

Uncovering the invisible giant: Amyloid beta plaques and their proposed association with waste removal in Alzheimer-affected human hippocampus

Ruth Fabian-Fine

rfabianfine@smcvt.edu

Saint Michael's College

Abigail G. Roman

Saint Michael's College

Melanie J. Winters

Saint Michael's College

Kyleena J. Lathram

Saint Michael's College

Carly H. Bennett

Saint Michael's College

Luwago K. Kipingi

Saint Michael's College

Sondre M. Brännare-Gran

Saint Michael's College

Abigail E Whitley

Saint Michael's College

Chloe M. Paul

Saint Michael's College

Lydia M. Altman

Saint Michael's College

Ian C. Carrillo

Saint Michael's College

Finn M. Joyce

Saint Michael's College

Lydia A. Kragh

Saint Michael's College

Theodore J. McKnight

Saint Michael's College

Calum J. Reding

Saint Michael's College

Leaf J. D. Reiderer

Saint Michael's College

Lesley J. Rivera

Saint Michael's College

Hannah A. Steen

Saint Michael's College

Adam L. Weaver

Saint Michael's College

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4 Ruth Fabian-Fine^{1*}, Abigail G. Roman¹, Melanie J. Winters¹, Kyleena J. Lathram¹, Carly H.
5 Bennett¹, Luwago K. Kipongi¹, Sondre M. Brännare-Gran¹, Abigail E Whitley¹, Chloe M. Paul¹, Lydia
6 M. Altman^{1,2}, Ian C. Carrillo^{1,2}, Finn M. Joyce^{1,2}, Lydia A. Kragh^{1,2}, Theodore J. McKnight^{1,2}, Calum
7 J. Reding^{1,2}, Leaf J. D. Reiderer^{1,2}, Lesley J. Rivera^{1,2}, Hannah A. Steen^{1,2}, and Adam L. Weaver¹

8 ¹ Department of Biology, Saint Michael's College, Colchester, VT 05439, USA

9 ² Students participating in the 2025 Developmental Biology Course-Based Undergraduate
10 Research Experience (CURE) course.

11

12 * Corresponding author

13 E-mail: rfabianfine@smcvt.edu

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17 **ABSTRACT**

18 According to the prevalent 'Amyloid Hypothesis,' the underlying cause for neurodegeneration in
19 Alzheimer Disease (AD) is attributed to the accumulation of misfolded Amyloid β and tau protein in
20 the form of extracellular sticky plaques and neurofibrillary tangles, respectively. These protein
21 accumulations are thought to be caused by impaired waste removal. In an alternative hypothesis,
22 we have proposed the existence of an extensive glial canal system that is likely formed by
23 myelinated aquaporin-4 (AQP4)-expressing tanycytes and removes cellular waste from the
24 hippocampal formation. Here, we demonstrate that tanycyte-derived waste-internalizing
25 receptacles are immunoreactive for A β and emanate from specialized nucleus-like organelles in
26 the following referred to as 'tansomes.' Utilizing RNA-scope in situ hybridization, we demonstrate
27 that these receptacle-forming tansomes express RNA for AQP4 and the A β -related genes,
28 amyloid precursor protein, and presenilin-1. These findings suggest that A β is likely synthesized
29 where receptacle formation is observed and that A β may play an important structural role in
30 receptacle formation. In AD-affected hippocampus, excessive amounts of A β -immunoreactive
31 waste receptacles emerge from tansomes and have the appearance of plaques in A β -
32 immunolabeled hippocampus. Moreover, we demonstrate that the same receptacle-forming
33 organelles exhibit strong immunolabeling for hyperphosphorylated tau protein in AD-affected
34 tissue. We postulate that both proteins may play important structural roles in waste uptake and that
35 hypertrophic swelling of impaired tanycytes in AD-affected brain may be due to obstructions of this
36 extensive interconnected glial canal system.

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Keywords: Aquaporin 4, Myelin, Tanycyte, Convective flow, Neurodegeneration

Introduction

Over 50 million people worldwide are believed to be affected by progressive neurodegenerative diseases (Asher and Priefer, 2022, Breitner, et al., 2022, Carlsson, 2008, Cummings, et al., 2021, Gustavsson, et al., 2023, Huang, et al., 2019, Sathyanarayana Rao and Jagannatha Rao, 2009, Tatulian, 2022). One of the most prevalent forms of neurodegeneration is associated with Alzheimer disease (AD) that affects roughly 6.9 million USA citizens over the age of 65, thus ranking within the leading causes of death (2024). However, despite massive international research efforts, the underlying causes for AD remain elusive. Our lack of insight into the cellular processes that trigger neurodegeneration may help explain the repeated setbacks the pharmaceutical industry has encountered in clinical trials (Asher and Priefer, 2022, Breitner, Dodge, Khachaturian and Khachaturian, 2022, Carlsson, 2008, Cummings, Lee, Zhong, Fonseca and Taghva, 2021, Huang, Shen and Zhao, 2019, Sathyanarayana Rao and Jagannatha Rao, 2009, Tatulian, 2022).

Over the past several decades, the ‘amyloid hypothesis’ has been the prevalent proposition regarding the underlying causes of AD. One prevalent theory postulates that the presence of

misfolded, 'amyloid beta (A β) plaques' in the brain of AD decedents may be the result of failed waste removal from the brain(Nedergaard, 2013, Pedersen, et al., 2023a, Pedersen, et al., 2023b)

Based on the amyloid hypothesis, these protein aggregates obstruct intra- and extracellular spaces thereby triggering neurodegeneration. This prevailing theory led to drugs designed to clear A β and hyperphosphorylated tau protein from the brain using monoclonal antibodies (2024, Asher and Priefer, 2022, Breitner, Dodge, Khachaturian and Khachaturian, 2022, Carlsson, 2008, Cummings, Lee, Zhong, Fonseca and Taghva, 2021, Gustavsson, Norton, Fast, Frolich, Georges, Holzapfel, Kirabali, Krolak-Salmon, Rossini, Ferretti, Lanman, Chadha and van der Flier, 2023, Huang, Shen and Zhao, 2019, Sathyanarayana Rao and Jagannatha Rao, 2009, Tatulian, 2022). However, these approaches have yielded limited cognitive benefits to patients with numerous trials failing in the clinical phase (Asher and Priefer, 2022). In addition to the suffering experienced by affected individuals and their families, the monetary implications of these failed drug trials are staggering. With an estimated annual cost of US\$ 300 billion in healthcare-related expenses (2024), these numbers highlight the paramount importance to re-evaluate past and current research findings and therapeutic approaches.

Why have we been unable to illuminate the underlying causes for neurodegeneration despite (i) massive international research undertakings, (ii) advanced scientific knowledge, and (iii) increasingly sophisticated biomedical technologies?

With these progress limitations in mind, we argue that all assumptions regarding AD etiology be critically revisited, and basic characterization of healthy and AD-affected cellular changes be re-investigated. In this study, we have revisited cellular structure utilizing high-resolution cell imaging, RNA-scope in situ hybridization, immunohistochemistry, and correlative light- and electron microscopy to investigate where A β -related genes are expressed in relation to A β plaques. Utilizing this knowledge, we studied how plaque-formation starts and how these plaques grow in AD-affected human hippocampus.

Due to the prevalence of rodent-models in AD research, we have also investigated mouse and rat tissue. Based on the findings presented here, we propose that the answer to the

above-stated question may be misidentification of cellular structure. Here, we provide a novel hypothesis that A β plaques in AD-affected hippocampus may not represent accumulations of misfolded A β protein deposits (Kepp, et al., 2023), but may show abnormal proliferation and swelling of proposed waste-internalizing receptacles that are formed by a recently-discovered glial canal system (Fabian-Fine, et al., 2024). We show that the A β -immunolabeled receptacles express the A β -related genes amyloid precursor protein (APP) and presenilin-1 (Pres1). Based on compelling structural evidence provided here, we postulate that the functional significance of A β along waste receptacles is likely their structural stabilization to prevent the collapse of these membranous receptacles during waste intake.

The preliminary findings presented here provide a new perspective into underlying causes for AD-related neurodegeneration. We encourage other researchers to exhaustively investigate and critique our hypotheses.

Materials & Methods

Brain tissue

The human brain tissue investigated here was obtained by Dr. John DeWitt (University of Vermont Medical Center) in the context of a previously published study (Fabian-Fine, Weaver, Roman, Winters and DeWitt, 2024). In brief, the tissue originated from autopsy examinations of four decedents at the University of Vermont Medical Center. The tissue was obtained with full written consent from the next of kin for biomedical research, diagnostic, and teaching purposes, and was fixed (see below) immediately after brain removal. All processes were in accordance with Vermont State law, the Health and Human services regulation 45 CFR 46.102(e), and the University of Vermont regulations regarding human subjects research. Tissue from two 86-year-old decedents (post-mortem intervals of 15 and 16 h) showed accumulations of amyloid β plaques and phosphorylated tau tangles, with ABC scores consistent with intermediate (A2B2C1) and high (A3B3C2) burdens of AD neuropathologic change. Tissue obtained from two 76- and 35-year-old decedents (45- and 14-hours post-mortem intervals) were negative for AD neuropathologic change.

The mouse brain originated from three adult (15-month-old) female wildtype mice (Cdh5-GCaMP8 strain). For ethical reasons and to avoid unnecessary animal sacrifices, the brains used in this study were obtained from surplus tissue. To prevent animal suffering, the animals were euthanized by a fully trained and licensed technician using Euthasol (Virbac). The tissue was obtained freshly upon dissection of the animals and fixed in ice-cold 4% paraformaldehyde in phosphate buffered saline 0.1 M, pH 7.4. IACUC approval was granted under PROTO202200018 (University of Vermont) and IACUC-2024-001 Fabian-Fine (Saint Michael's College). The rat brain was obtained from two healthy three-month-old male Sprague Dawley control rats and embedded into Araldite as described below.

Immunohistochemistry

Brain tissue processed for immunolabeling was fixed in freshly prepared 4% paraformaldehyde (EMS 15710) overnight in the fridge. The tissue was embedded in 4% Agarose (Sigma # A 9539) and sectioned into 70 μ m vibratome sections using a Leica S 1000 vibratome. The sections were washed 4x5 min in 0.1 M phosphate buffered saline pH 7.4 (PBS) prior to immersion in incubation and blocking medium (IBM; 0.25% Bovine Serum Albumin (Sigma A4503), 5% Normal goat serum (Sigma G9023) in 1% Triton-X/PBS) for 20 min. The sections were incubated with the primary antibodies overnight at 6 °C. The primary antibodies utilized were rabbit anti-AQP4 (BiCell #20104), Mouse anti-APP-C99; Sigma Aldrich MABN380, goat-anti mouse anti-Vimentin (AMF-17b-s DSHB, IOWA) and mouse anti-Myelin 6-4H2 DSHB, IOWA, mouse anti-APP-C99; Sigma Aldrich MABN380, and rabbit anti-Presenilin-1 ZRB1614 Sigma Aldrich at dilutions of 1:100 in 10% IBM and 90% PBS.

Subsequently the preparations were washed in PBS 5x10 min, incubated in IBM (20 min) and incubated in the secondary antibodies overnight 6 °C. The secondary fluorochrome-coupled antibodies were Cy3 goat anti-rabbit, Jackson ImmunoResearch Laboratories 111-165-003, and FITC goat anti-mouse Jackson ImmunoResearch Laboratories 115-096-072. Hoechst Blue nuclear stain was used to visualize nucleic acids at a dilution of 1:3000 in PBS for 20 min. After thorough washing in ice cold PBS for 90 min, the vibratome sections were mounted on glass slides and

embedded in Mowiol (Sigma 81381). To avoid bleaching of the secondary antibodies all steps were performed in the absence of sunlight.

The sections were analyzed using a confocal Zeiss AxioImager MZ with Apotome.

Fluorochrome uptake experiments in living mouse brain

To investigate whether waste swell-bodies and associated receptacles in living mouse brain internalize Cy3 fluorochromes, we have exposed freshly dissected mouse brain to these fluorochromes as described previously (Fabian-Fine, et al., 2025).

Immunoperoxidase stain

Immunoperoxidase stain was carried out in the histology lab of the University of Vermont Medical Center. For A β and Tau protein using paraffin-embedded 5 μ m-thick brain sections. The detailed procedure using the Leica Bond-3 auto staining system was described previously (Fabian-Fine, Weaver, Roman, Winters and DeWitt, 2024). The primary antibodies used were β Amyloid 1-42 (mOC 64, AbCam) and Tau AT8 directed against Phospho-Tau Serine 202 and Threonine 205 (AT8, Thermo Scientific # MN1020).

Tissue preparation for electron microscopy

Brain tissue that was processed for electron microscopy was fixed in freshly prepared 4% paraformaldehyde containing 2.5% glutaraldehyde (EMS 16019) in PBS. The brains were embedded in 4% agarose and sectioned into 70-300 μ m vibratome sections. After washing in PBS the sections were post-fixed for 30 min in 1.0% osmium tetroxide (Electron Microscopic Sciences 19150). After rinsing in PBS (2x5 min), the sections were dehydrated using a graded series of ethanol according to standard electron microscopy protocols. The preparations were transferred into propylene oxide (Electron Microscopic Sciences 20401), slowly infiltrated with Araldite (Electron Microscopic Sciences 13900), and polymerized overnight at 60 °C, according to manufacturer's instructions.

174

175 **Ultrathin sectioning**

176 The araldite embedded tissue was trimmed and sectioned with an 8-mm Diatome histo-knife using
177 a Leica Ultracut E at a thickness of 65-nm. To prevent wrinkles, we utilized wooden toothpicks that
178 were soaked with chloroform (Electron Microscopic Sciences, #12540) and held over the sections
179 at approximately 2 mm distance to stretch them. It is important not to touch the sections with the
180 toothpicks as it will damage them. The sections were collected on pioloform-coated single-slot
181 copper or nickel grids (EMS# G2010CU). Contrasting was carried out using aqueous 1.5% uranyl
182 acetate (6 min) and Reynold's lead citrate (5 min) according to standard protocols. Electron
183 microscopic examination was conducted using a JOEL 1400 electron microscope operated at 80
184 kV.

185 **RNAscope in-situ hybridization**

186 We utilized *in-situ* hybridization for the detection of AQP4, Amyloid Precursor Protein and
187 Presenilin-1 gene expression as described earlier using formalin-fixed 5- μ m paraffin embedded
188 human hippocampal sections (Fabian-Fine, Weaver, Roman, Winters and DeWitt, 2024). The
189 complementary probes were designed by Bio-Techne. The paraffin sections were obtained from
190 the histology lab of the University of Vermont Medical Center. These experiments were carried out
191 in the University of Vermont Center for Biomedical Shared Resources.

192 **Controls**

193 Overall RNA integrity was assessed using *in situ* hybridization for the low level expressed
194 'housekeeping' gene peptidylpropyl isomerase B. For negative controls, we utilized the bacterial
195 gene dapB. We routinely assessed RNA integrity (PPIB-positive and dapB-negative) for all
196 samples.

197 **Luxol H&E staining of autopsy tissue**

This staining was performed on paraffin-embedded brain sections (5-10 μ m-thick) following previously described standard protocols (Fabian-Fine, Weaver, Roman, Winters and DeWitt, 2024).

Quantitative analysis

To evaluate the percentage of swell-body stages I-V in both healthy and AD-affected human hippocampus, blinded tissue sections were used to avoid bias. Images of swell-bodies were taken from all areas examined here including the alveus, stratum oriens, and stratum pyramidale. Images of swell-bodies throughout the CA2 and 3 regions were captured and projected onto a ~200 cm x ~110 cm smart board. The swell-body stages were determined and recorded collectively by the participating research team. The group attempted to evaluate comparable numbers of swell-bodies (non-AD: 314; AD: 336). To achieve these comparable swell-body numbers, one additional slice was evaluated for non-AD (4 vs. 3).

A Chi-square test was used to evaluate whether there was a significant association between healthy vs. Alzheimer disease state and the five swell-body stages. Statistics were calculated and the graph was created using Prism 10.5.0 (GraphPad Software).

Image acquisition

Images of the Luxol H&E, anti-A β , and anti-tau immunolabeled paraffin sections were taken using an Olympus compound microscope with digital image acquisition capabilities.

Extended depth of field

To merge microscopic features across multiple focal depths, images were taken with an Olympus BX60 microscope with motorized stage in focal steps of 0.125–0.5 μ m to form an image stack. The stage was computer controlled using Micro-Manager (Edelstein, et al., 2014) and Micro-Magellan (Pinkard, et al., 2016). The image stack was then processed to remove jitter with the Linear Stack Alignment with SIFT rigid transformation (Lowe, 2004), and finally the stack was combined into an output image with the EPFL Extended Depth of Field/Focus (Forster, et al., 2004) plugin [Easy Mode, Quality Setting] in FIJI (Schindelin, et al., 2012).

Image processing

Confocal images were exported from the ZEN Blue program using the 'Image export' function. Pictures and Figures from confocal, histological, and ultrastructural preparations were created using Adobe Photoshop.

Results

Hippocampal tanycytes form a vast network of myelinated cell processes that project into the stratum pyramidale

Ependymal tanycytes reside in the ventricular lining of mouse, rat and human hippocampus that borders on the temporal horn of the lateral ventricles. Their long, slender processes form a vast network that projects into the brain parenchyma in the hippocampal formation (Figure 1a, b). Tanycyte processes also project into the brain parenchyma of surrounding areas (Figure 1). The findings on human brain presented in the following were investigated and appeared consistent throughout all areas where tanycyte processes were observed as indicated in Figure 1a. The Luxol blue H&E stain of tanycyte processes is indicative of their myelinated nature (Figure 1). Individual tanycyte processes form electron-lucent 'swell-bodies,' each containing between one and six tanycyte-associated nucleus-like organelles in the following referred to as 'tansomes' (Figure 1c-g). Swell-bodies can easily be distinguished from cell-somata, due to their lack of cytoplasmic content or organelles (Figure 1h) typically found in cell bodies (Figure 1g, mouse hippocampal neuron). In contrast to cell nuclei, tansomes are associated with tanycyte processes (Figure 1e-h) and give rise to receptacles that emanate from (i) the tansome, and (ii) surrounding myelinated tanycyte profiles (Figures 1g, h; 2a). These receptacles often project into neuronal somata (Figures 1j, 2). Interestingly, in both human and mouse hippocampus the tanycytes contain myelinated ring structures that swell, thereby enlarging the inner lumina of tanycyte processes in areas where these 'luminal rings' are inflated (Figure 1k, l *Luxol blue-stain*; Figure 1m, n *ultrastructural depiction*). Immunolabeling shows the aquaporin4- and myelin immunoreactive nature of tanycyte processes and reveals the periodic spacing of these myelin-immunolabeled luminal rings at 3-5 μm intervals (Figure 1o). The varicose appearance of these myelinated processes is consistently observed in

mouse, rat, and human brain at both the light- and electron-microscopic levels and distinguishes tanyocyte profiles from neuronal axons (Figure 1u-x). As demonstrated at the ultrastructural level in rat hippocampus, ependymal tanyocytes are connected through cytoplasmic canals (Figure 1r). This syncytial network sends vast numbers of varicose myelinated cell processes into the underlying hippocampal formation where they form receptacles that project into neurons and surrounding tissue. Interestingly, swelling tanyocyte projections are frequently observed contacting neurons at the axon hillock (Figure 1u-w).

Tanyocyte-derived receptacles internalize electron-dense waste within the neuronal cytoplasm

Luxol blue H&E-stained neurons reveal that myelinated tanyocytes project into neuronal somata and form numerous circular receptacles (Figure 2). The receptacles either emanate from tanyocyte processes directly (Figure 2a, b), or from tansomes that contact the neuronal soma (Figure 2c). In Alzheimer affected brain both, the receptacles and their associated tanyocyte processes show hypertrophic swelling that results in obstruction and gradual depletion of affected neurons and their outer perimeter due to swelling varicosities that emanate from affected tanyocytes (Figure 2a-d, r, s). Interestingly, individual tanyocyte processes can be seen to contact several adjacent neurons in a serial fashion explaining hypertrophic swelling in adjacent neurons (Figure 2d, see also below). Varicosity-forming, myelinated tanyocyte processes can be distinguished from neuronal axons due to their smaller diameters and different staining patterns that are clearly visible in the Luxol H&E-stained preparations (Figure 2e). Neuronal axons lack translucent varicosities that are characteristic to ependymal tanyocytes (Figure 2e-g). In non-AD affected toluidine blue-stained semithin sections through human hippocampus the tanyocyte-receptacles appear yellow (Figure 2h). Ultrastructural examination of healthy brain shows that this discoloration is due to the internalization of electron-dense material into centrally located

repositories. At the subcellular level forming receptacles can be seen to emanate from myelinated tanyocyte processes that project into the cytoplasm of adjacent cells (Figure 2j) consistent with our observations in Luxol H&E-stained preparations (Figure 2k). The formation of varicose tanyocyte processes that project into a neuronal soma is clearly visible in vibratome sections through AD-affected brain tissue that contains intact neuronal somata (2k). At the ultrastructural level receptacles consist of lateral electron-lucent swellings that have openings to the neuronal cytoplasm and contain internalized vesicular structures (Figure 2l-n). Interestingly, the electron-dense center of waste receptacles contains a circular opening that internalizes vesicular structures that resemble those observed in the surrounding electron-lucent receptacles (Figure 2l, m). Immunolabeling for the tanyocyte marker vimentin and AQP4 show vimentin-immunolabeled receptacles that are arranged around a centrally located AQP4-immunoreactive varicosity similar to the ultrastructural appearance of waste receptacles (Figure 2l; inset). Anti-A β immunolabeling demonstrates that these intraneuronal receptacles stain for this protein (Figure 2o-q). Interestingly, the A β -immunoreactivity along intraneuronal receptacles is observed in healthy neurons and stains centrally located toroid-shaped canal and tubular structures that resemble canal openings (insets in 2o-q). Compared to healthy human neurons Figure 2h-q), degenerating neurons in AD-affected human brain show severe structural degradation (Figure 2r-t). Proliferating and excessively swelling tanyocyte protrusions explain the gradual replacement of neuronal cytoplasm by hypertrophic tanyocyte processes and swelling waste receptacles (Figure 2r, s). The emergence of swelling waste receptacles from myelinated tanyocyte processes that replace the neuronal cytoplasm can be seen at the ultrastructural level (Figure 2s). In advanced degeneration only the neuronal nucleus and some waste receptacles remain, whereas the rest of the neuron appears depleted (Figure 2t).

Tanycyte-derived swell-bodies differentiate waste-internalizing toroids and receptacles that show strong immunolabeling for anti-A β and anti-tau protein.

Light- and electron-microscopic investigation of swell-bodies in human, non-AD-affected hippocampus shows that each swell-body contains one or more nucleus-like organelles that stain for nuclear stain and are referred to as 'tansomes' (Figure 3). Three primary features distinguish tansomes from conventional cell nuclei. (i) The lumina of swell-bodies are void of cytoplasm and appear electron-lucent at both light and electron-microscopic levels (Figure 3a, b). (ii) tansomes are penetrated by myelinated tanycyte processes (Figure 3c, d) through clearly visible pores that can reach diameters of $>2\ \mu\text{m}$ (Figure 3c, see also below). (iii) tansomes give rise to myelin-derived receptacles that internalize electron-dense material consistent with cellular waste (Figures 3; 4; see also below). We have identified five distinct stages of receptacle differentiation in swell-bodies investigating both non-AD and AD-affected brain tissue. *Stage I* swell-bodies are $\sim 6\text{-}20\ \mu\text{m}$ in diameter, have electron-lucent lumina and contain one tanycyte-associated tansome (Figure 3f, *Stage I*). *Stage II* tansomes give rise to either donut-shaped 'toroids,' several smaller receptacles or both. The receptacles stain for Luxol-blue, a histological marker for myelin. Interestingly, both toroids and receptacles also show immunolabeling for anti-A β protein in both AD and non-AD-affected hippocampus. In AD-affected tissue, these structures are also labeled for anti-tau protein (Figure 3f, *Stage II*). In *Stage III* swell-bodies, the toroids swell and separate from the tansomes but remain connected to the tansomes via thin translucent connections. Increasing numbers of waste receptacles can be observed in individual swell-bodies (Figure 3f, *Stage III*). Both toroids and receptacles continue to increase in size and number in *Stage IV* swell-bodies (Figure 3f, *Stage IV*) and project out of the swell-bodies in *Stage V*. The anti-tau and anti-A β immunolabeling observed in AD-affected tissue appears more prominent in *Stage V* compared to Stages I-IV and is consistently associated with both receptacles and toroidal structures (Figure 3f). The results of the Chi-square test ($\chi^2(4, N = 314\text{ [healthy]}, 336\text{ [Alzheimer]}) = 45.75, p < 0.0001$) indicate a significant association between disease state and swell-body stages (Figure 3g), although the small number of replicates are only suggestive of a trend that should be further investigated systematically.

Tanysomes and associated receptacles and toroids express the A β -related genes amyloid precursor protein (APP) and presenilin-1 (Pres1) and give rise to A β -plaques

Immunolabeling of AD-affected human hippocampus for A β , anti-Tau protein, the A β -related protein APP in addition to the AQP4 water channel demonstrate the association of these proteins with toroids and waste receptacles in swell-bodies (Figure 4). The appearance of immunolabeled swell-bodies and receptacles is consistent in size, shape, and location with Luxol blue H&E-stained preparations (Figure 3). Furthermore, gene expression patterns for APP, Pres1, and AQP4 are consistent with the expression of these proteins in swell-bodies (Figure 4i-o; Figure 5a-h) and support our hypothesis that A β and AQP4 may play important functional roles in waste uptake into tanysome-derived waste receptacles (see discussion). Immunolabeling for A β (Figure 4p), AQP4, and APP (Figure 4q-s) reveals a vast network of smaller receptacles that emanate from swell-bodies and tanycyte processes (see also below). These smaller immunolabeled sites may easily be dismissed as background labeling. However careful examination of brain sections using confocal microscopy and Differential Interference Contrast microscopy consistently show that these smaller immunolabeled sites that surround swell-bodies depict numerous translucent receptacles whose circular centers are stained for AQP4, APP, and A β (Figure 4p-s).

Progressive formation of A β -plaques

Figure 5I-Ai demonstrates the progression from Stage II swell-bodies to extremely hypertrophic Stage V swell-bodies in AD-affected A β -immunolabeled human brain. *Stage II* swell-bodies (Figure 5i) progressively transition into *Stage III* where the toroids and emanating receptacles separate from their associated tanysomes (Figure 5j-m). As the toroids and associated receptacles continue to swell and transition into *Stages III* and *IV*, the A β -immunolabeling appears more prominent (Figure 5n-q). As these swell-bodies progress into *Stage V* (Figure 5r-ai), the areas surrounding affected swell-bodies are densely obstructed with excessively forming A β -immunolabeled receptacles that emerge from tanysomes (e.g., Figure 5y) and associated toroids (e.g., Figure 5u, v). *Stages IV* and *V* are particularly abundant in the ventricular lining. Other swell-bodies in AD-affected brain, predominantly in and near the ventricular lining, show increasingly hypertrophic

swell-bodies that consist of swelling toroids with forming receptacles around their periphery (Figure 5o-t). The physical association of the A β -immunolabeled toroids and receptacles with tansomes is clearly visible (Figure 5o-q; Figure 6). Larger A β -plaques often form around (i) several adjacent swell-bodies, or (ii) individual swell-bodies that contain multiple tansomes that are often located in different optical focal planes. In these plaques, large amounts of swelling receptacles can be seen adjacent to or surrounding tansome-containing swell-bodies (Figure 5u-ai, Figure 6a-m). In Figure 6c, we have provided a schematic drawing of the stage V plaque depicted in Figure 6d to illustrate the formation of smaller, translucent receptacles that emerge from peripheral 'chimney-like' canals (corresponding arrows in 6c and d). Figure 6e shows the peripheral area of a larger plaque demonstrating the abundance of small, translucent receptacles that emerge from strongly A β -immunolabeled canals. Such peripheral receptacles contain faintly stained smaller receptacles and appear surrounded by a membranous compartment (Figure 6m). These observations are consistent with ultrastructural investigations that demonstrate the electron dense nature of the toroidal structure contained within these peripheral receptacles (inset in 6m, Figure 7). The emergence of translucent waste receptacles is also demonstrated in Figure 7 where we have performed correlative ultrastructural investigations of these receptacles.

Amyloid β -plaques consist of interconnected star-shaped receptacles that can also be observed in Luxol H&E

High magnification examination of individual A β -plaques shows that they consist of star-shaped receptacles each comprised of a central receptacle from which peripheral canal-like extension emanate that interconnect adjacent receptacles (Figure 6k-s). To demonstrate the abundance and consistency by which large amounts of A β -immunolabeled, and translucent receptacles emerge from tansomes and toroids in AD-affected human hippocampus, we have included a larger than usual number of images that show this process (Figures 5-7). Ultrastructural investigations of waste receptacles in AD-affected hippocampus demonstrate the association of both tansomes and myelinated cell profiles with strands of electron-dense receptacles consistent with the hypothesis that these receptacles internalize cellular waste (Figure 7). Our ultrastructural investigations show the association of numerous waste receptacles with myelinated profiles within and outside of swell-

bodies. These observations are consistent with Luxol H&E-stained preparations that show blue coloration of forming receptacles within swell-bodies indicative of myelination (Figure 7j, m).

Anti-Tau immunolabeling of AD-affected human hippocampus is associated with tansomes and emanating receptacles and toroids

Investigation of anti-Tau immunolabeled AD-affected tissue demonstrates that this protein is also associated with swell-bodies and forming receptacles (Figure 8a-o).

Interestingly, the appearance of anti-Tau immunolabeled swell-bodies in human AD-affected brain shows strong resemblance to swell-bodies in living mouse brain that fluoresce after exposure to Cy3 fluorochrome. These preliminary functional observations are consistent with our hypothesis of waste internalization by these structures from their surroundings (Figure 8d). Anti-Tau immunoreactive neurons that are considerably larger compared to immunoreactive swell-bodies (Figure 8p) are often contacted by swell-bodies whose tansome-derived receptacles project into the neuronal somata (Figure 8q).

Extended depth of field microscopy visualizes the interconnected, reticular nature of toroid-forming tancyte processes in AD-affected human hippocampus

Investigation of serial paraffin sections through AD-affected human hippocampus stained for A β , Luxol H&E and Tau protein in an alternating fashion demonstrates that Luxol-stained tancyte processes reside in the ependymal lining and project into the stratum oriens and stratum pyramidale within the anteromedial part of the hippocampal formation (Figure 9a). The same area shows A β -immunoreactive structures known as A β plaques (Figure 9b). Immunolabeling of this area for the A β -related proteins Pres1 and APP show strong labeling for both proteins along tancyte processes and associated swell-bodies throughout the hippocampus and adjacent brain areas (Figure 9c-h). In Luxol H&E-stained alveus the interconnected nature of this tancyte network consisting of numerous forming swell-bodies and toroids is clearly visible (Figure 9). This toroid-forming network appears A β -immunolabelled, whereby the immunolabeling is consistently associated with tansome-containing swell-bodies (Figure 9). This interconnected nature is

particularly apparent using Extended Depth of Field-Microscopy (Figure 9k-m). Immunolabeling for AQP4, Anti-Tau and A β shows that toroids emerging from tanycyte processes are immunolabeled for all three proteins, consistent with our previously published postulation that this cell network is likely formed by AQP4-expressing myelinated tanycytes that shows hypertrophic swelling in AD-affected hippocampus (Figure 9t-x). These observations were not restricted to the hippocampal formation but were consistent throughout all investigated brain areas shown in Figure 1a.

Summary

As summarized in Figure 10, the findings presented here are indicative of a vast network of myelinated tanycyte processes that project into the brain parenchyma and internalize intra- and extracellular waste (Figure 10a). We provide ultrastructural evidence that myelin-derived waste receptacles differentiate within (i) specialized swell-bodies, or (ii) emanate from myelin-derived tanycyte protrusions that project into both neuronal somata and extracellular areas where they mature and internalize electron-dense material consistent with cellular waste. The receptacles are strongly immunoreactive for A β and tau protein suggestive of a functional/structural role of these proteins in the waste-uptake process. The presence of APP, Pres1, and AQP4 mRNA within swell-bodies is indicative of protein translation in areas where receptacle formation is observed. We provide strong evidence that hypertrophic swelling of tanycyte processes and associated receptacles in AD affected human brain leads to neuronal obstruction and hypertrophic proliferation of A β -lined receptacles that emanate from tansomes within swell-bodies and have the appearance of A β plaques (Figure 10a).

DISCUSSION

Here, we provide the structural and biochemical foundation that has informed our hypothesis of the existence of a tanycyte-derived glial canal system that internalizes cellular waste from the brain parenchyma. We demonstrate that waste-internalizing receptacles differentiate along tanycyte

processes and within swell-bodies where AQP4 and the A β -related genes APP and Pres1 are expressed. These findings together with the prominent anti-tau immunoreactivity observed in AD-affected swell-bodies support our postulation that both tau and A β proteins may play important roles in the structural and functional architecture of waste receptacles. Moreover, the expression of AQP4 within tanycytes provides a compelling explanation for the different swelling patterns observed along tanycyte processes, swell-bodies and associated receptacles. Our postulation that tanysome-derived receptacles internalize cellular waste is corroborated by our correlative ultrastructural investigations that show the accumulation of electron-dense material within receptacles. Based on the findings presented here we propose an additional functional significance for tanycytes besides their previously proposed metabolic functions (Bolborea and Dale, 2013, Dali, et al., 2023, Ebling and Lewis, 2018, García-Cáceres, et al., 2019, Rodriguez, et al., 2005)

Evidence in support of the 'glial canal hypothesis' and proposed functional model

Based on the findings presented here, we postulate that A β -plaques show hypertrophic waste receptacles that emanate from tanycyte-associated swell-bodies. This postulation contradicts the prevalent 'amyloid hypothesis' that proposes that A β -plaques are due to the accumulation of misfolded A β protein (Behl, 2024, Kepp, Robakis, Hoiland-Carlson, Sensi and Vissel, 2023).

We favor the recently proposed 'glial canal hypothesis' (Fabian-Fine, Weaver, Roman, Winters and DeWitt, 2024). Based on the structural and biochemical foundation provided here, we propose the following, testable functional model:

waste-internalizing structures require structural stabilization for this process. An analogous anatomical example are cartilaginous rings of trachea that are vital to keep airways open during inspiration (Lambert, et al., 1991, Rains, et al., 1992). We propose that this stabilization may be the functional significance of the luminal rings shown here. As these rings swell, they likely expand and stabilize the inner lumina of the myelinated processes. In this context, the previously proposed circadian dynamic of waste removal (Hablitz, et al., 2020) that may regulate the periodic swelling of these canals provides a compelling mechanism.

How is waste channeled into the inner receptacles shown here? This process requires: (i) a driving force into the receptacles, (ii) openings to the neuronal cytoplasm through which waste

can enter the receptacles, (iii) stabilization of the receptacle membranes and their openings to prevent their collapse during waste intake, and (iv) the dynamic and quantitatively appropriate formation of such waste-internalizing structures. We propose that the driving force is exerted by the expressed AQP4 that is likely located central to the receptacles, thus creating an inward flow into the receptacles. This postulation is supported by the central location of AQP4-immunoreactivity in waste receptacle aggregations shown here. We postulate that waste internalization is through receptacle openings shown here at the ultrastructural level. We propose that A β may play an important role in the required stabilization of the receptacle membranes and their openings to prevent their collapse. Amyloid β is a lipophilic, structurally stable insoluble protein that is thus uniquely suitable for the stabilizing function of cell membranes (Tycko, 2015). Observations of healthy brain tissue demonstrate that the tanycyte-derived waste receptacles shown here and previously (Fabian-Fine, Weaver, Roman, Winters and DeWitt, 2024) contain structures that are consistent with canal-like openings and are strongly immunoreactive for A β . We thus postulate that the observed (lipophilic) A β lines these receptacle-membranes to provide the required structural stabilization.

We argue that transport of these proposed functional (AQP4) and structural (A β) proteins through narrow tanycytes to areas where dynamic receptacle formation is required would likely be inefficient and risk blockage of narrow tanycyte processes. We propose that these proteins may be translated where receptacle formation takes place, thus explaining the presence of mRNA that encodes for these proteins in swell-bodies where receptacle formation is observed. This proposal is comparable to siding on a house. The components of the siding are made in the production facility (equivalent to mRNA). However, the siding is not assembled at the production facility but rather at the house as it is built. We propose that this is the role of tansomes in swell-bodies. As demonstrated here and in a previous study, these organelles contain mRNA for AQP4, APP, Pres1 and glial-fibrillary acid protein (Fabian-Fine, Weaver, Roman, Winters and DeWitt, 2024) and likely other key proteins such as Pres2 that remain to be tested. We propose that this mRNA is translated during the formation of waste receptacles and that the functional (AQP4) and

structural proteins (A β) are incorporated into the forming receptacles (i.e., the siding is assembled around the house where it is built). This proposed model would ensure efficient and dynamic (activity-dependent) formation of waste receptacles where they are needed.

This postulation is based on the observed immunolabeling for the RNA-encoded proteins APP, AQP4, and Pres1 (Fabian-Fine, Roman and Weaver, 2025) together with A β along toroids and receptacles that emanate from tansomes. The AQP4-expression in these receptacles explains the swelling nature of tancyte-derived receptacles, processes, and swell-bodies. Swelling of AQP-expressing cells is well established and would explain the electron-lucent nature and lack of cytoplasmic structure observed in swell-body lumina (Preston, et al., 1992, Wang, et al., 2023). This swelling would furthermore explain why neuronal somata in AD-affected brain tissue are often densely obstructed with receptacles which results in cytoplasmic depletion and cell death (Fabian-Fine, Weaver, Roman, Winters and DeWitt, 2024). Until now, such intraneuronal receptacles have been described as 'lipofuscin' that has been proposed to contain toxic waste that remains in neurons indefinitely (Gray and Woulfe, 2005). Considering our findings that myelin-derived tancytes form receptacles that project into neuronal somata [see also (Fabian-Fine, Roman and Weaver, 2025)], we encourage the re-investigation of myelin and myelin-derived structures in the vertebrate brain. The proposed myelin-derived origin of waste receptacles would explain the prominent intraneuronal myelin-specific Luxol-stain observed in neurons of Australian cattle dogs diagnosed with neuronal ceroid lipofuscinosis, a disease that is characterized by progressive neurodegeneration (Schmutz, et al., 2019). We postulate that the structures identified as lipofuscin depict intraneuronal tancyte receptacles that clear waste from healthy neurons similar to those shown here in human brain. We suggest that pathological swelling of these receptacles may be due to hypertrophic impairment of the AQP4-expressing tancytes that obstructs neuronal somata resulting in neurodegeneration (Fabian-Fine, Roman and Weaver, 2025, Fabian-Fine, Weaver, Roman, Winters and DeWitt, 2024, Sauv  , et al., 2022). Until now, the concept of an AQP4-mediated convective flow in context of waste removal from the brain has been postulated in context with astrocytes and the proposed glymphatic system (Iliff, et al., 2012).

Proposed functional significance of tau protein

In context of the proposed waste removal system introduced here we argue that an additional structural requirement for this glial canal system is 'dynamic adaptability.' To ensure adequate waste removal, the quantitatively appropriate release of waste receptacles is imperative. Too many receptacles pose the risk of neuronal obstruction and depletion; too few receptacles may cause waste buildup within the neurons. Previous investigations on spider brain have shown that neurodegeneration is preceded by pathological unraveling of adjacent myelinated glial cells (Fabian-Fine, Weaver, Roman, Winters and DeWitt, 2024). As a consequence, in areas where the glial cells unravel, we have observed excessive formation of waste-internalizing glial canals that lead to the catastrophic depletion of affected neurons (Fabian-Fine, et al., 2023, Fabian-Fine, Weaver, Roman, Winters and DeWitt, 2024). Interestingly, in this arachnid model system, the forming glial canals are anchored to microtubules. This observation led us to propose that the dynamic instability of microtubules is a uniquely suitable mechanism to enzymatically and dynamically control the quantitatively appropriate release of waste-internalizing glial canals. Triggering mechanisms may include mechanical or biochemical signals such as increasing hydrogen concentrations caused by lysosomal activity that produces cellular waste (Fabian-Fine, Weaver, Roman, Winters and DeWitt, 2024). Interestingly, tau protein is well known to stabilize microtubules in the mammalian brain (Barbier, et al., 2019). As demonstrated here, its hyperphosphorylated form is abundant in AD-affected swell-bodies in which excessive receptacle proliferation is observed. It is thus feasible that microtubules may play a similar role in controlling the quantitatively appropriate release of waste receptacles and that this mechanism may fail in AD. This idea would explain the increased formation of waste receptacles that coincides with immunolabeling for hyperphosphorylated tau protein shown here.

Tanycytes and their proposed roles

Tanycytes are ependymal glial cells that have predominantly been described along the ventricular lining of the third and fourth ventricles and are currently subdivided into four major types based on their location alpha-1 and -2, beta-1 and -2. However, the presence of additional tanycyte types

has been proposed (Fabian-Fine, Weaver, Roman, Winters and DeWitt, 2024, Pasquettaz, et al., 2021). These ependymal glial cells that label for glial fibrillary acid protein and vimentin send long, slender processes into the hypothalamic parenchyma and are proposed to have metabolic functions (Bolborea and Dale, 2013, Bolborea, et al., 2020, García-Cáceres, Balland, Prevot, Luquet, Woods, Koch, Horvath, Yi, Chowen and Verkhatsky, 2019). Interestingly, the formation of varicose protrusions along tanycytes that contact neurons have been observed in the hypothalamus of mice (Pasquettaz, Kolotuev, Rohrbach, Gouelle, Pellerin and Langlet, 2021). The degradation of tanycytes in AD-affected brain has previously been reported (Sauvé, Ternier, Dewisme, Lebouvier, Dupré, Danis, Rasika, Kim, Ciofi, Giacobini, Buée, Landrieu, Pasquier, Maurage, Nogueiras, Schwaninger and Prevot, 2022). The authors propose that tanycyte impairment may play a prominent role in neurodegeneration. Based on our findings here, we concur with this postulation.

Conclusions

This study provides novel insight into the histopathology of AD-affected brain tissue and offers a new hypothesis for the formation of A β plaques and neurofibrillary tau tangles and intraneuronal obstructions with waste-containing receptacles. We are acutely aware that we are unable to address other important topics in this study. This includes: (i) the striking resemblance of brain structure identified as astrocytes in brain disease (Verkhatsky, et al., 2023) with *Stage III-V* swell-bodies shown here, (ii) the roles of myelin and glial fibrillary acid protein in the brain, (iii) the possible role of tanycytes with regard to gliomas, (iv) possible biochemical pathways that trigger receptacle formation in swell-bodies, and (v) underlying causes that lead to excessive receptacle formation. We have addressed the latter to some degree here and in previous publications where we propose a link between AD and possible viral, fungal, and bacterial infections whose impact is increasingly recognized (Muzambi, et al., 2020). It is feasible that structural proteins of such pathogens are not sufficiently catabolized prior to uptake into the glial canal system and may lead to blockage (Fabian-Fine, Roman and Weaver, 2025, Fabian-Fine, Weaver, Roman, Winters and DeWitt, 2024). The resulting increase in intracellular turgor would explain the excessive release of receptacles from

these AQP4-expressing organelles. These and other topics must be addressed in more detail in future studies. We concur with previously voiced suggestions to critically re-evaluate the 'amyloid hypothesis' (Kepp, Robakis, Hoiland-Carlsen, Sensi and Vissel, 2023) and consider alternative hypotheses, one of which is presented here.

AUTHOR CONTRIBUTIONS

Conceptualization: RFF
Funding acquisition: RFF, ALW
Methodology: RFF
Project administration: RFF
Supervision: RFF
Investigation: RFF, ALW, AGR, MJW, KJL, CHB, LKK, SMB, AEW, CMP, LMA, ICC, FMJ, LAK, TJM, CJR, LJDR, LJR, HAS
Visualization: RFF, ALW, AGR, MJW, KJL, CHB, LKK, SMB, AEW, CMP, LMA, ICC, FMJ, LAK, TJM, CJR, LJDR, LJR, HAS
Formal Analysis: RFF
Resources: RFF, ALW
Validation: RFF
Writing – original draft: RFF
Writing – review & editing: RFF, AGR, ALW

Data availability

All data presented in this study are available from the first author upon request.

Competing Interests

Dr. Fabian-Fine together with Saint Michael's College holds a patent that directly resulted out of this research. The other co-authors on this paper have no conflict of interest.

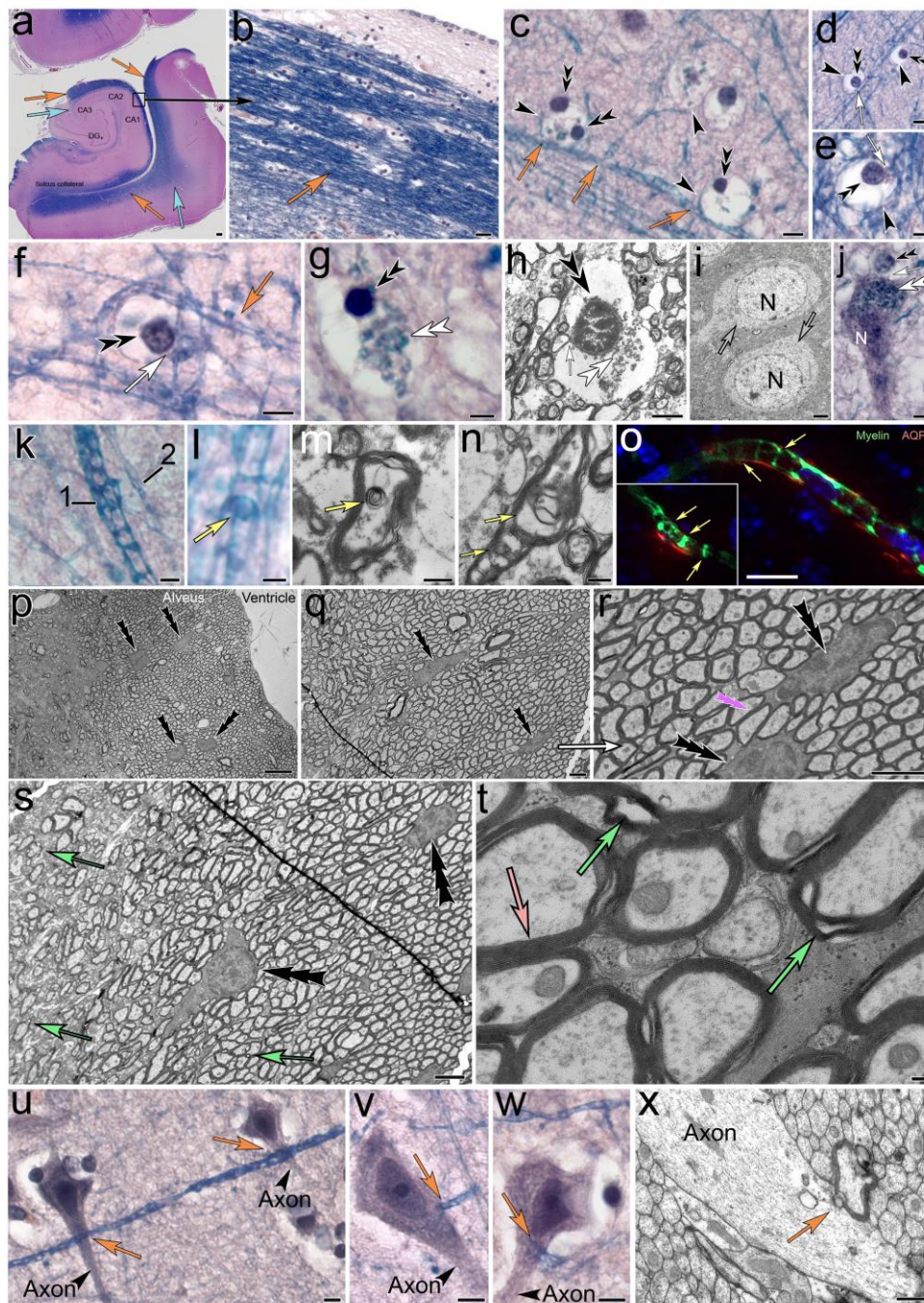
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Legends



715

716 **Figure 1. Hippocampal tanycytes form myelinated, varicose cell processes that project into**
 717 **the stratum pyramidale. (a, b) Luxol H&E-stained 4- μ m section shows the location and**
 718 **appearance of myelinated tanycyte processes (orange arrows) in the ventricular lining of the**
 719 **hippocampal formation (a). The tanycyte processes (a, b) project into the brain parenchyma (blue**

arrows in a). **(c-g)** Individual tanycyte processes give rise to swell-bodies with electron lucent lumina
 (black arrowheads) that contain one or more nucleus-like tanysomes (black double arrowheads).
 Each tanysome is physically associated with myelinated tanycyte processes (white arrows) and
 give rise to receptacles (white double arrowheads; g, h). The lumina of swell-bodies appear
 translucent (h) and lack the typical cytoplasmic organelles and structure of cell bodies (i, mouse
 hippocampal neurons, N). **(j)** Tanysome-derived receptacles often project into neuronal somata
 (white double arrowheads in h, human hippocampal neuron, N). **(k-n)** Tanycytes show different
 swell patterns (k, 1: swelling process; 2: normal process). The lumina of tanycyte processes form
 translucent varicosities that have the appearance of myelinated ring structures (yellow arrows l-n)
 that often show increased swelling in AD-affected human brain (m compared to n). **(o)**
 Immunolabeling of mouse brain for Aquaporin-4 (red) and myelin (green) show similar anti-myelin
 immunoreactive ring structures in AQP4 double-labeled tanycyte processes. **(p-t)** Ultrastructural
 depiction of the hippocampal alveus in rat brain shows somata of ependymal tanycytes (triple
 arrowheads) that give rise to vast numbers of varicosity-forming myelinated cell processes that
 project into the hippocampal formation. The syncytial nature of this tanycyte network is
 demonstrated by the cytoplasmic connections (pink triple arrowhead in r) formed between adjacent
 somata (black triple arrowheads in r). Please note the typical varicose nature of myelinated
 processes that exit the alveus and project into the hippocampal formation (green arrows in s).
 Higher magnification shows the myelinated nature of tanycyte processes within the alveus (light
 pink arrow in t) that contain electron-lucent varicosities (green arrows in t). **(u-w)** Neuronal axons
 in Luxol H&E-stained human AD-affected hippocampus lack the varicose nature of the Luxol-
 stained tanycyte processes that often contact neuronal somata near the axon hillock (orange
 arrows). (u) Similar observations of myelinated, varicose cell profiles (orange arrow) transecting
 into neuronal axons can be observed at the ultrastructural level in rat hippocampus. **Scale bars:**

(a) 500 μm , (b) 20 μm ; (c, d) 10 μm ; (e) 5 μm ; (f, g) 5 μm ; (h, i) 2 μm ; (j) 5 μm ; (k) 1 μm , (l) 0.5 μm ;
 (m, n) 500 nm; (o) 10 μm ; (p) 5 μm ; (q-s) 2 μm ; (t) 100 nm; (u-w) 5 μm ; (x) 500 nm.

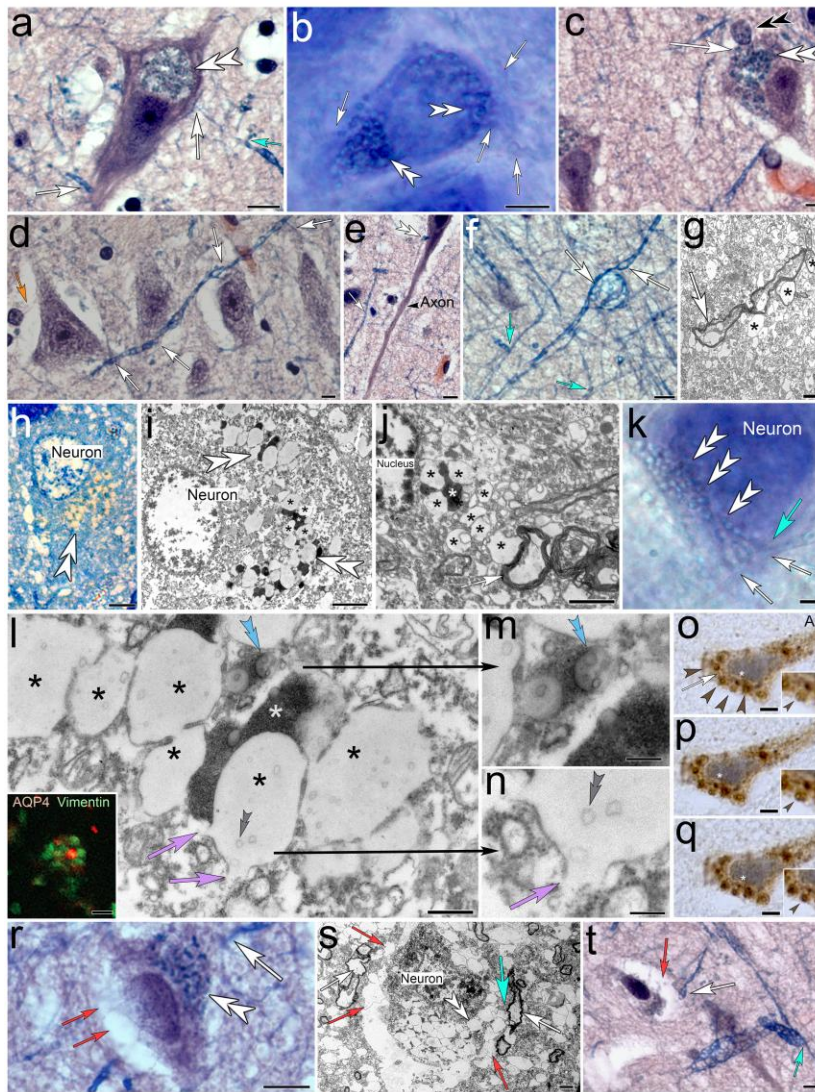


Figure 2. Histological, ultrastructural and immunohistochemical characterization of tanyocyte-derived waste receptacles in human hippocampal neurons.

(a) Neuronal soma in a Luxol blue H&E-stained 10 μm paraffin section that is densely obstructed with abnormally swelling receptacles (*white double arrowheads*). Luxol blue-stained tanyocytes processes project into the soma (*white arrows*). Extracellular perpendicular protrusions emerge from the tanyocyte process that projects into the neurons (*turquoise arrow*). (b) Toluidine-blue stained 70- μm vibratome section demonstrates the magnitude of cytoplasmic obstruction by waste receptacles (*white double arrowheads*) throughout an un-sectioned Alzheimer affected neuron. The

755 hypertrophic, blue-stained receptacles are near translucent tanycyte processes that transect into
 756 the soma (*white arrow*). **(c)** Neuronal soma that is contacted by a tanysome (*black double*
 757 *arrowhead*). Receptacles that emerge from the tanysome (*white arrow*) project into the cell soma
 758 (*white double arrowhead*). **(d)** A Luxol blue-stained varicose tanycyte process contacts adjacent
 759 neurons (*white arrows*). Please note the electron-lucent nature of a tanycyte process that emerges
 760 from a tanysome and projects into an adjacent axon hillock (*orange arrow*) **(e-f)** Luxol-blue stained
 761 myelinated tanycyte processes form numerous electron-lucent varicosities and larger swell-bodies
 762 (*white arrows f, g*) that appear distinctly different from neuronal axons (*e*). The latter lack varicose
 763 swellings and appear purple in Luxol blue H&E-stained brain tissue. Please note the varicose
 764 perpendicular protrusions that emerge from tanycyte processes (*turquoise arrows in f*). **(h)**
 765 Toluidine-blue stained 1- μ m semithin-section through a healthy neuron where waste receptacles
 766 appear yellowish (*white double arrowhead*). **(i)** Ultrastructural depiction of a healthy neuron with
 767 intact, structured cytoplasm and nucleus shows clusters of receptacles that consist of electron-
 768 dense compartments (*white asterisk*) that are associated with membrane-bound electron lucent
 769 protrusions (*black asterisks*). **(j)** A myelinated tanycyte process (*white arrow*) gives rise to electron
 770 lucent varicosities (*black asterisks*) that project into an adjacent cell soma where the receptacles
 771 internalize electron-dense material (*white asterisk*). **(k)** Toluidine-blue stained 70- μ m vibratome
 772 section that contains an un-sectioned Alzheimer affected neuron. The receptacle-formation along
 773 perpendicular protrusions (*white double arrowheads*) that emerge (*turquoise arrow*) from an
 774 adjacent tanycyte process (*white arrow*) and project into the soma are clearly visible. **(l)**
 775 Ultrastructural depiction of waste receptacles in a healthy neuron shows membrane-bound,
 776 interconnected electron lucent compartments (*black asterisks*) that have openings to the
 777 surrounding cytoplasm (*purple arrows, higher zoom in n*) and contain vesicular structures (*grey*
 778 *double arrowheads*). Associated electron-dense compartments can be seen to internalize vesicular
 779 structures through circular openings (*blue double arrowheads, higher zoom in m*). **(o-q)** Different
 780 focal planes through an A β -immunolabeled neuron in non-AD brain tissue. Serially aligned
 781 immunoreactive receptacles (*brown arrowheads in o*) are connected by a cell process (*yellow*
 782 *arrow*). The receptacles contain circular openings that show dark A β -immunolabeling (*arrowheads*

in insets); *Asterisks*: areas shown in insets. **(r-s)** Light- (*r*) and electron microscopic (*s*) depiction of degenerating neurons shows their dense obstruction with proliferating, swelling waste receptacles (*white double arrowheads*) that emerge from (*turquoise arrow*) associated myelinated tanycyte profiles (*white arrows*). The neuronal cytoplasm and surrounding areas are gradually replaced by spongiform swelling of membrane-bound tanycyte protrusions (*red arrows*). **(t)** Neuron contacted by a swelling tanycyte process (*white arrow*) with advanced cytoplasmic depletion (*red arrow*) with only the neuronal nucleus remaining. An adjacent tanycyte projection that emerges perpendicular from the same tanycyte processes (*turquoise arrow*) shows severe hypertrophic swelling. **Scale bars:** (*a*) 10 μm ; (*b-d*) 5 μm ; (*e*) 10 μm ; (*d*) 10 μm ; (*f*) 5 μm (*g*) 2 μm , (*h*) 2.5 μm ; (*i-k*) 2 μm ; (*l*) 500 nm, inset 2 μm ; (*m, n*) 200 nm; (*o-q*) 3 μm ; (*r*) 3 μm ; (*s*) 2 μm ; (*t*): 5 μm

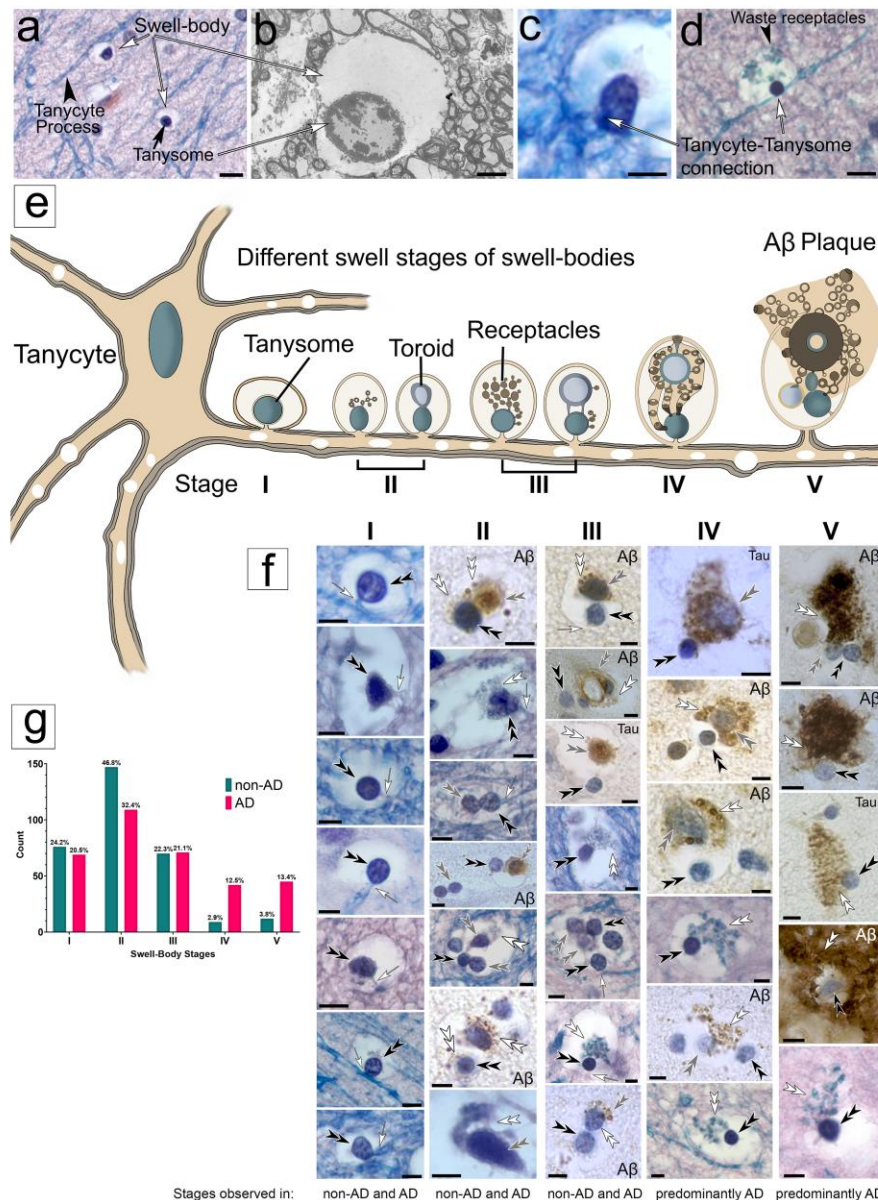


Figure 3. Histological, anatomical, and immunohistochemical characterization of tanyocyte-derived swell-body stages in human hippocampus. (a) Electron-lucent swell-bodies containing nucleus-like tanyosomes form along varicose Luxol-blue stained tanyocyte processes in Luxol H&E-stained preparations. (b) The electron-lucent nature of the tanyosome lumen is apparent at the ultrastructural level. (b, d) Luxol H&E-stained swell-bodies with associated tanyosomes. Please note the physical connection between tanyosome and tanyocyte processes (*white arrows*). *Black arrowhead*: Luxol-stained receptacles within swell-body. (e) Schematic drawing of tanyocyte with swell-bodies that show different stages of receptacle formation (*Stage I-V*). For clarity, only one

804 tancyte process with swell-bodies is drawn. **(f)** Histological and immunohistochemical images
 805 corresponding to the schematic stages shown above each vertical image column. *Stage I* swell-
 806 bodies show predominantly clear lumina that contain tancyte-associated tansomes; Stage II
 807 swell-bodies contain tansomes that give rise to receptacles or larger toroids (schematic drawing
 808 left and right, respectively). Please note the anti-A β and anti-tau immunolabeled nature of the
 809 toroids and receptacles. In Stage III swell-bodies, both receptacles and toroids swell and detach
 810 from the tansomes, while remaining connected through slender cell processes. Swelling toroids
 811 form receptacles around their periphery that are strongly A β -immunolabeled. This swelling
 812 increases in Stage IV. In Stage V, both toroids and receptacles project out of the swell-bodies into
 813 the surrounding tissue or adjacent cells. Both toroids and receptacles show increasingly strong
 814 anti-A β and anti-tau immunolabeling. *White double arrowheads*: receptacles; *black double*
 815 *arrowheads*: tansomes; *grey double arrowheads*: toroids; *white arrows*: connective cell processes
 816 between tansome and tancytes. All histological images are stained for Luxol-H&E. **(g)** The results
 817 of the Chi-square test ($\chi^2(4, N = 314$ [healthy], 336 [Alzheimer]) = 45.75, $p < 0.0001$) indicate a
 818 significant association between disease state and swell-body stages. Percentages above the bars
 819 indicate the total percentage of a given stage in relation to the total number of evaluated swell-
 820 bodies within a given test group (non-AD: N=314; AD: N=336). **Scale bars:** (a) 10 μm ; (b) 2 μm ;
 821 (c) 5 μm ; (d) 10 μm ; (f) 5 μm . *Schematic not drawn to scale.*

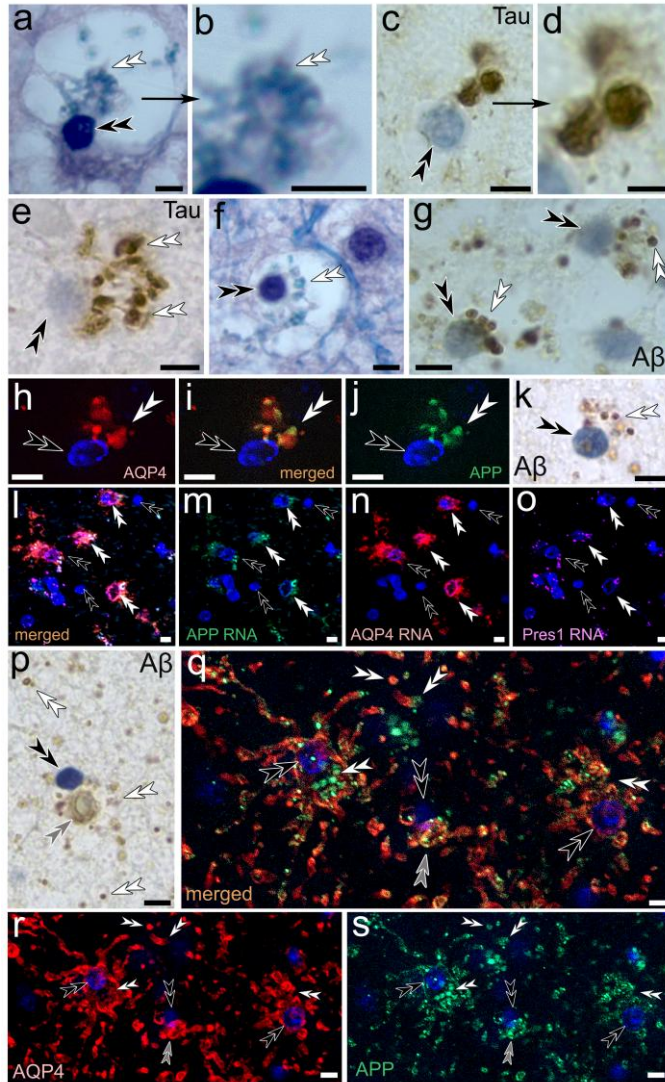


Figure 4. Immunohistochemical characterization of human tansome-associated waste receptacles in swell-bodies. (a-g) Receptacles emanating from tansomes stain for Luxol blue indicative of myelin (a, *higher zoom in b, f*), anti-hyperphosphorylated tau protein (c, *higher zoom in d, e*), and anti-Aβ (g). (h-o) Immunolabeling for aquaporin-4 (h, i) and amyloid precursor protein (i, j) labels receptacles that emanate from tansomes (*Hoechst blue nuclear stain*). The observed APP-immunolabeling is consistent with the anti-Aβ immunolabeling observed on tansome-derived receptacles (k). (l-o) RNA-gene expression for APP (*green, l, m*), AQP4 (*red, l, n*) and Presenilin-1 (*magenta, l, o*) observed along tansomes and associated toroids and receptacles are consistent with the proposed formation of Aβ-lined AQP4 expressing receptacles in swell-bodies. (p-s)

Tanyosome-associated receptacles show immunolabeling for A β (*p*) and APP (*green, q, s*) in AQP4-immunoreactive (*red, q, r*) swell-bodies. Please note restriction of immunoreactivity to tanyocyte processes and receptacles and the presence of numerous immunoreactive receptacles outside of swell-bodies that is consistently observed in both A β and AQP4/APP immunolabeled human brain of both AD and non-AD-affected hippocampus (*white double arrowheads p, q*). *White double arrowheads*: receptacles; *black double arrowheads*: tanyosomes; *grey double arrowheads*: toroids. **Scale bars**: (a) 5 μ m; (b) 2 μ m; (c) 5 μ m; (d-e) 2 μ m; (f-s) 5 μ m.

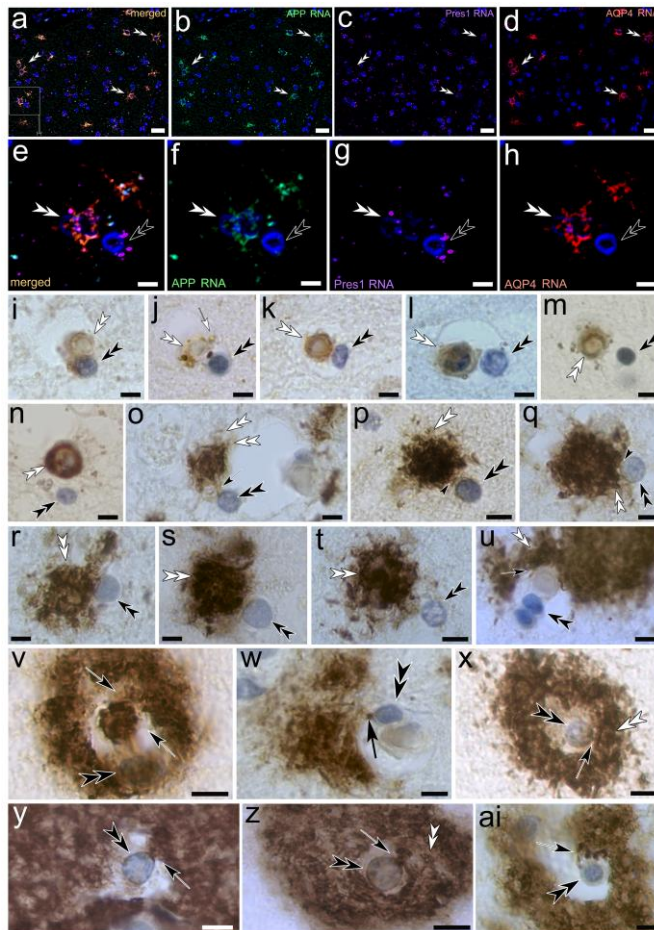


Figure 5. Progressive formation of amyloid beta plaques in AD-affected human hippocampus. (a-h) Swell-bodies in human hippocampus show RNA-expression for amyloid precursor protein (APP, green), presenilin-1 (Pres1, magenta) and aquaporin-4 (AQP4, red); Blue signal: DAPI nuclear stain, higher zoom shows the gene-expression along tanyosomes (black double arrowheads) and associated toroids and receptacles (white double arrowheads). (i-m) These

findings are consistent with the modestly A β -immunolabeled nature of swell-body derived toroids and receptacles in AD-unaffected brain tissue that is void of A β -plaques. **(n-t)** In AD-affected brain tissue, both receptacles and toroids start to proliferate and swell. **(u-ai)** Investigation of larger A β plaques shows the emergence of numerous A β -immunolabeled waste receptacles from tansomes and associated toroids (*black arrows*). **Scale bars:** (a-d) 15 μ m; (e-ai) 5 μ m.

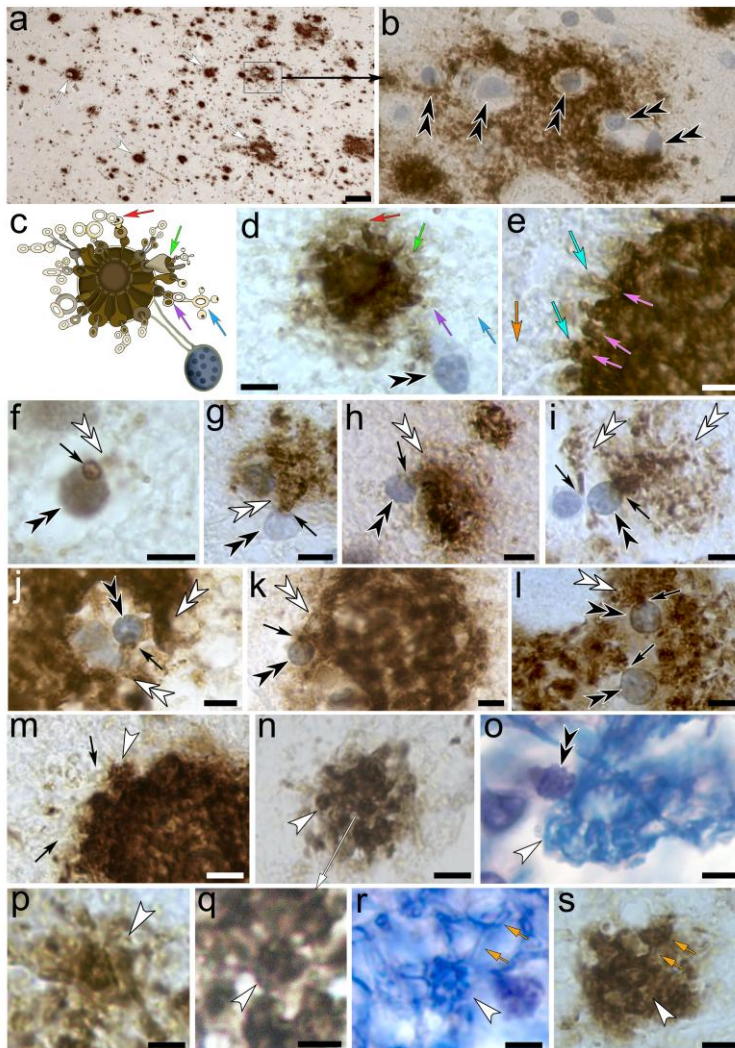


Figure 6: Anatomical features of A β plaques in AD-affected human hippocampus. (a, b) Dense accumulations of A β plaques (*white arrows*) in the hippocampal alveus are associated with tansome-containing swell-bodies. The boxed area in *a* shown at higher magnification in *b* reveals the accumulation of tansome-derived A β -immunoreactive waste receptacles (*black double arrowheads*). **(c, d)** Schematic drawing of the tansome-associated toroid in *d* illustrates the

emergence of translucent waste receptacles from peripheral canals (*colored arrows show*
respective areas in c and d). Note the translucent nature of newly forming receptacles (*blue arrow*
in d) **(e)** Partial image of a larger plaque shows strongly A β -immunolabelled 'chimney-like'
peripheral canals (*pink arrows*) from which large numbers of forming translucent receptacles
emerge (*turquoise arrows*). Please note the faint A β labeling of receptacles that emerge from the
plaque (*turquoise arrows*) compared to the unstained nature of more distant receptacles (*orange*
arrow). **(f-l)** Tansomes (*black double arrowheads*) form A β -immunolabeled pores (*arrow in f*) from
which large numbers of A β -immunolabeled toroids and receptacles emerge (*white double*
arrowheads) in areas where A β plaques are found. **(m, n)** Toroid-shaped plaques consisting of a
centrally located toroid from which peripheral receptacles emerge (*white arrowhead*). The latter in
turn give rise to receptacles that appear translucent at the light microscopic level (*pink arrows in*
m) and contained within a membranous compartment (*pink arrowheads in m*). *Inset:* At the
ultrastructural level this membranous compartment is clearly visible (*pink arrowhead*). The toroidal
structures contained within the depicted compartment appear electron dense, consistent with
internalization of cellular waste (*pink arrow*). **(o)** Tansome-associated toroid in Luxol H&E-stained
human alveus shows a centrally located toroid that contains Luxol-blue stained circular receptacles
and ring structures (*white arrowhead*). **(p-s)** Higher zoom of star-shaped receptacles (*white*
arrowheads) observed in A β -plaques and Luxol H&E-stained alveus. Radial extensions from the
centrally located toroidal structure form additional receptacles (*orange arrows*). Image in *q* is a
higher zoom of the plaque shown in image *n*. **Scale bars:** (*a*) 60 μ m; (*b, d-o*) 5 μ m; *inset in m*: 1
 μ m; (*p-s*) 2.5 μ m. *Schematic not drawn to scale.*

Figure 7. Correlative light and electron-microscopic characterization of tansome-derived waste receptacles in AD-affected human hippocampus. (a) Amyloid β -immunolabeled swell-body with receptacle-forming tansome. Please note the strong circular immunolabeling in the center of forming receptacles. **(b)** Ultrastructural depiction of a swell-body with centrally located tansome and waste receptacles. Some receptacles are filled with electron-dense material (*white arrowheads*) while others appear less electron-dense (*beige arrowhead*). Receptacles also

884 emanate from myelinated tanyocyte profiles that border the outer periphery of the swell-body (*blue*
 885 *arrowhead*) and can be seen to form connections to both tanysomes and surrounding myelinated
 886 cell profiles (*green arrowheads*).**(c, d)** Tanysome with associated A β -immunolabeled toroid
 887 (outlined by grey double arrowheads) from which lateral strands of waste receptacles emanate
 888 (white double arrowhead, higher zoom in d). **(e)** Luxol H&E-stained tanyocytes in the alveus show
 889 Luxol-stained receptacles emanating from tanysomes. **(f-i)** Amyloid β -immunolabeled toroids from
 890 which translucent waste receptacles emanate (*black arrows, g and i depict higher zooms of images*
 891 *f and h respectively*). Please note the strong immunolabeling of the pore from which receptacles
 892 emanate in *f, g (white arrows)*. **(j, k)** Tanysome-associated toroids with emanating waste
 893 receptacles. **(l-n)** Higher zoom of peripheral waste receptacles (*l*) indicated in *k* show emanating
 894 peripheral receptacles similar to the one shown in Luxol H&E-stained swell-body. Correlative
 895 electron-microscopy depicts a myelinated receptacle associated with electron-dense material
 896 consistent with internalization of cellular waste. **(o-t)** Electron-micrographs of waste-internalizing
 897 receptacles that emanate from myelinated tanyocyte profiles. *White double arrowheads:*
 898 *receptacles; black double arrowheads: tanysomes; grey double arrowhead: toroid; black*
 899 *arrowhead: areas in which electron-dense waste receptacles emanate from myelinated tanyocyte*
 900 *profiles or tanysomes. Scale bars: (a) 5 μ m; (b) μ m; (c-h) 5 μ m; (i) 1 μ m; (j, k) 5 μ m; (l) 2 μ m; (m)*
 901 *5 μ m; (n-t) 500 nm.*

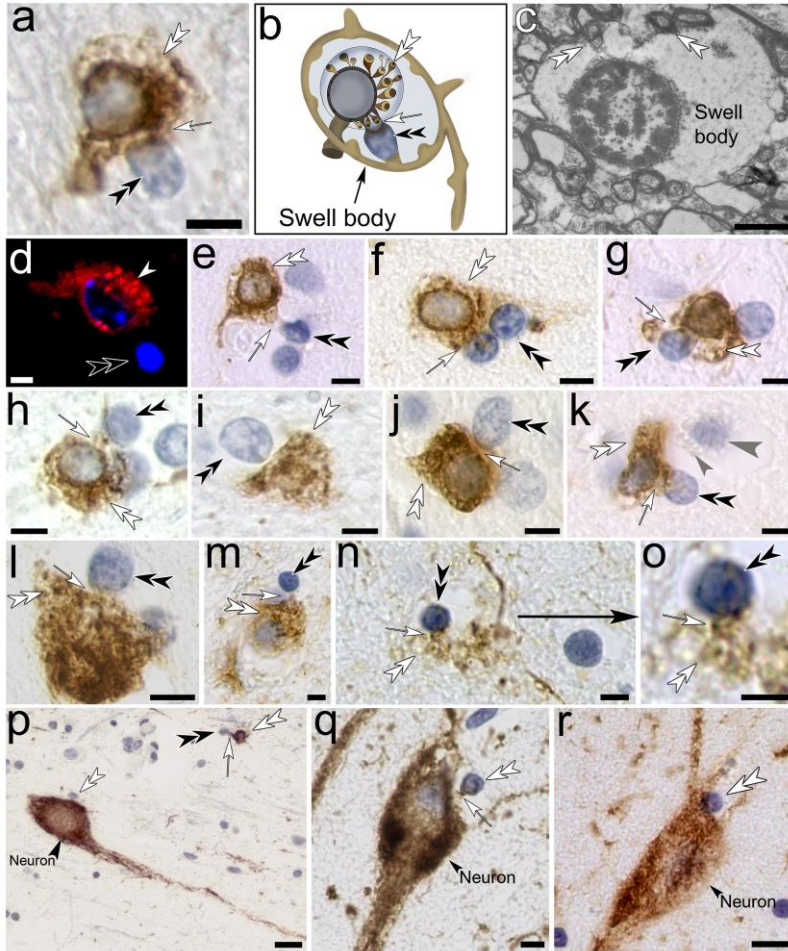
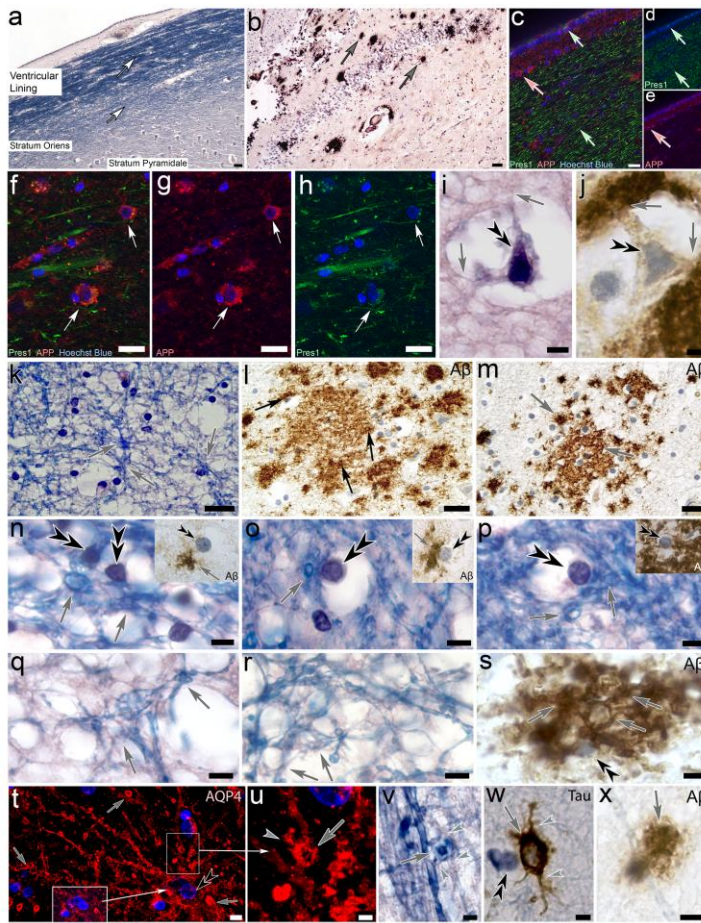


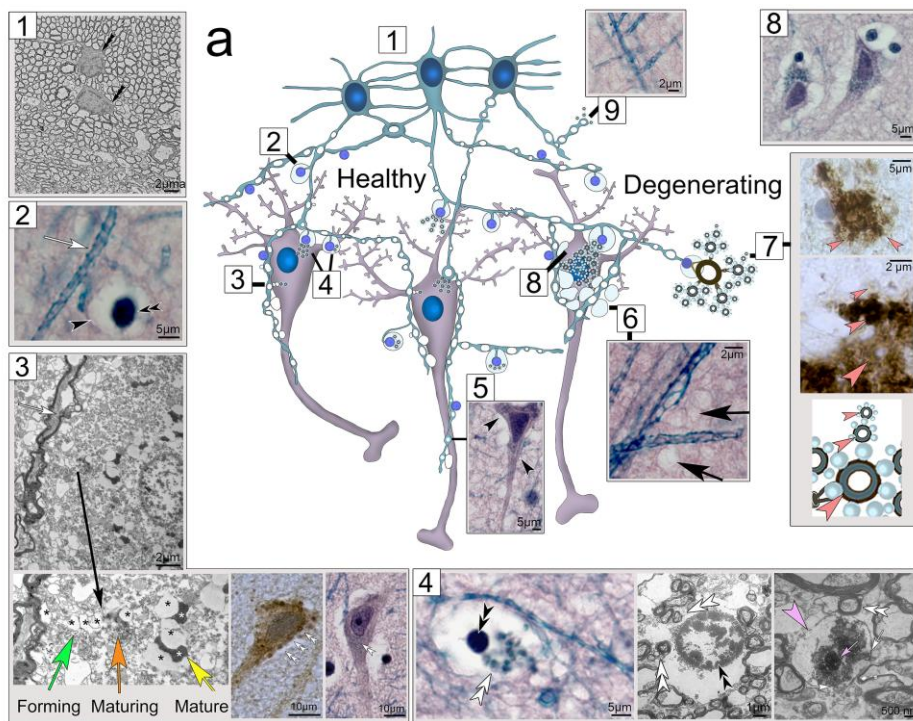
Figure 8. Anti-Tau immunolabeling of AD-affected human hippocampus. (a) Swell-body with receptacle-forming toroid shows that the anti-tau labeling is associated with receptacle formation. (b) Schematic drawing of swell-body in a. (c) Electron-micrograph of swell-body with forming myelinated receptacles. (d) Swell-body in living mouse hippocampus that was exposed to Cy3 fluorochrome shows brightly fluorescing receptacles consistent with the hypothesis of fluorochrome uptake into receptacles (*white arrowhead*). (e-o) Swell-bodies that show the consistency of tau-immunolabeling associated with receptacle formation from both toroids (e, f, g, h, i, j, m) and tansomes (k, l, n with higher zoom in o). Please note the unlabeled swell-body with forming receptacles (*small grey arrowhead*) that protrude from nuclear-stained receptacle shaped structures (*large grey arrowhead*) in k. (p-r) Anti-Tau immunolabeled neurons that are contacted by tansomes are distinctly larger compared with swell-bodies (p). *White double arrowheads*: receptacles; *black double arrowheads*: tansomes, *white arrows*: connection between tansomes

915 and receptacles or toroids. **Scale bars:** (a) 5 μ m; (c) 2 μ m; (d-o) 5 μ m; (p) 15 μ m; (q) 5 μ m; (r) 10
 916 μ m. Schematic not drawn to scale.



917
 918 **Figure 9. Histological and immunohistochemical depiction of AD-affected human**
 919 **hippocampal alveus and stratum oriens. (a)** Luxol H&E-stained paraffin section demonstrates
 920 the location of myelinated tanycyte processes in the ventricular lining of the anteriomedial
 921 hippocampus above the CA3 area (*white arrows*), within the stratum oriens and stratum pyramidale.
 922 **(b)** A β -immunolabelling demonstrates the accumulation of A β -immunoreactive swell-bodies and
 923 associated toroids (*grey arrows*). **(c-e)** Immunolabeling of the alveus for presenilin-1 (*Pres1*, *green*,
 924 *green arrows*) and amyloid precursor protein (*APP*, *red*, *red arrows*) demonstrates the expression
 925 of both proteins by ependymal tanycytes. **(f-h)** Swell-bodies in the stratum oriens immunolabeled
 926 for Pres1 and APP show double labelling associated with tanysome-containing swell-bodies and
 927 associated tanycyte processes (*white arrows*). Nuclear stain: *Hoechst blue*. **(i, j)** Two swell-bodies

928 in subsequent Luxol H&E-stained (*i*) and A β -immunolabeled (*j*) sections through the same area in
 929 the stratum oriens show the formation of toroid-shaped tanyocyte processes (*grey arrows*).(*k-m*)
 930 Extended depth of field microscopy in which several focal planes are merged shows the
 931 interconnected nature of tansomes (*black double arrowheads*) with the toroid-forming (*grey*
 932 *arrows*) tanyocyte network in both Luxol blue H&E-stained and A β -immunolabeled sections. (**n-s**)
 933 Luxol blue H&E-stained and A β -immunoreactive toroids (*insets in n-p*) show the association of
 934 tansomes (*black double arrowheads*) with the surrounding toroid-forming (*grey arrows*) network of
 935 tanyocyte processes. (**t, u**) Aquaporin 4-immunolabeled swell-body that is associated with strongly-
 936 immunoreactive, toroid-forming tanyocyte processes (*grey arrows, right inset higher zoom in u*).
 937 Upregulation of the Hoechst-blue nuclear stain signal demonstrates the presence of three-nuclear
 938 stained organelles within the lumen of the swell-body (left *inset in t*). (**v-x**) Swelling toroids (*grey*
 939 *arrows*) that form along tanyocyte processes stained for Luxol Blue H&E (*v*), anti-Tau (*w*) and A β
 940 (*x*). Grey arrowheads: Cellular processes that emanate from the toroids and form an interconnected
 941 reticulum of tanyocyte processes. **Scale bars:** (*a, b*) 30 μ m; (*c-h*) 20 μ m; (*i-j*) 5 μ m; (*k-m*) 20 μ m,
 942 (*n-t*) 5 μ m; (*u*) 4 μ m; (*v*) 2 μ m, (*w*) 4 μ m; (*x*) 3 μ m.



943

Figure 10. Depiction of the proposed glial canal system in rodent and human hippocampus.

(a) In healthy brain tissue a vast interconnected syncytium of ependymal tanycytes form long varicose processes into the brain parenchyma (1). Tanycyte processes form (i) swell-bodies that express aquaporin 4 and amyloid beta related genes (2) and (ii) varicose protrusions that differentiate into waste-internalizing receptacles (3). These receptacles are myelin-derived and project into both neuronal somata and extracellular spaces (4). Electron-dense accumulations within waste receptacles are consistent with the appearance of cellular waste (4). Tanycyte processes also contact neuronal axons, particularly at the axon hillock (5). In Alzheimer-affected brain tanycyte-derived varicosities appear hypertrophic compared to healthy brain tissue leading to an increasingly spongiform appearance of affected brain tissue (6). Tanycyte-derived waste receptacles proliferate through the emergence of new receptacles from existing receptacles (*orange arrowheads in 7*). The swelling of these receptacles together with their strong A β deposits gives them the appearance of A β plaques (7). Hypertrophic swell-bodies and associated receptacles that transect into neuronal somata cause dense obstruction of affected neurons with swelling receptacles and gradual replacement of cytoplasmic content with swelling, electron lucent tanycyte protrusions (8). Spongiform abnormalities of adjacent tissue are caused by the proliferation and swelling of tanycytes and their perpendicular projections (6 and 9).