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Reduction of the immunostainable length of the hippocampal dentate granule cells' primary cilia in 3xAD-transgenic mice producing human $A\beta_{1-42}$ and tau

Balu Chakravarthy [a,*], Chantal Gaudet [a], Michel Ménard [a], Leslie Brown [a], Trevor Atkinson [a], Frank M. LaFerla [b], Shingo Ito [a], Ubaldo Armato [c], Ilaria Dal Prà [c], James Whitfield [a]

[a] Human Health Therapeutics, National Research Council of Canada, Ottawa, ON, Canada
[b] Department of Neurobiology and Behavior, University of California, Irvine, CA, United States
[c] Department of Life and Reproduction Sciences, University of Verona Medical School, Verona, Italy

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ABSTRACT

The hippocampal dentate gyrus is one of the two sites of continuous neurogenesis in adult rodents and humans. Virtually all dentate granule cells have a single immobile cilium with a microtubule spine or axoneme covered with a specialized cell membrane loaded with receptors such as the somatostatin receptor 3 (SSTR3), and the p75 neurotrophin receptor ($p75^{NTR}$). The signals from these receptors have been reported to stimulate neuroprogenitor proliferation and the post-mitotic maturation of newborn granule cells into functioning granule cells. We have found that in 6–24-months-old triple transgenic Alzheimer's disease model mice (3xTg-AD) producing both $A\beta_{1-42}$ and the mutant human tau protein tau_{P301L}, the dentate granule cells still had immunostainable SSTR3- and $p75^{NTR}$-bearing cilia but they were only half the length of the immunostained cilia in the corresponding wild-type mice. However, the immunostainable length of the granule cell cilia was not reduced either in 2xTg-AD mice accumulating large amounts of $A\beta_{1-42}$ or in mice accumulating only a mutant human tau protein. Thus it appears that a combination of $A\beta_{1-42}$ and tau protein accumulation affects the levels of functionally important receptors in 3xTg-AD mice. These observations raise the important possibility that structural and functional changes in granule cell cilia might have a role in AD.

1. Introduction

Each hippocampal granule cell in the mature rodent has a single signaling antenna—a 'primary' cilium [1]. This organelle consists of a spine (axoneme) of 9 microtubule doublets that serve as trackways for the traffic of components to and from the basal body by the intraflagellar transport (IFT) machinery. Primary cilia are wrapped in a specialized plasma membrane bearing various receptors and their signaling machinery, the signals from which are believed to drive diverse functions ranging from neurogenesis, maturation to memory encoding [2–8]. One of these receptors is the SSTR3 (somatostatin type 3 receptor) which is specifically localized to primary cilia in various types of cell including the dentate granule cells [4,9–14]. SSTR3 receptors are loaded by maturing granule cells into their primary cilia to send signals to drive functions such as memory formation as measured by the ability to recognize novelty [4]. Another receptor is $p75^{NTR}$ which we have recently shown to also be localized to the granule cell cilia along with SSTR3 [15] where it likely provides signals to drive granule cell progenitor proliferation in the dentate sub-granular zone (SGZ), but it may also have other roles because virtually all postmitotic, mature granule cells have $p75^{NTR}$-loaded cilia [8].

In the present study we show that the accumulation of human amyloid ß ($A\beta_{1-42}$) and mutant tau protein, the dual initiators and drivers of the events leading to AD, together reduce the immunostainable length of the SSTR3/$p75^{NTR}$-bearing primary cilia in the mouse dentate gyri of AD-model mice. In 2xTg-AD mice accumulating only Aβ cleaved from a mutant human APP, and in tau-Tg mice producing only a mutant human tau_{P301S} protein [16,17], the cilial length was the same (i.e., 4–5-μm average) as in the corresponding wild type mice. However, the $p75^{NTR}$-SSTR3-bearing cilia are much shorter in 3xTg-AD mice accumulating human $A\beta_{1-42}$ and mutant human tau protein. Thus, this is hopefully a heuristic example of $A\beta_{1-42}$ and mutant tau collaborating to cause the severely reduced the cilium-based signaling for neurogenesis, memory and other mature functions in these AD-model mice [18].

* Corresponding author. Address: National Research Council, 1200 Montreal Road, Building M-54, Institute for Biological Sciences, Ottawa, ON, Canada K1A 0R6. Fax: +1 613 941 4475.
E-mail address: Balu.Chakravarthy@nrc-cnrc.gc.ca (B. Chakravarthy).

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2. Materials and methods

2.1. Animals

Age-matched (6–8, 14–18, 20–24 months-old) female sv129/C57/BL6 wild-type, AD-triple transgenic (3xTg-AD) mice harboring APP_{Swe}, $PS1_{M148V}$ and tau_{P301L} transgenes (from the Department of Neurobiology and Behaviour, University of California, Irvine, CA, bred and maintained in-house), 6–8-months old female wild-type C57/BL6 and double transgenic (B6.Cg-Tg) mice harboring APP_{Swe} and PSEN1dE9 transgenes (from the Jackson Laboratory, Bar Harbor, ME, bred and maintained in-house) and 3–4-months old female wild-type and tau-transgenic mice expressing mutant human tau (B6.C3-Tg, MAPT P301S, from Jackson Laboratory) were used in these studies. All animal studies were approved by NRC Institute's Animal Care Committee.

2.2. Reagents

Rabbit polyclonal antibody against somatostatin receptor, SSTR3, was obtained from Gramsch Laboratories (Schwabhausen, Germany). $p75^{NTR}$ rabbit polyclonal antibody directed against the receptor's extra-cellular domain (amino acids 188–203) was obtained from Alomone Labs Ltd. (Jerusalem, Israel). The selectivity of the antibody in labeling $p75^{NTR}$ was confirmed by the total blockade of antibody immunostaining by the antigen peptide, CEEIPGRWITRSTPPE Alomone Labs), against which the anti-$p75^{NTR}$ antibody was raised (data not shown).

The mouse monoclonal tau antibody recognizing the 159–163 (PPGQK) sequence of human tau was obtained from Pierce Biotechnology Inc (Rockford, IL). Alexa Fluor 488 goat anti-rabbit IgG was from Invitrogen Canada (Burlington, ON). Peroxidase conjugated anti-mouse IgG was purchased from Sigma Chemical Co. (St. Louis, MO). Amyloid ß (Aß) ELISA kit was from Biosource (Invitrogen). PVDF membrane and Western Lightning Chemiluminiscence reagent were obtained from Perkin Elmer (Mississauga, ON, Canada).

2.3. Tissue extraction

Anesthetized age-matched female wild-type and the AD transgenic mice (three to five animals in each group) were decapitated, their brains rapidly removed and split into two equal halves (hemi-brains). One hemi-brain was frozen immediately and stored at −80 °C for immunohistochemical analysis. Hippocampi from the other hemi-brains were immediately removed and placed in ice-cold PBS, snap frozen on dry-ice, and stored at −80 °C until needed. The hippocampi were homogenized in ice-cold Tris–HCl buffer (20 mM, pH 7.4, and protease inhibitors cocktail, 5 μg/ml AEBSF, 0.8 μg/ml aprotonin, pepstatin, and leupeptin) and the $Aß_{1-42}$ levels in the homogenates were measured with an ELISA kit (Biosource/Invitrogen-Medicorp Inc. Montréal, Quebec) as described before [19].

2.4. Immunoblot analysis

Hippocampal homogenates were centrifuged at 600*g* for 5 min at 4 °C, the supernatants (tissue homogenates) were centrifuged further at 100,000*g* for 30 min at 4 °C in a Beckman TL-100 ultracentrifuge to separate particulate fraction (total membranes) and subjected to Western blot analysis as described before [19]. Briefly, solubilized membrane proteins were separated on 10% polyacrylamide gels and transferred to PVDF membranes. The immunoblots were then blocked with 5% non-fat milk in TBST (10 mM Tris pH 8.0, 150 mM NaCl, 0.05% Tween 20) for 1 h and incubated with primary tau antibody. After three washes in TBST, immunoblots were incubated with peroxidase-conjugated secondary antibody (1:5000 dilution) in TBST containing 1% non-fat milk for 30 min at room temperature. Western blots were visualized using Western Lightning Chemiluminescence Reagent Plus kit.

2.5. Immunohistology

The frozen hemi-brain was embedded in OCT and 10-μm sections were prepared using a Jung CM 3000 cryostat and stored at −80 °C until use. Tissue sections were fixed in acetone and then permeabilized in 0.1% TritonX-100, 2% normal goat serum, 0.02% Na Azide, 10 mg/ml BSA. After blocking with a solution of 5% normal goat serum, 0.02% Na Azide, 10 mg/ml BSA for 1 h, sections were incubated overnight at 4 °C with various primary antibodies diluted in 2% normal goat serum, 0.02% Na Azide, 10 mg/ml BSA at indicated dilutions: 1:700 dilution for SSTR3, and 1:100 dilution for $p75^{NTR}$ antibodies. After incubation with primary antibodies, sections were washed in PBS and incubated with Alexa Fluor 488 goat anti-mouse IgG at 1:400 dilution for 1 h at room temperature. All sections were then washed and cover-slipped with Dako fluorescent mounting medium containing DAPI. The cilia in the various preparations were measured blindly in randomly chosen fields. Cilial measurements (160–180 cilia for each hippocampus, a total of 480–540 cilia for 3 hippocampi from three animals in each group) were made using a fluorescence microscope (Olympus) and quantifications were carried out using ImagePro Plus soft-ware (Media Cybernetics Inc, Bethesda, MD). To obtain the length, the tracing tool in the Measurements Command was used by moving the cursor from the base to the tip of primary cilia in the digital image. The software was calibrated to convert the number of pixels into micrometers. The measurement scale of the software was calibrated using values obtained with a stage micrometer (Olympus).

Data were statistically analyzed with factorial ANOVA (Statistica software) followed by Fisher's LSD *post hoc* test. The p values are indicated in the figure legends. Values of $p < 0.05$ were considered statistically significant.

3. Results

Somatostatin receptor 3 (SSTR3) has been convincingly shown to be localized to primary (solitary) cilia and should be considered a cilial biomarker [4,9,11,14]. As expected immunostained SSTR3 receptors were localized to cilia in the dentate granular cell layer (Fig. 1). We also found another cilial marker, adenylyl cylclase III (ACIII), in the granule cell cilia (data not shown).

The cells (90 ± 2%) in the granule cell layers of hippocampi of both wild-type and 3xTg-AD mice had primary cilia (Fig. 1A and C). There were no changes in the fraction of ciliated dentate granule cells in either the C57/BL6 wild-type or the 3xTg-AD mice derived from them as they aged from 6 to 24 months as indicated by the mean 0.90 ± 0.03 SEM ratio of primary cilia to nuclei over the 18 months (Fig. 1C). Thus the granule cells in both strains of mouse did not lose their cilia with age. While the immunostainable lengths of the cilia protruding from the granule cells in the C57/BL6-wild-type mice remained around an average of 4 μm in animals 6–24 months of age (6–8 months, 4.08 ± 0.29 μm; 14–18 months, 4.33 ± 0.07 μm; and 20–24 months, 4.5 ± 0.53 μm, means ± SEMs), the cilial length in the $Aβ_{1-42}$ plus tau_{P301L}-producing C57/BL6-derived 3xTg-AD mice had dropped significantly from an average of 4 μm to an average of 2.2 μm in the same age groups (6–8 months, 2.54 ± 0.51, $p < 0.01$; 14–18 months, 2.05 ± 0.10; $p < 0.001$ and 20–24 months, 2.04 ± 0.05, $p < 0.001$; mean ± SEM) (Fig. 1A, B and D). It is highly unlikely that the shortened cilia could have been trivial artifacts of the cilia being more curved or bent in

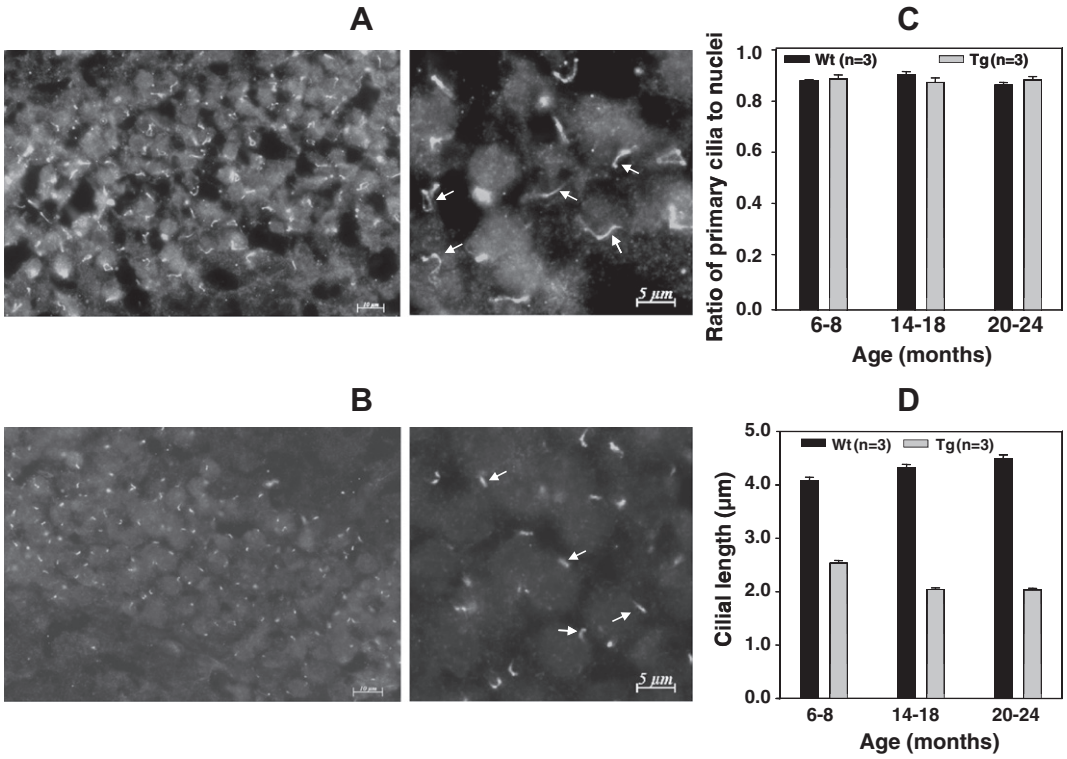

Fig. 1. Somatostatin receptor 3 (SSTR3) bearing primary cilia of the hippocampal dentate gyral cells of triple transgenic-AD mice are shorter than that seen in the corresponding wild-type mice. Brains from 7 to 8 months old female wild-type sv129/C57BL6 (Wt) mice (A) and triple transgenic (3xTg) mice (B) derived from them were removed, split into two halves (hemi-brains) and stored at −80 °C. One of the hemi-brains was subjected to immunohistological studies with SSTR3-specific antibody (A and B) as described in Section 2. Figure represents a typical of samples from 3 separate mice of different age groups (6–24 months). The ratio of cilia to nuclei (C) and SSTR3-bearing cilial length (D) in the dentate gyral region were determined by individually counting neurons (606 ± 157 cells) and measuring the cilial length as described in Section 2. Bar graph heights are the means ± SEMs of the values from 3 hemi-brain sections from 3 separate mice for each age group. The SSTR3-bearing cilial length was significantly shorter in the Tg mice than those in Wt animals at all age groups tested (6–8 months, $p < 0.01$; 14–18 months, $p < 0.001$; 20–24 months, $p < 0.001$). Arrows: long, curved/bent cilia.

the 3xTg-AD mice. In these mice it was only the very few longer cilia that could have been bending over and still be traceable.

The decreased lengths of SSTR3-bearing cilia in the granule cells of the 3xTg-AD mice could have been due to a selectively reduced SSTR3 content because of the reduced SST level in the brains of AD-mice and AD humans [20,21]. Alternatively, it could have been due to an overall reduction of cilial factors loading. This was tested by immunostaining against an entirely different receptor, reasoning that an identically reduced cilial length using a different label would more likely indicate an overall loading impairment of the organelle in the 3xTg-AD mice. We have recently shown using $p75^{NTR}$ selective antibodies that $p75^{NTR}$ receptors are also localized to the primary cilia of hippocampal granule cells [15]. As shown in Fig. 2 the same reductions in cilial length in 3xTg-AD granule cells compared with wild-type cilia were observed when stained with anti-$p75^{NTR}$ antibody as seen with anti-SSTR3 antibody.

There was a progressive accumulation with age of both $A\beta_{1-42}$ and human tau protein in the 3xTg-AD animals, with a significant amount of tau having accumulated by 6–8 months (data not shown). Since tau is normally structurally and functionally linked to axonal microtubules, an abnormally distributed mutant tau like that in 3xTg-AD mice and human AD brains might disrupt microtubular structures such as primary cilia. So was it the mutant tau that decreased length of granule cell cilia in the 3xTg-AD mice?

To find out whether it was one or both of the two agents that reduced the granular cells' cilial length in 3xTg-AD mice we compared the hippocampal tissues from 2xTg-AD mice only producing large amounts of $A\beta_{1-42}$ and tauopathy model mice producing only a mutant human tau. As can be seen in Fig. 3A and B, there was no difference between the mean granule cell cilial length in the $A\beta_{1-42}$-accumulating 2xTg-AD mice compared to the granule cell cilial length in the dentate gyri of the corresponding C57/BL6 wild-types at 6–8 months of age when immunostained with anti-SSTR3 antibody. Identical results were obtained when primary cilia were immunostained with anti-$p75^{NTR}$ antibodies (data not shown). The $A\beta_{1-42}$ levels in the hippocampi of these 2xTg-AD mice were actually 15–20-times higher than in the 3xTg-AD mice! Thus, for example, the $A\beta_{1-42}$ levels at 6–8 months of age in 3xTg mice were 8.3 ± 2.1 ng/mg ($n = 3$) and 127.4 ± 37.2 ng/mg ($n = 3$) in double transgenic mice. Similarly, no significant changes in the cilial length or gross morphology with respect to the corresponding wild-type cilia were seen in the granule cells of 3–4 months old tau-Tg mice producing only the tangle-producing mutant P301S human tau protein [17] and no $A\beta_{1-42}$ when immunostained with SSTR3 (Fig. 4A) or $p75^{NTR}$ antibody (data not shown). Indeed the lengths of the cilia protruding from the dentate granule cells in the human tau-producing B6.C3H-Tg mice were the same as, or even slightly longer than the granule cell cilia in the wild-type B6.C3H mice (Fig. 4A) although B6.C3H tau-Tg mice produced significant amount of human tau protein (Fig. 4B). Importantly, the three principal strains (3xTg-AD, 2xTg-AD and tau-Tg) used in this study all had the same C57/BL6 background and all had ~4-μm wild-type cilia. Thus the shorter cilia in 3xTg-AD mice cannot be dismissed because these mice were derived from mice with 'dwarf' wild-type cilia. Thus, both $A\beta_{1-42}$ and tau appear to be required to shorten the granule cell cilia in 3xTg-AD mice.

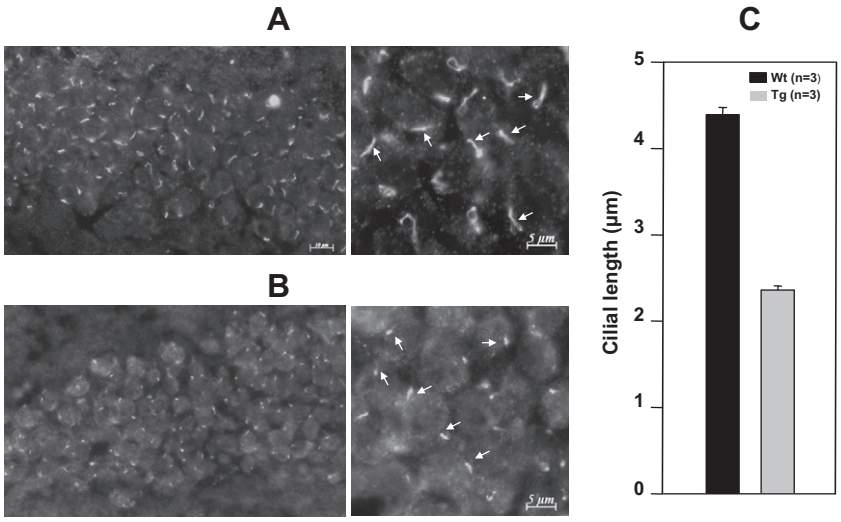

Fig. 2. The p75^{NTR} is localized to primary cilia and the p75^{NTR} bearing cilia in the dentate gyral cells of triple transgenic-AD mice are shorter compared to that in the wild-type. Hemi-brains from 7 to 8 months old female wild-type (A) and triple transgenic (B) mice were immunostained with p75^{NTR} antibody as described for Fig. 1 and hippocampal dendate gyral subfields were assessed for immunostaining. Cilial length (C) was determined as described in the legends for Fig. 1. Bar graph heights are the means ± SEMs of the values from 3 hemi-brain sections from 3 separate mice for each group. As seen with SSTR3, the p75^{NTR}-bearing clilal length was also significantly shorter in the Tg animals compared to Wt animals ($p < 0.01$). Arrows: Long, curved/bent cilia.

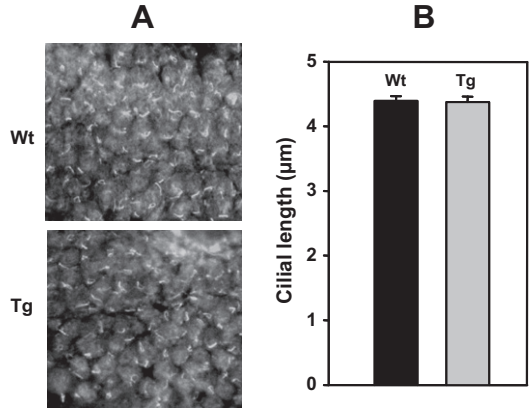

Fig. 3. The apparent length of SSTR3 expressing primary cilia of the dentate gyral cells did not change in the double transgenic-AD mice accumulating only Aß$_{1-42}$. Hemi-brains from 6 to 8-months old female wild type C57BL6 (Wt) and double transgenic (Tg) mice were immunostained with SSTR3-specific antibody (A) as described in Section 2. The Figure is typical of samples from 3 different mice. Cilial length (B) in the granular layer of the dentate gyrus was measured as described in Section 2. Bar graph heights are the means ± SEMs of the values in 3 hemi-brain sections from 3 different mice for each group.

4. Discussion

Hippocampal dentate granules cells have primary cilia which have p75^{NTR} receptors [15] along with SSTR3 receptors which have been known to be localized to cilia [11]. It is most likely that these cilia are somehow shortened structurally instead of by depletion of their two receptors in the 3xTg-AD mice producing both Aβ_{1-42} and tau$_{P301L}$ because they were still intensely immunostained ~2 μm remnants instead of faded immunostained ~4 μm full-length cilia. By contrast, the immunostained cilia in the 2xTg-AD mice producing only Aβ_{1-42} were as long as the wild-type cells' cilia. Since the 2xTg-AD mice with normal cilia accumulated 15.3 times more Aβ_{1-42} than their 3xTg-AD relatives, and that the 3xTg-AD mice with shorter immunostained cilia accumulate a large amount of human tau$_{P301L}$ as well as Aβ_{1-42}, we reasoned that over-production of mutant human tau protein in mice might play a role in reducing cilial length. Indeed we have shown an example with these AD-model mice of the two principal AD drivers collaborating to produce a pathology like they collaborate to produce AD in humans.

As it turned out, human tau protein, like Aβ_{1-42}, did not by itself affect the length of anti-p75^{NTR}/anti-SSTR3 immunostained cilia. Cilia were not shorter in the tau-Tg mice accumulating human tau mutant protein P301S when compared to the cilia of their parental wild-type B6.C3H mice or to the cilia of C57/BL6 wild-type mice and their 2xTg-AD derivatives. Evidently both Aβ_{1-42} and an accumulating mutant tau were needed to produce granule cells with short p75^{NTR}/SSTR3-bearing cilia. The fact that the human tau proteins P301L in the 3xTg AD and the P301S in the B6.C3H-tau-Tg mice are different mutants cannot affect this conclusion because both proteins are mutant in the same P301 region and are known produce the similar severe tau pathology [17,22]. Another example of Aβ_{1-42} and tau collaboration is the ability of injecting fibrillar Aβ_{1-42} into the brains of tau-transgenic mice expressing only tau$_{P301L}$ to enhance tau pathology [23]. Moreover, King et al., [24] have shown that soluble Aβ_{1-42} oligomers sensitize microtubules to normal human tau-induced disassembly, a fact relevant to cilia with their microtubular structure.

We can only speculate at this early stage how accumulating human Aβ_{1-42} and mutant tau might produce shortened cilia. According to a recent study by Zempel et al. [25], accumulating Aβ_{1-42} oligomers and Ca^{2+} surges in primary neurons delocalize tau from the axons and phosphorylated tau enters dendrites, destroys microtubules and dendritic spines and their synapses. By analogy, the accumulating Aβ_{1-42} and mutant human tau in the 3xTg-AD mice might have directly destabilized the microtubular cores of the granule cell cilia and affected its length. Alternatively Aβ_{1-42} and the mutant tau might have reduced cilial lengthwise growth and maintenance by impeding the continuous trafficking of BBS (Bardet-Biedl syndrome) protein complexes, BBSomes, carrying structural components and receptors such as SSTR3 and p75^{NTR} to the cilial basal body for delivery to the intraflagellar transport machinery [9,26–32].

Very important questions arising from this study are: do human dentate granule cells have cilia; if so, do they contain p75^{NTR} and SSTR3 receptors; and are they also abnormally short in the dentate gyri of human AD brains? In this connection it is important to note that the Bardet-Biedl syndrome, a proven "ciliopathy", includes hippocampal shrinkage with cognitive impairments in humans

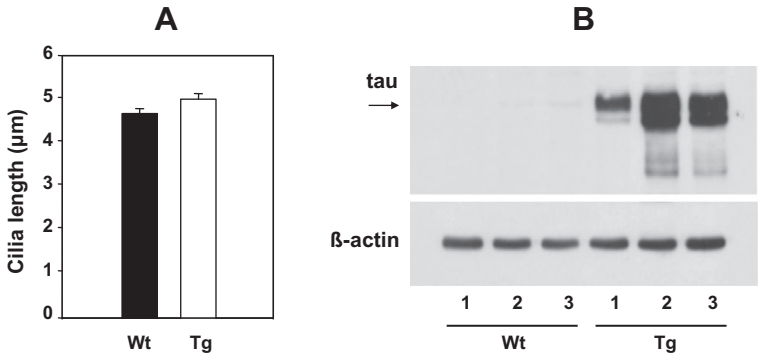

Fig. 4. The apparent length of SSTR3 expressing primary cilia of the dentate gyral cells did not change in the tau transgenic-AD mice. Hemi-brains from 3 to 4-months old female wild-type B6.C3H (Wt) and MAPT P301S tau-expressing transgenic B6.C3 mice were isolated and one of the hemi-brains was immunostained with SSTR3-specific antibody and the cilial length in the granular layer of the dentate gyrus was measured as described in Section 2. Bar graph heights (A) are the means ± SEMs of the values in 3 hemi-brain sections from 3 different mice for each group. The other hemi-brain from 3 wild-type and 3 Tg mice each was homogenized and subjected to Western blot analysis with tau-specific antibody (B) as described in Section 2. After probing with tau antibody the same immunoblots were probed with ß-actin antibody to confirm equal protein loading.

and a mouse model of this disease [33–35]. In this syndrome, mutant BBSomes cannot carry structural and signaling components such as SSTR3 into the cilium thus causing cilial shrinkage and signal silencing [4,9,13,32].

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