbs with statistically significant difference ($p < 0.005$ - 0.001). We also confirmed deprivation in all occluded animals by using TH immunolabeling, which was diminished in the GL (e.g., Supplemental Fig. 3B, C). Overall, specific densities of plaque-related BACE1 ($p < 0.0001$; $df=3$; $F=33$, 159) and A β ($p < 0.0001$; $df=3$; $F=11$, 72) immunoreactivities in the piriform cortex were significantly increased from 9 to 24 month of age (Supplemental-Fig. 1G, H), and were greater in the deprived side based on two-way ANOVA analysis. In contrast, specific densities of BACE1 and A β labelings measured over the frontal cortex (defined using the anterior ventricle as a landmark in dorsal forebrain sections) did not show differences ($p > 0.05$, student-t tests, $n=4$ /per age group) between the ipsilateral and contralateral sides to the occluded naris at 18 and 24 month of age (Supplemental-Fig. 1G, H). Specific densities of BACE1 and A β labelings and corresponding statistical analyses at individual age points will be detailed further below.

Consistent with previous reports (Hsiao et al., 1996; Sarsoza et al., 2009), a few amyloid plaques became detectable discretely in the cortex and hippocampal formation in 9 month-old animals. A small number of localized BACE1 and A β immunoreactive profiles resembling plaques were found in the piriform cortex at this age (Fig. 2A-F). These profiles were noticeably denser in the deprived relative to the non-deprived sides (Fig. 2D-F). In some ventral-level sections, individually-labeled profiles appeared to occur only in the deprived side, especially around layer I (Fig. 2A-C). Quantitatively, specific densities of the plaque-like BACE1 labeling were 156 ± 18 (DLU/mm², mean $\pm$ S.D., same below) in the deprived and 66 ± 19 in the non-deprived piriform cortex ($p=0.010$, ANOVA paired mean comparison, $n=4$, same below). Similarly, specific densities of A β deposits were 117 ± 39 and 49 ± 16 in the deprived and non-deprived sides, respectively, and were significantly different between the two sides ($p=0.033$). Plaque-like profiles were essentially absent in the bulb at this age. Comparing immunolabelings between adjacent sections, it appeared that large BACE1 and A β labeled profiles spatially colocalized (Fig. 2D, E, arrows).

In 12 month-old transgenics, more A β immunoreactive plaques occurred in the piriform cortex relative to 9-month-old animals, with a few plaques also sparsely seen in SEZ of the bulb (therefore not quantified for this age group) (Fig. 2G). Amyloid plaques in the piriform cortex varied in size and were mainly localized to layers I and II (Fig. 2H, I). Densitometry indicated that more amyloid deposition existed in deprived side (352 ± 41) than the non-deprived side (249 ± 42) ($p=0.027$). Similarly, specific densities of plaque-like BACE1 reactivity were significantly higher in the deprived (334 ± 54) than non-deprived (253 ± 34) piriform cortex ($p=0.030$) (images not shown).

In 18 and 24 month-old Tg2576 mice, plaque-like BACE1 immunoreactive profiles appeared to be further increased in the piriform cortex (Supplemental Fig. 1H). Meanwhile, many labeled profiles occurred in the olfactory bulb at these ages over the GCL and SEZ (Fig. 3A-E). In 18 month-old transgenics, specific BACE1 densities in the GCL and SEZ were 135 ± 18 and 77 ± 9 , respectively, in the deprived and non-deprived bulbs ($p=0.011$) (Fig. 3F). In 24 month-old animals, specific BACE1 densities in these layers were also higher in the deprived (235 ± 37) than the non-deprived (171 ± 19) bulbs ($p=0.028$). In the piriform cortex, BACE1 densities were

higher in the deprived side relative to non-deprived side at 18 month (488 ± 49 vs 373 ± 32 , $p=0.010$) and 24 month (707 ± 79 vs 582 ± 90 , $p=0.015$) (Fig. 3F).

A β immunoreactive plaques appeared denser in the olfactory centers at 18 and 24 month relative to younger ages (Supplemental Fig. 1G), with more labeled profiles seen in the deprived side (Fig. 4A-E). Specific A β densities in the piriform cortex (deprived vs non-deprived sides) were 415 ± 58 vs 350 ± 73 at 18 month ($p=0.045$), and 543 ± 80 vs 490 ± 95 at 24 month ($p=0.012$). Specific A β densities in the GCL and SEZ of the deprived vs non-deprived bulbs were 122 ± 21 vs 80 ± 15 at 18 month ($p=0.010$) and 179 ± 42 vs 134 ± 32 at 24 month ($p=0.060$) (Fig. 4F).

Elevated BACE1 protein levels, enzymatic activity and products in the deprived bulbs

Biochemical analyses were carried out to confirm elevations of BACE1 protein, activity and products in the deprived olfactory bulbs at representative (i.e., 18 and 24 month-old) post-occlusion time points. Consistent with previous characterization in adult rats (Yan et al., 2007), glycosylated (70 kd) and deglycosylated (46 kd) BACE1 proteins were significantly elevated in the deprived bulbs in 24 month-old mice, in average about 185% for the 70 kd ($p=0.044$) and 267% for the 46 kd ($p=0.032$) BACE1 forms, relative to counterparts (Fig. 5A, B). Levels of APP β -site cleavage C-terminal fragments (APP β -CTF) were also increased in the deprived bulb extracts at this age, to $\sim 333\%$ in average ($p=0.028$). Levels of A β 40 and A β 42 were assessed by western blot for the 24 month-old group (not reliably detectable at younger ages), and were $\sim 155\%$ ($p=0.066$) and $\sim 182\%$ ($p=0.026$) in the deprived bulbs, respectively, relative to counterparts (Fig. 5A, B). In contrast, levels of putative human transgenic APP, as blotted with the 6E10 antibody, were comparable between the deprived and non-deprived bulbs in tissue homogenates (Fig. 5A, B) ($p=0.24$). In line with western blot data, elevated β -site APP cleavage ($\sim 400\%$, $p=0.0001$) and A β levels (2-3 fold, $p<0.01$) were detected in the deprived relative to non-deprived bulbs in 18 month-old transgenics as assayed with sensitive enzyme activity assay and ELISA (Fig. 5C, D).

BACE1 elevation coincident with axonal pathology and local A β accumulation

The plaque-like BACE1 immunoreactivity in the bulb and piriform cortex in older animals (Figs. 3A-F; 6A-F) was apparently “de nova” compared to the diffuse neuropil-like reactivity in pre-plaque Tg2576 mice (Fig. 1A, E). These locally-labeled elements exhibited heavy reactivity comparable to that in the glomeruli (Fig. 6A). The smallest profiles were discrete spherical structures with a diameter around a few microns (Fig. 6C, E, indicated by arrows). Medium-sized profiles appeared to be clusters of swollen processes arranged in a rosette-like fashion, some with a grape-like head extending towards periphery (Fig. 6C, E, arrowheads). Larger profiles appeared to be densely packed swollen processes also largely arranged in a radiation fashion, with a central paler zone seen in some profiles (Fig. 6F).

In double immunofluorescence, BACE1 labeled processes colocalized spatially with 3D6 labeling at low magnification, (Fig. 7A-C). Importantly, numerous discretely-distributed small spherical structures were simultaneously labeled for BACE1 and 3D6, and they appeared in identical size and shape (Fig. 7B-F). Around large labeled profiles or established plaques, 3D6 reactivity appeared somewhat diffuse relative to BACE1 reactivity, and exhibited a star-fish appearance in some cases. In merged images, 3D6 immunoreactivity appeared to coexist with BACE1 labeling (appearing yellow), but also occurred around or between BACE1 labeled elements (Fig. 7G-I). It should be emphasized that the heavy BACE1 reactivity in the glomeruli was not associated with any specific 3D6 labeling in either the main bulb or the accessory olfactory bulb (Fig. 7A-F).

Multiple double immuno- and histochemical labelings were carried out to determine BACE1 immunoreactive swollen processes being axon terminals and axonal dystrophic neurites, and

to identify their potential neuronal origins, as described in detail in our recent study in two other transgenic models of AD (Zhang et al., 2009). These BACE1 labeled profiles were found to colocalize with presynaptic terminal markers synaptophysin and growth-associated protein-43, but not with dendritic marker microtubule associated protein-2 (data not shown). In both the olfactory bulb and piriform cortex, BACE1 labeled processes clearly co-labeled for vesicular glutamate transporter-1 (VGLUT-1), a marker for glutamatergic presynaptic terminals (Fig. 7J-R). In addition, BACE1 labeled swollen processes also colocalized with distinct reactivities for APP (Supplemental-Fig. 2A-F) and likely presenilin-1 (Supplemental-Fig. 2G-I). In contrast, BACE1 immunoreactive swollen processes rarely colocalized with markers of mature and immature interneurons in the bulb and piriform cortex, including TH (Supplemental-Fig. 3A-C), NADPH-d (Supplemental-Fig. 3D-H) and doublecortin (Supplemental-Fig. 3I-N), GAD67 and calcium binding proteins (data not shown).

Discussion

Neuronal activity down-regulates BACE1 at synaptic terminals in the olfactory system

The olfactory system has been used as a classic *in vivo* model to address neurobiological issues regarding activity-dependent synaptic/neuronal modulation (Johnson and Leon, 2007; Oka et al., 2009; Wessen et al., 2009). Naris-occlusion per se does not cause direct structural damage to the system, and the experimental effect can be efficiently assessed in the same brain between the deprived side and its internal control, the non-deprived side. Blockage of olfactory stimulation by this procedure reduces neuronal activity and oxidative metabolism on the pathway (Cullinan and Brunjes, 1987; Jin et al., 1996; Philpot et al., 1997). Using this model and several BACE1 antibodies (Yan et al., 2007), we find strong evidence indicating that neuronal activity down-regulates BACE1 *in vivo* especially at synaptic terminals. BACE1 labeling in the olfactory centers is localized generally to neuronal terminal laminae, particularly distinct in the glomeruli and piriform cortical layer I, major targets of the first and second-order olfactory projection neurons (de Olmos et al., 1978). Functional deprivation leads to significant BACE1 elevation in these neuronal terminal fields in the bulb and piriform cortex. Thus, these findings implicate that neuronal (sensory) activity may lower BACE1 expression trans-synaptically on the olfactory pathway, hence down-regulate β -site APP cleavage and probably A β production at synaptic terminals under physiological conditions.

Functional deprivation promotes amyloid plaque formation in the olfactory pathway

The current anatomical and biochemical study shows that functional deprivation can promote the age-dependent plaque pathogenesis in Tg2576 mouse olfactory centers, which is featured by coincident formation of BACE1 immunoreactive swollen axon terminals associated with local A β accumulation. Several points regarding this finding are of note. First, the deprivation effect on BACE1/A β labeling is detectable around, but not apparently before, the age of global plaque onset in Tg2576 mouse brain. Therefore, this experimental effect is contingent upon age-dependent factor(s) governing plaque formation. Second, BACE1 labeled neurites and A β deposition occur first in the piriform cortex then in the bulb, and functional deprivation does not alter this overall spatiotemporal order. Third, the co-occurrence of localized BACE1 and A β labelings with age in Tg2576 mouse olfactory centers appears to implicate a key role for axonal A β production and secretion in plaque formation, as we have recently characterized in the forebrain of two other transgenic models of AD (Zhang et al., 2009).

Because naris-occlusion caused apparent and correlated elevations of BACE1 proteins, activity and products, it is plausible that BACE1 upregulation plays a major role in the increased plaque pathogenesis seen in the deprived olfactory centers. However, olfactory deprivation also causes other cellular changes that potentially affect plaque pathogenesis. For instances, sensory deprivation enhances apoptosis in the developing and adult olfactory bulb (Najbauer et al.,

2002; Xiong et al., 2008). Deprivation also activates glial astrocytes in the olfactory bulb, which may be suggestive of increased cellular or oxidative stress (Martínez García et al., 1991). Both conditions may facilitate amyloidogenesis, including by stimulating BACE1 expression (Velliquette et al., 2005; Dong et al., 2009; Xiong et al., 2007). Moreover, one might argue the concurrent elevation of the amyloidogenic proteins (APP, BACE1 and PS1), along with other molecules (e.g., VGLUT1 as shown in this study), simply a part of axonal/neuritic pathology that is somehow enhanced by deprivation or under conditions of neuronal hypoactivity. Therefore, the increased plaque pathogenesis in the deprived olfactory centers perhaps should be better considered as a synergetic effect of multiple cellular abnormalities.

Olfactory neurons are differentially involved in plaque-related axonal pathology

Dystrophic axonal neurites around amyloid plaques in most forebrain structures may derive from multiple neuronal phenotypes either intrinsic (e.g., cortical principal and interneurons) or extrinsic (e.g., subcortical cholinergic or catecholaminergic neurons) to the plaque-containing regions, as characterized in diseased human and non-human primate cerebral cortex, and in various transgenic models of AD (Struble et al., 1987; Walker et al., 1988; Quinn et al., 2001; Bell et al., 2006; Rodrigo et al., 2004; Perez et al., 2007; Liu et al., 2008; Zhang et al., 2009). However, to our surprise, BACE1 labeled swollen/dystrophic neurites in Tg2576 mouse olfactory centers appear to be mostly glutamatergic axon terminals. Thus, BACE1 immunoreactive axonal neurites might originate largely from mitral cells (and tufted cells), the glutamatergic projection neurons in the bulb (Nakamura et al., 2005). Mitral cell axons project chiefly to the ipsilateral piriform cortex, especially the superficial part of layer I, with their collaterals also terminating in the inner bulb layers (internal plexiform layer, GCL, SEZ) (de Olmos et al., 1978). BACE1-labeled swollen axons and A β deposits (together forming neuritic plaques) in Tg2576 mice are largely present around these terminal fields of the mitral cell axons. Therefore, the earlier occurrence of neuritic plaques in the piriform cortex relative to the bulb might implicate a greater vulnerability of the distal/major mitral cell axon terminals to plaque pathogenesis, relative to their axonal collaterals.

One puzzling finding of this study is that no apparent axonal pathology and A β deposition occur around the glomerular layer, despite a heavy constitutive BACE1 expression and a dramatic deprivation-induced increase of the enzyme at this location. BACE1 expression at the glomeruli appears to occur largely in presynaptic terminals from the olfactory sensory neurons based on its colocalization with VGLUT1 and synaptophysin (Nakamura et al., 2005; Yan et al., 2007, and this study). Thus, despite the same glutamatergic phenotype, the first and the second-order olfactory projection neurons appear to be fundamentally different with regard to their vulnerability to AD-like axonal dystrophy. Also surprising is that BACE1 labeling around the neuritic plaques rarely colocalizes with immature and mature interneuron markers in the bulb and piriform cortex. Why are the olfactory sensory neurons and interneurons less prone to AD-like axonal pathology? For the immature and mature granule cells, it is perhaps simply because they are axonless, hence would not exhibit axonal pathology. As for the olfactory sensory neurons and periglomerular interneurons, their rare or infrequent axonal pathology might potentially relate to the fact that these neurons are renewable and highly plastic (Calof et al., 1996; Lledo and Saghatelian, 2005). Thus, perhaps the relatively short life span of these neurons does not allow enough time for the development of full-range axonal pathology. Alternatively, because of their dynamic plasticity, these neurons might possess a great capability to eliminate unhealthy/diseased axonal elements before the latter evolve into swollen/dystrophic neurites.

In summary, this study further confirms a negative modulation of BACE1 expression by neuronal activity in rodent olfactory system. We show a promotive effect of functional deprivation on age-dependant neuritic plaque development in Tg2576 mouse olfactory centers.

Our double labeling data support a potential role of axonal A β genesis in neuritic plaque formation in Tg2576 mouse forebrain, as with other transgenic models of AD (Zhang et al., 2009).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This study was supported in part by Illinois Department of Public Health (X.X.Y., R.W.C., R.G.S.), and National Institute of Health (1R21NS056371 to P.R.P., X.X.Y.), intramural program of the National Institute on Aging (H.C.) and Hunan Natural Science Foundation (07JJ5026 to X.K.). We thank Elan, Drs. H. Mori and S. Gandy for providing A β and PS1 antibodies.

References

- Adlard PA, Perreau VM, Pop V, Cotman CW. Voluntary exercise decreases amyloid load in a transgenic model of Alzheimer's disease. *J. Neurosci* 2005;25:4217–4221. [PubMed: 15858047]
- Bell KF, Ducatenzeiler A, Ribeiro-da-Silva A, Duff K, Bennett DA, Cuello AC. The amyloid pathology progresses in a neurotransmitter-specific manner. *Neurobiol. Aging* 2006;27:1644–1657. [PubMed: 16271419]
- Billings LM, Green KN, McGaugh JL, LaFerla FM. Learning decreases A beta*56 and tau pathology and ameliorates behavioral decline in 3xTg-AD mice. *J. Neurosci* 2007;27:751–761. [PubMed: 17251414]
- Braak H, Braak E. Evolution of the neuropathology of Alzheimer's disease. *Acta Neurol. Scand* 1996;165:3–12.
- Cai H, Wang Y, McCarthy D, Wen H, Borchelt DR, Price DL, Wong PC. BACE1 is the major beta-secretase for generation of A beta peptides by neurons. *Nat. Neurosci* 2001;4:233–234. [PubMed: 11224536]
- Calof AL, Hagiwara N, Holcomb JD, Mumm JS, Shou J. Neurogenesis and cell death in olfactory epithelium. *J. Neurobiol* 1996;30:67–81. [PubMed: 8727984]
- Cullinan WE, Brunjes PC. Unilateral odor deprivation: effects on the development of staining for olfactory bulb succinate dehydrogenase. *Brain Res* 1987;432:35–42. [PubMed: 2820549]
- de Olmos J, Hardy H, Heimer L. The afferent connections of the main and the accessory olfactory bulb formations in the rat: an experimental HRP-study. *J. Comp. Neurol* 1978;181:213–244. [PubMed: 690266]
- Dong Y, Zhang G, Zhang B, Moir RD, Xia W, Marcantonio ER, Culley DJ, Crosby G, Tanzi RE, Xie Z. The common inhalational anesthetic sevoflurane induces apoptosis and increases beta-amyloid protein levels. *Arch. Neurol* 2009;66:620–631. [PubMed: 19433662]
- Fratiglioni L, Wang HX. Brain reserve hypothesis in dementia. *J. Alzheimers Dis* 2007;12:11–22. [PubMed: 17851191]
- Fukumoto H, Cheung BS, Hyman BT, Irizarry MC. Beta-secretase protein and activity are increased in the neocortex in Alzheimer disease. *Arch. Neurol* 2002;59:1381–1389. [PubMed: 12223024]
- Holsinger RM, McLean CA, Beyreuther K, Masters CL, Evin G. Increased expression of the amyloid precursor beta-secretase in Alzheimer's disease. *Ann. Neurol* 2002;51:783–786. [PubMed: 12112088]
- Hsiao K, Chapman P, Nilsen S, Eckman C, Harigaya Y, Younkin S, Yang F, Cole G. Correlative memory deficits, A beta elevation, and amyloid plaques in transgenic mice. *Science* 1996;274:99–102. [PubMed: 8810256]
- Jankowsky JL, Melnikova T, Fadale DJ, Xu GM, Slunt HH, Gonzales V, Younkin LH, Younkin SG, Borchelt DR, Savonenko AV. Environmental enrichment mitigates cognitive deficits in a mouse model of Alzheimer's disease. *J. Neurosci* 2005;25:5217–5224. [PubMed: 15917461]

- Jin BK, Franzen L, Baker H. Regulation of c-Fos mRNA and fos protein expression in olfactory bulbs from unilaterally odor-deprived adult mice. *Int. J. Dev. Neurosci* 1996;14:971–982. [PubMed: 9010739]
- Johnson BA, Leon M. Chemotopic odorant coding in a mammalian olfactory system. *J. Comp. Neurol* 2007;503:1–34. [PubMed: 17480025]
- Kemppainen NM, Aalto S, Karrasch M, Nägren K, Savisto N, Oikonen V, Viitanen M, Parkkola R, Rinne JO. Cognitive reserve hypothesis: Pittsburgh Compound B and fluorodeoxyglucose positron emission tomography in relation to education in mild Alzheimer's disease. *Ann. Neurol* 2008;63:112–118. [PubMed: 18023012]
- Kim HH, Puche AC, Margolis FL. Odorant deprivation reversibly modulates transsynaptic changes in the NR2B-mediated CREB pathway in mouse piriform cortex. *J. Neurosci* 2006;26:9548–9559. [PubMed: 16971539]
- Laurin D, Verreault R, Lindsay J, MacPherson K, Rockwood K. Physical activity and risk of cognitive impairment and dementia in elderly persons. *Arch. Neurol* 2001;58:498–504. [PubMed: 11255456]
- Lazarov O, Robinson J, Tang YP, Hairston IS, Korade-Mirnic Z, Lee VM, Hersch LB, Sapolsky RM, Mirnic K, Sisodia SS. Environmental enrichment reduces A β levels and amyloid deposition in transgenic mice. *Cell* 2005;120:701–713. [PubMed: 15766532]
- Li R, Lindholm K, Yang LB, Yue X, Citron M, Yan R, Beach T, Sue L, Sabbagh M, Cai H, Wong P, Price D, Shen Y. Amyloid beta peptide load is correlated with increased beta-secretase activity in sporadic Alzheimer's disease patients. *Proc. Natl. Acad. Sci. U.S.A* 2004;101:3632–3637. [PubMed: 14978286]
- Liu Y, Yoo MJ, Savonenko A, Stirling W, Price DL, Borchelt DR, Mamounas L, Lyons WE, Blue ME, Lee MK. Amyloid pathology is associated with progressive monoaminergic neurodegeneration in a transgenic mouse model of Alzheimer's disease. *J. Neurosci* 2008;28:13805–13814. [PubMed: 19091971]
- Lledo PM, Saghatelian A. Integrating new neurons into the adult olfactory bulb: joining the network, life-death decisions, and the effects of sensory experience. *Trends Neurosci* 2005;28:248–254. [PubMed: 15866199]
- Martínez García MC, Cuervas-Mons Finat M, Latorre Macarrón E, Bullón Sopelana MM, Gayoso Rodríguez MJ. Responses of the astroglia in sensory deprived olfactory bulb of developing rats. *Histol. Histopathol* 1991;6:235–239. [PubMed: 1802122]
- Najbauer J, Yan XX, Leon M. Internucleosomal DNA fragmentation during deprived and non-deprived olfactory development. *Brain Res* 2002;926:118–125. [PubMed: 11814413]
- Nakamura K, Hioki H, Fujiyama F, Kaneko T. Postnatal changes of vesicular glutamate transporter (VGLUT)1 and VGLUT2 immunoreactivities and their colocalization in the mouse forebrain. *J. Comp. Neurol* 2005;492:263–288. [PubMed: 16217795]
- Nestor PJ, Fryer TD, Smielewski P, Hodges JR. Limbic hypometabolism in Alzheimer's disease and mild cognitive impairment. *Ann. Neurol* 2003;54:343–351. [PubMed: 12953266]
- Oka Y, Takai Y, Touhara K. Nasal airflow rate affects the sensitivity and pattern of glomerular odorant responses in the mouse olfactory bulb. *J. Neurosci* 2009;29:12070–12078. [PubMed: 19793965]
- Perez SE, Dar S, Ikonovic MD, DeKosky ST, Mufson EJ. Cholinergic forebrain degeneration in the APP^{swe}/PS1^{DeltaE9} transgenic mouse. *Neurobiol. Dis* 2007;28:3–15. [PubMed: 17662610]
- Perneczky R, Hartmann J, Grimmer T, Drzezga A, Kurz A. Cerebral metabolic correlates of the clinical dementia rating scale in mild cognitive impairment. *J. Geriatr. Psychiatry Neurol* 2007;20:84–88. [PubMed: 17548777]
- Philpot BD, Foster TC, Brunjes PC. Mitral/tufted cell activity is attenuated and becomes uncoupled from respiration following naris closure. *J. Neurobiol* 1997;33:374–386. [PubMed: 9322155]
- Quinn J, Davis F, Woodward WR, Eckenstein F. Beta-amyloid plaques induce neuritic dystrophy of nitric oxide-producing neurons in a transgenic mouse model of Alzheimer's disease. *Exp. Neurol* 2001;168:203–212. [PubMed: 11259108]
- Reiman EM, Caselli RJ, Chen K, Alexander GE, Bandy D, Frost J. Declining brain activity in cognitively normal apolipoprotein E epsilon 4 heterozygotes: A foundation for using positron emission tomography to efficiently test treatments to prevent Alzheimer's disease. *Proc. Natl. Acad. Sci. U.S.A* 2001;98:3334–3339. [PubMed: 11248079]

- Rodrigo J, Fernández-Vizarr P, Castro-Blanco S, Bentura ML, Nieto M, Gómez-Isla T, Martínez-Murillo R, Martínez A, Serrano J, Fernández AP. Nitric oxide in the cerebral cortex of amyloid-precursor protein (SW) Tg2576 transgenic mice. *Neurosci* 2004;128:73–89.
- Roe CM, Xiong C, Miller JP, Cairns NJ, Morris JC. Interaction of neuritic plaques and education predicts dementia. *Alzheimer Dis. Assoc. Disord* 2008;22:188–193. [PubMed: 18525294]
- Sarsoza F, Saing T, Kaye R, Dahlin R, Dick M, Broadwater-Hollifield C, Mobley S, Lott I, Doran E, Gillen D, Anderson-Bergman C, Cribbs DH, Glabe C, Head E. A fibril-specific, conformation-dependent antibody recognizes a subset of Aβ plaques in Alzheimer disease, Down syndrome and Tg2576 transgenic mouse brain. *Acta Neuropathol* 2009;118:505–517. [PubMed: 19360426]
- Selkoe DJ. Toward a comprehensive theory for Alzheimer's disease. Hypothesis: Alzheimer's disease is caused by the cerebral accumulation and cytotoxicity of amyloid beta-protein. *Ann. N.Y. Acad. Sci* 2000;924:17–25. [PubMed: 11193794]
- Struble RG, Powers RE, Casanova MF, Kitt CA, Brown EC, Price DL. Neuropeptidergic systems in plaques of Alzheimer's disease. *J. Neuropathol. Exp. Neurol* 1987;46:567–584. [PubMed: 2442313]
- Tyler SJ, Dawbarn D, Wilcock GK, Allen SJ. α- and β-secretase: profound changes in Alzheimer's disease. *Biochem. Biophys. Res. Commun* 2002;299:373–376. [PubMed: 12445809]
- Velliquette RA, O'Connor T, Vassar R. Energy inhibition elevates β-secretase levels and activity and is potentially amyloidogenic in APP transgenic mice: possible early events in Alzheimer's disease pathogenesis. *J. Neurosci* 2005;25:10874–10883. [PubMed: 16306400]
- Walker LC, Kitt CA, Cork LC, Struble RG, Delovade TL, Price DL. Multiple transmitter systems contribute neurites to individual senile plaques. *J. Neuropathol Exp. Neurol* 1988;47:138–144. [PubMed: 2828554]
- Wang L, Shim H, Xie C, Cai H. Activation of protein kinase C modulates BACE1-mediated β-secretase activity. *Neurobiol. Aging* 2008;29:357–367. [PubMed: 17157415]
- Wesson DW, Verhagen JV, Wachowiak M. Why sniff fast? The relationship between sniff frequency, odor discrimination, and receptor neuron activation in the rat. *J. Neurophysiol* 2009;101:1089–10102. [PubMed: 19052108]
- Xiong K, Cai H, Luo XG, Struble RG, Clough RW, Yan XX. Mitochondrial respiratory inhibition and oxidative stress elevate β-secretase (BACE1) proteins and activity in vivo in the rat retina. *Exp. Brain Res* 2007;181:435–346. [PubMed: 17429617]
- Xiong K, Luo DW, Patrylo PR, Luo XG, Struble RG, Clough RW, Yan XX. Doublecortin-expressing cells are present in layer II across the adult guinea pig cerebral cortex: partial colocalization with mature interneuron markers. *Exp. Neurol* 2008;211:271–82. [PubMed: 18378231]
- Yan XX, Xiong K, Luo XG, Struble RG, Clough RW. β-Secretase expression in normal and functionally deprived rat olfactory bulbs: inverse correlation with oxidative metabolic activity. *J. Comp. Neurol* 2007;501:52–69. [PubMed: 17206602]
- Yang LB, Lindholm K, Yan R, Citron M, Xia W, Yang XL, Beach T, Sue L, Wong P, Price D, Li R, Shen Y. Elevated β-secretase expression and enzymatic activity detected in sporadic Alzheimer disease. *Nat. Med* 2003;9:3–4. [PubMed: 12514700]
- Yu F, Kolanowski AM, Strumpf NE, Eslinger PJ. Improving cognition and function through exercise intervention in Alzheimer's disease. *J. Nurs. Scholarsh* 2006;38:358–365. [PubMed: 17181084]
- Zhang XM, Cai Y, Xiong X, Cai H, Luo XG, Feng JC, Clough RW, Struble RG, Patrylo PR, Yan XX. BACE1 elevation in transgenic mouse models of Alzheimer's disease is associated with synaptic/axonal pathology and amyloidogenesis: Implication for neuritic plaque development. *Eur. J. Neurosci.* 2009 in press.

Fig. 1.

Inverse modulation between β -secretase-1 (BACE1) and cytochrome c oxidase (CO) reactivities in Tg2576 mouse olfactory bulb and piriform cortex following functional deprivation. Panels A-F are coronal sections with the left and right olfactory structures matched precisely, from a 7-month-old animal 1 month post unilateral naris-occlusion stained for BACE1 and CO. BACE1 immunoreactivity is clearly stronger on the deprived side in the glomerular layer (GL) and the external plexiform layer (EPL) in the bulb (A), and in layer I (especially its superficial part or lamina Ia) in the piriform cortex (C, E, representing two coronal levels). In contrast, CO reactivity is clearly lighter in these same laminae on the deprived side (B, D, F). Similar opposite alterations in BACE1 (E) and CO (F) reactivities are noticeable in layers II/III of the piriform cortex in the deprived side relative counterpart. Densitometric data from animals surviving 1 (n=3) and 2 (n=3) months post occlusion (combined) indicate statistically significant increase of BACE1 (G) and decrease of CO (H) reactivities in the GL, EPL and layer I (Pir-I) in the deprived relative to the non-deprived olfactory centers. lot: lateral olfactory tract; *: $p < 0.001$; #: $p < 0.01$. Scale bar in A = 200 μ m applying to for B-D and 500 μ m for E, F.

Fig. 2.

Occurrence of localized BACE1 reactivity and amyloid deposition in the piriform cortex in 9 (A-F) and 12 (G-I) month-old Tg2576 mice occluded at 6 month of age, with noticeably increased labelings in the deprived side relative to the non-deprived side. Antibodies 3D6 and Ter-40 show comparable pattern and amount of Aβ immunoreactivity in the forebrain. Panel A is a low-power view of a ventral forebrain section showing dramatically stronger BACE1 labeling in the glomerular layer in the deprived bulb. At higher magnification, small and discretely-distributed BACE1 immunoreactive profiles are seen in the deprived (B, framed area and enlarged insert) but not in the non-deprived (C) piriform cortex. Large BACE1 and Aβ immunoreactive profiles appear matchable in location between adjacent sections (D-E, arrows). Panel G is a representative image of Aβ labeling with Nissl counterstain from a 12 month-old animal showing increased plaques in the deprived piriform cortex relative to counterpart (H, I). Amyloid plaques are occasionally seen around the core of the bulb at this

age (small arrows in G). lot: lateral olfactory tract; Arab numbers and short bars indicate the lamination of the piriform cortex. Scale bar in A = 1 mm, equal to 200 μm for B-F. Scale bar in J = 1 mm, equal to 100 μm for K and L.

Fig. 3.

Increased BACE1 reactivity in the deprived olfactory centers in 18 (A, B, F) and 24 (C-F) month-old occluded Tg2576 mice. Shown are 2 (A, B) and 3 (C-E) levels of sections parallel to the ventral surface of the olfactory bulbs (OB). Plaque-like BACE1 immunoreactive profiles occur in both the piriform cortex and bulb, with more labeled profiles on the deprived side. The constitutive BACE1 reactivity in the glomerular layer is dramatically heavier in the deprived side at all levels. Bar graph (G) shows increased specific densities of BACE1 labeling in the deprived than the non-deprived bulb and piriform cortex (Pir). ST: striatum; *: $p < 0.05$. Scale bar (in A) = 1mm for all image panels.

Fig. 4. Increased Aβ reactivity in the deprived relative to non-deprived olfactory centers in 18 (A, B, F) and 24 (C-F) month-old Tg2576 mice. Representative images are at 2 and 3 different ventrodorsal brain levels. Aβ reactivity exhibits typical amyloid plaque morphology, and labeled profiles are denser in the deprived piriform cortex (Pir) and bulb (OB) relative to counterparts (F). *: $p < 0.05$. Scale bar (in A) = 1mm for all image panels.

Fig. 5.

Elevations of BACE1 proteins, enzymatic activity and immediate and end products of β -cleavage pathway of amyloid precursor protein (APP) in deprived olfactory bulbs relative to counterparts. Western blot analyses (A, B) show increased levels [normalized to β -tubulin optical density (o.d.) in the same sample] of the glycosylated 70 kd and deglycosylated 46 kd BACE1 proteins, β -cleavage APP C-terminal fragments (β -CTF) as well as A β 40 and A β 42 in the deprived bulbs from 24 month-old Tg2576 mice. No difference in the levels of putative human APP proteins (blotted with 6E10) is detectable in tissue homogenate between the deprived and non-deprived bulbs. Panels C and D show increased β -site APP cleavage activity, and elevated levels of A β 42 and A β 40, respectively, in the deprived relative to non-deprived bulbs from 18 month-old animals, as determined using enzyme activity assay and ELISA. Asterisks (*) indicate statistically significant difference between deprived sample group relative to internal control.

Fig. 6.

Morphology of BACE1 immunoreactive neuritic elements in the olfactory bulb (A-C) and piriform cortex (D-F) from a 24 month-old animal. BACE1 labeled neurites vary in size and shape, occur mostly in the GCL and SEZ in the bulb, and commonly in layers I and II in the piriform cortex. These labeled elements may exhibit strong reactivity comparable to that in the glomeruli (A). The smallest profiles are lightly-stained and isolated spherical structures (arrows). Small to medium-sized profiles are clusters of swollen processes arranged in a rosette-like fashion (arrowheads). Large profiles consist of a few or several closely apposed rosette-like neuritic clusters. Long and thin neuronal processes are sometimes visible around, and appear to join, the neuritic clusters (double arrows in F). Scale bar=100 μ m in A applying to D, equal to 25 μ m for B, C, E and F.

Fig. 7.

Colocalization of BACE1-labeled swollen neurites with A β (3D6 antibody reaction, A-I) and vesicular glutamate transporter-1 (VGLUT-1) (J-R) immunoreactivities in the bulb and piriform cortex (from a 12 month-old animal). BACE1 and 3D6 labelings appear to fully colocalize at low magnification (A-C). At higher magnification, BACE1 and A β reactivity is seen in small isolated spherical elements as well as neuritic clusters in varying sizes (examples are marked with frames and enlarged insets) (D-F). Around established neuritic plaques, A β reactivity may colocalize with as well as spread between or around BACE1 labeled neuritic profiles (G-I). BACE1 and VGLUT-1 reactivities colocalize in discretely-distributed small swollen axons (arrows) as well as densely-packed large neuritic clusters in the GCL and SEZ

of the bulb or over layers I and II in the piriform cortex. Strong and distinct VGLUT-1 reactivity is present in the glomeruli (K). Scale bar (in A) = 500 μm for A-C, J-L; 100 μm for D-F, P; and 50 μm for G-I, M-O, Q, R.

Table 1
Primary antibodies used in the present study

Antibody	Source	Product #	Dilution
mouse anti-A β 1-5, 3D6	Elan	Lot#1132	1:3000
mouse anti-A β 1-16, 6E10	Signet	39320	1:4000
rabbit anti-A β 35-40	H. Mori	Ter-40	1:3000
rabbit anti-A β 38-42	H. Mori	Ter-42	1:3000
mouse anti-amyloid precursor protein, 22C11	Millipore	MAB348	1:1000
rabbit anti-amyloid precursor protein	Serotec	AHP538	1:1000
rabbit anti-BACE1 (a.a. 46-163)	H. Cai	anti-BACE1 α	1:2000
rabbit anti-BACE1	Chemicon	AB5940	(1:400)
rabbit anti-glutamic acid decarboxylase (GAD) 65/67	Millipore	AB1511	1:2000
mouse anti-GAD67	Millipore	MAB5406	1:4000
mouse anti-growth- associated protein	Sigma	G9264	1:4000
mouse anti-microtubule associated protein-2	Sigma	M9942	1:1000
rabbit anti-presenilin-1	S. Gandy	ab14	1:500
mouse anti-parvalbumin	Sigma	P3088	1:4000
mouse anti-synaptophysin	Millipore	MAB329	1:4000
rabbit anti- β -tubulin-III	Sigma	T2200	1:10000
mouse anti-tyrosine hydroxylase	Sigma	T2928	1:4000
mouse anti-vesicular glutamate transporter-1	Millipore	MAB5502	1:2000