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TAG-1 is an inhibitor of TGFβ2-induced neuronal death via amyloid β precursor protein

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ABSTRACT

Our earlier studies indicated that TGFβ2-induced neuronal cell death by binding to the extracellular domain of amyloid β precursor protein (APP) on the cell surface and by triggering an intracellular death signal pathway, mediated by a heterotrimeric G protein Go, Rac1/cdc42, ASK1, JNK, NADPH oxidase, and caspases in this order. Recently, transient axonal glycoprotein-1 (TAG-1), a glycophosphatidylinositol-linked protein, was identified as another natural ligand of APP. TAG-1 increases APP intracellular domain release and triggers FE65-dependent transcriptional activity in a γ-secretase-dependent manner by binding to APP. In this study, we show that TAG-1 inhibits TGFβ2-mediated neuronal cell death via APP by attenuating the binding of TGFβ2 to APP in a γ-secretase-independent manner. TAG-1 is expressed in murine hippocampal neurons at 8 weeks of age, but its expression is reduced at 8 and 20 months. These findings suggest that an age-related reduction of TAG-1 expression may predispose neurons to cell death, induced by the binding of TGFβ2 to APP. This mechanism may contribute to the onset and the progression of Alzheimer's disease-relevant neuronal cell death.

1. Introduction

APP, a type I transmembrane protein, is a putative receptor for ligands and mediates some biological activities in non-neuronal cells and neuronal cells. Treatment with an antibody to APP or the multimerization of APP, which mimics the binding of an unknown relevant ligand, leads to the increase in phosphorylation levels of APP and Src and in production of proinflammatory mediators and adhesion molecules in endothelial cells [1].

Accumulating evidence has indicated that the enforced expression of the familial Alzheimer's disease (AD)-linked APP mutant V642I-APP causes neuronal cell death [2–4] by triggering apoptotic signaling cascades [5–7]. Based on this finding, Hashimoto et al. showed that TGFβ2 was a ligand for cell surface APP and induces neuronal cell death by triggering an intracellular death signal transduction pathway, mediated by a heterotrimeric G protein Go, Rac1 or cdc42, ASK1, JNK, NADPH oxidase, and caspases. Therefore, familial AD-causative mutations in APP may activate the APP-mediated neuronal cell death signal cascade constitutively without TGFβ2 binding [8].

The death of neurons in the cerebral cortex and certain subcortical regions, including the hippocampus, causes cognitive impairment in AD [9,10]. Of multiple hypotheses on the mechanism concerning neuronal death associated with AD, the TGFβ2 hypothesis centers on neuronal death, caused by TGFβ2 binding to APP [11]. Levels of TGFβ2 are upregulated in AD brains [12]. Amyloid beta (Aβ) increases the expression of TGFβ2 mRNA in astrocytes and neurons [13]. The upregulated TGFβ2 may contribute to the onset and progression of AD-relevant neuronal cell death by binding to APP and activating the cell death cascade [8,11].

Axonin-1/Contactin-2/transient axonal glycoprotein-1 (TAG-1) is a large glycophosphatidylinositol-linked protein anchored to the outer leaflet of the plasma membrane [14,15]. TAG-1, expressed in neurons and glial cells, plays a role in axon outgrowth, migration and fasciculation during development. TAG-1 was found to be another natural ligand of APP. By binding to APP, it increases the generation of the APP intracellular domain (AICD) in a γ-secretase-dependent manner, which in turn regulates neurogenesis, in collaboration with a transcription factor FE65 [16].

TAG-1 is expressed during adulthood as well as during development in the juxtaparanodal region of axon fibers myelinated by oligodendrocytes and Schwann cells [17,18]. Despite the identification of TAG-1 function in neurogenesis, its function in adult neurons is unclear. A recent study suggested that autoantibodies to TAG-1 may lead to the progression of multiple sclerosis-like demyelination in the cortex and spinal cord [19].

In this study, we show that TAG-1 attenuates TGFβ2-induced cell death via APP, by competitively inhibiting the binding of TGFβ2 to APP. TAG-1 is expressed in hippocampal neurons in adult mice.

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Hippocampal expression of TAG-1 is reduced in mice at ages of 8 months and older mice, as compared with mice at an age of 8 weeks. These results suggest that the age-related downregulation of TAG-1 may contribute to the onset and progression of AD-related hippocampal neuronal death.

2. Materials and methods

2.1. Cell lines, genes, recombinant proteins, and antibodies

Neurohybrid F11 cells, wt-APP and V642I-APP cDNAs in the pcDNA3 vector, were as described [8,20]. The cDNA fragment encoding the most part of the extracellular domain of mouse APP695 (APP-ED) corresponding to amino acids 1–590, fused in frame to the cDNA encoding the Fc region of human IgG [21], were inserted into the pEF-BOS plasmid (pEF-APP-ED/Fc). The extracellular domain of TAG-1 fused to the immunoglobulin Fc portion in the Pig1 vector (Pig1-TAG-1-ED/Fc) was provided by Dr. Domna Karagogeos. pEF-1-TAG-1-ED encoding C-terminally MycHis-tagged TAG-1-ED was constructed by inserting the extracellular domain of TAG-1 into the pEF-1 vector. Recombinant human TGFβ2 and six histidine-tagged human TAG-1 extracellular domain (rTAG-1) were purchased from R&D Systems (Minneapolis, MN). Antibodies to TGFβ2, APP (22C11), and Myc were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), Chemicon (Temecula, CA), and Sigma (St. Louis, MU), respectively. Mouse TAG-1 monoclonal antibody clone 4D7 was purchased from Developmental Studies Hybridoma Bank at the University of Iowa (IA).

2.2. Transfection procedure, cell death assay, and cell-viability assay

The transient transfection procedures were as described [8,20]. At 24 h after transfection, F11 cells were treated with recombinant TGFβ2 in serum-free Ham's F-12 with N2 supplement. In some experiments, various amounts of recombinant TAG-1 were added in addition to TGFβ2. At 72 h after transfection, representative microscopic views were taken and the WST-8 assay was performed as cell-viability assays [8,20].

2.3. Co-immunoprecipitation analysis

F11 cells, co-transfected with indicated vectors, were harvested at 24 h after the onset of transfection for immunoprecipitation analysis with 15 μl of 1:1 slurry of protein G Sepharose and immunoblot analysis with antibodies to APP and to Myc.

2.4. TAG-1 competition assay

COS-7 cells were transfected with pEF-APP-ED/Fc. At 24 h after transfection, cells were cultured in serum-free DMEM. At 48 h after the onset of transfection, cultured conditioned media were collected for co-immunoprecipitation analysis. Five hundred microliters of the conditioned medium containing about 20–30 nM APP-ED/Fc was mixed with 10 picomoles of TGFβ2, and 15 μl of 1:1 slurry of protein G Sepharose 4B and rotated in the presence or the absence of recombinant TAG-1 at 4 °C overnight before immunoblot analysis.

2.5. Immunoblot analysis

Cell lysates (20 μg/lane) or pulled-down precipitates were subject to SDS–PAGE, and fractionated proteins were transferred onto polyvinylidene difluoride membranes. Visualization of the immunoreactive bands was performed by ECL (Amersham Pharmacia Biotech, Uppsala, Sweden).

2.6. Immunohistochemistry

Eight weeks, 8 months, and 20 months old male C57-BL6 mice were anaesthetized by diethylether. After fixed with phosphate buffer (pH 7.4) containing 4% paraformaldehyde, brains were embedded in O.C.T. Compound (Sakura, Tokyo, Japan) and cut by 10-μm thickness. The sagittal sections of hippocampi were prepared on MAS-coated slide glasses (Matsunami, Osaka, Japan). The sections were washed with TBS-T and treated with 0.3% H_2O_2 for 30 min to abolish endogenous peroxidase activity and rinsed with TBS-T. Blocking and immunostaining were processed with M.O.M. basic kit (Vector Laboratories, Burlingame, CA, USA). The sections were incubated with TAG-1 monoclonal antibody clone 4D7 (1:2 dilution) as the first antibody at 4 °C overnight. They were then rinsed in TBS-T and incubated with biotinylated goat anti-mouse IgM (Vector Laboratories) at room temperature for 30 min, processed with the avidin–biotin–peroxidase method using the ABC Elite kit (Vector Laboratories). Immunoreactivity was visualized using Tyramide-FITC (TSA kit; NEN-Perkin-Elmer). Fluorescence was observed with a laser scanning, confocal microscope LSM 510 (Carl Zeiss, Germany).

2.7. Statistical analyses

All cell-viability experiments were done with $n = 3$. All values in the experiments are mean ± SD. Statistical analyses were carried out by the one-way ANOVA followed by a post hoc test (Fisher's PLSD test). $p < 0.05$ was assessed as significant. $^{*}p < 0.05$, $^{***}p < 0.001$, n.s., not significant.

3. Results

3.1. TAG-1 inhibits TGFβ2-induced death of F11 cells overexpressing wild-type (wt)-APP

Considering that both TAG-1 and TGFβ2 are ligands for the extracellular domain of APP [8], we examined whether the expression of TAG-1 affected TGFβ2-induced neuronal death via APP (TGFβ2-induced cell death via APP). Of multiple *in vitro* TGFβ2-induced death assays, we employed an assay by TGFβ2-induced death of F11 neurohybrid cells overexpressing wt-APP because F11 cells are easily transfected with exogenous genes [8]. F11 cells were co-transfected with a wt-APP-encoding pcDNA3 vector or its backbone vector in association with a Pig1 vector encoding an immunoglobulin Fc portion-tagged extracellular domain of TAG-1 (TAG-1-ED/Fc) or its backbone vector encoding immunoglobulin Fc. Co-incubation with 20 nM TGFβ2-induced death for F11 cells overexpressing wt-APP and reduced the number of cells attached to the dishes (Fig. 1A, panels 3 and 4). However, co-expression of TAG-1-ED/Fc attenuated the death of F11 cells overexpressing wt-APP (Fig. 1A, panels 1 and 2). In agreement, WST-8 cell-viability assays showed that co-incubation with 20 nM TGFβ2 reduced the viability of F11 cells overexpressing wt-APP (Fig. 1B, columns 3 and 4) and the co-expression of TAG-1-ED/Fc attenuated TGFβ2-induced reduction of viability of F11 cells overexpressing wt-APP (Fig. 1B, columns 1 and 2).

Ma et al. showed that TAG-1 increased AICD generation in γ-secretase-dependent manner and reduced APP levels [16]. Immunoblot analysis showed that the co-expression of TAG-1-ED/Fc only marginally reduced levels of overexpressed wt-APP (Fig. 1C, compare lane 1 with lane 3 or lane 2 with lane 4). This result suggested that TAG-1 inhibited the death of F11 cells, induced by the binding of TGFβ2 to wt-APP.

In addition, we found that TGFβ2 treatment significantly reduced the WST-8 values of the cells, co-transfected with the

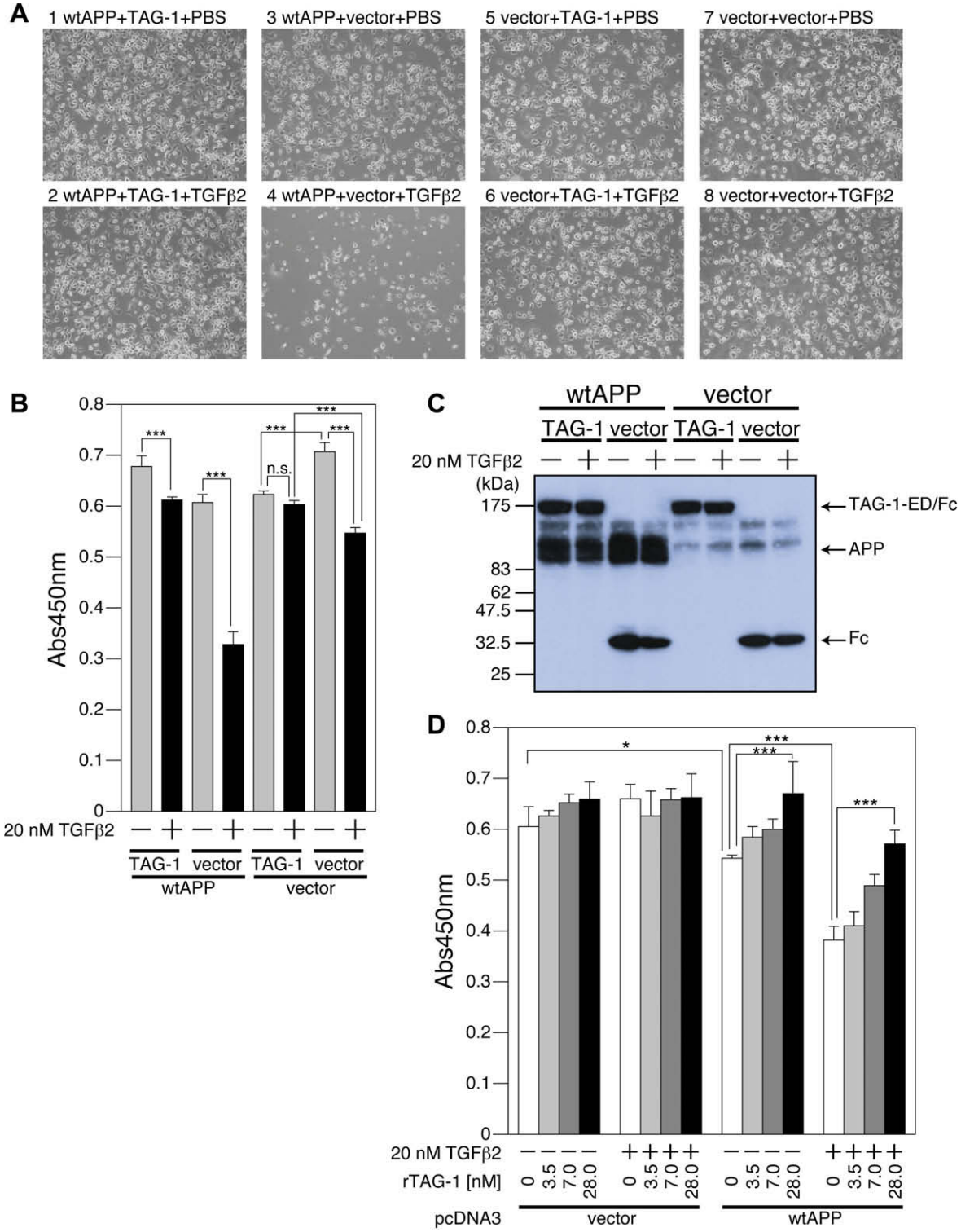

Fig. 1. TAG-1 inhibits TGFβ2-induced death of F11 cells overexpressing wt-APP. (A, B) F11 cells, seeded on 6-well plates at 7×10^4 cells/well and co-transfected with 0.5 μg of pcDNA3-wt-APP or the vector together with 0.5 μg of Pig1-TAG-1-ED (TAG-1-ED/Fc) or the vector (Fc), were treated with 20 nM of TGFβ2. Seventy-two hours after transfection, cell mortality was assessed by microscopic views of cells attached to the dishes (A) and the WST-8 cell-viability assays (B). (C) Cell lysates (20 μg in each lane), fractionated by SDS–PAGE, were examined by immunoblot analysis with antibody to APP and HRP-conjugated protein G (for the detection of TAG-1-ED/Fc and Fc). (D) TAG-1 inhibits TGFβ2-induced neuronal cell death via APP in a dose–responsive fashion. F11 cells, seeded on 6-well plates at 7×10^4 cells/well and transfected with 0.5 μg of pcDNA3-wt-APP or the vector, were treated with 20 nM of TGFβ2 in the absence or the presence of indicated concentrations of recombinant TAG-1 (rTAG-1). Seventy-two hours after transfection, cell mortality was assessed using the WST-8 cell-viability assays.

pcDNA3 vector and the Pig1 vector (encoding Fc), to limited extents but significantly (Fig. 1B, columns 7 and 8). This result suggests that TGFβ2 may reduce the viability of F11 cells possibly by binding to endogenous APP. Overexpression of TAG-1-ED/Fc marginally but significantly attenuated the TGFβ2-induced reduction of viability of F11 cells not overexpressing wt-APP (Fig. 1B, col-

umns 5 and 6). We also recognized that the overexpression of TAG-1-ED/Fc reduced the viability of F11 cells (Fig. 1B, columns 5 and 7) although its mechanism remains unknown.

3.2. TAG-1 inhibits TGFβ2-induced death in a dose–responsive fashion

We examined whether TAG-1 inhibited TGFβ2-induced cell death via APP in a dose–responsive fashion. Co-incubation with recombinant TAG-1 inhibited the TGFβ2-induced death of F11 cells overexpressing wt-APP in a TAG-1-dose–responsive fashion (up to 28 nM) (Fig. 1D, columns 13–16). Note that TAG-1 also inhibited the decrease in viability of F11 cells, induced by overexpression of wt-APP without co-incubation with TGFβ2, in a TAG-1-dose–responsive fashion (Fig. 1D, columns 9–12). These results suggest that TAG-1 antagonizes the death of F11 cells, not only induced by the binding of exogenously added TGFβ2 to overexpressed wt-APP but also by the binding of endogenously synthesized TGFβ2 to overexpressed wt-APP. In this particular experiment, TGFβ2 was unable to reduce the WST-8 values of cells not overexpressing wt-APP (Fig. 1D, compare columns 1–4 with columns 5–8) as it did in the experiments in Fig. 1A (panels 7 and 8). We suggest that differences in the transfection condition and the absence or the presence of Fc may affect the sensitivity of F11 cells to TGFβ2-induced death via APP.

3.3. TAG-1 does not inhibit V642I-APP-induced neuronal death

Next, we investigated the mechanism underlying TAG-1-mediated inhibition of TGFβ2-induced cell death via APP. Three possible mechanisms were examined. First, TAG-1 binding to APP may alter APP conformation and weaken the APP-mediated activation of Go. Second, the TAG-1 binding to APP may inhibit the binding of TGFβ2 to APP. Third, TAG-1-induced generation of AICD may lead to non-specific inhibition of neuronal cell death in a FE65-dependent and γ-secretase-dependent manner [16].

Our earlier studies indicated that low-level enforced expression of V642I-APP, the London-type familial AD-linked mutant APP, induced the death of F11 cells via the same signal transduction pathway as TGFβ2-induced cell death via APP [8,11]. The mutation is thought to allow APP to trigger the death signal in a death ligand-independent manner. A co-immunoprecipitation analysis indicated that TAG-1 bound to V642I-APP as well as to wt-APP in a similar manner (Fig. 2A). If TAG-1 inhibits TGFβ2-induced cell death via APP by altering APP conformation and weakening the APP-mediated activation of Go or if TAG-1-induced increase in the generation of AICD leads to the non-specific inhibition of neuronal cell death, it is highly likely that TAG-1 inhibited V642I-APP-induced death of F11 cells in a similar manner. However, as shown in Fig. 2B and C, co-expression of TAG-1-ED/Fc did not inhibit

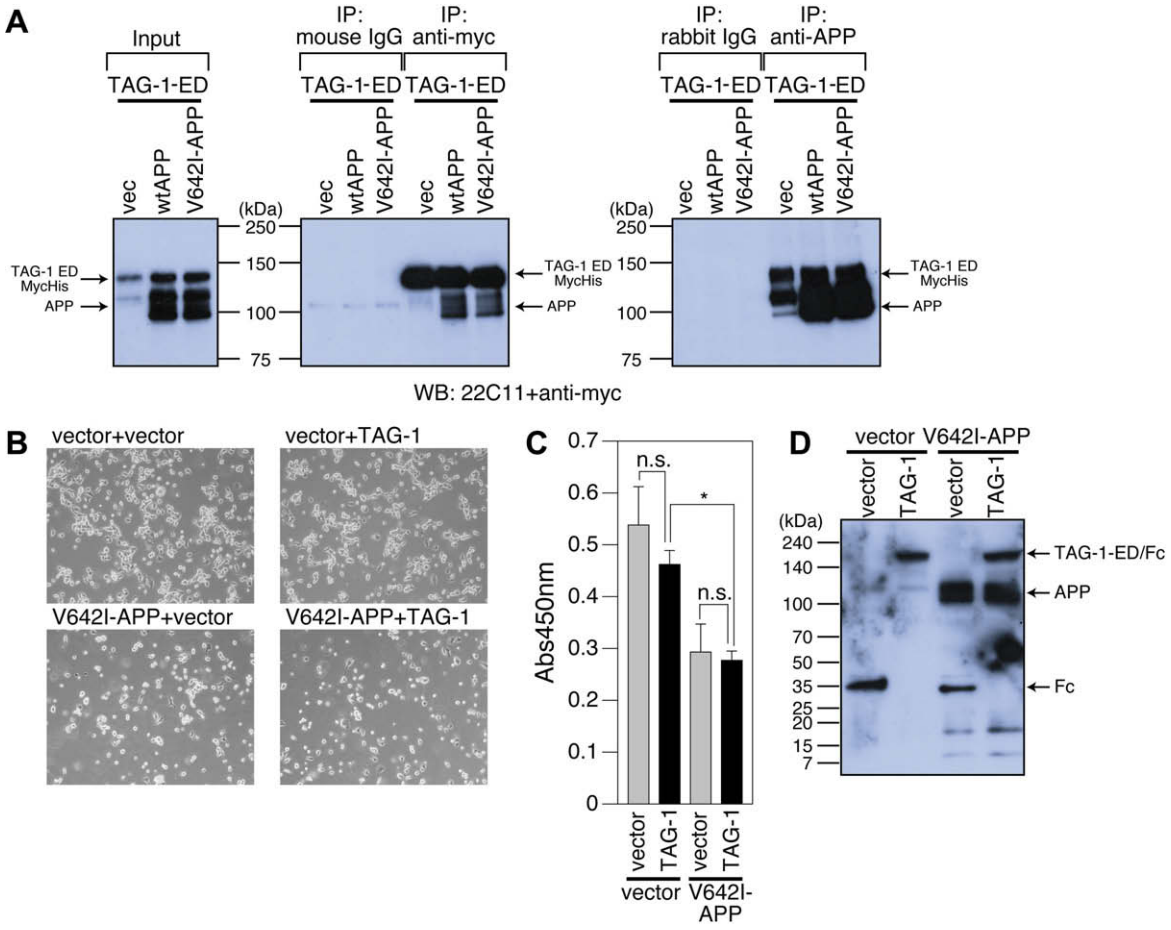

Fig. 2. TAG-1 does not inhibit V642I-APP-induced neuronal death. (A) TAG-1 binds to V642I-APP as well as wt-APP in a similar fashion. F11 cells, seeded on 6-well plates at 7×10^4 cells/well, were co-transfected with 0.5 μg of pcDNA3-V642I-APP, pcDNA3-wt-APP, or the vector, together with 0.5 μg of pEF-1-TAG-1-ED (encoding TAG-1-ED/MycHis) or the vector. Twenty-four hours after transfection, cells were harvested for co-immunoprecipitation analysis with antibodies to Myc (or control mouse IgG) and APP (or control rabbit IgG). Cell lysates and immunoprecipitates were used for immunoblot analysis with antibodies to Myc and APP (22C11). (B, C) F11 cells were co-transfected with 0.5 μg of pcDNA3-V642I-APP or the vector together with 0.5 μg of Pig1-TAG-1-ED (encoding TAG-1-ED/Fc) or the vector (Fc). Seventy-two hours after transfection, cell mortality was assessed by microscopic views (B) and the WST-8 cell-viability assays (C). (D) Cell lysates (20 μg in each lane) were examined for immunoblot analysis with an antibody to APP and HRP-conjugated protein A (for the detection of TAG-1-ED/Fc and Fc).

V642I-APP-induced death significantly as it did inhibit the TGFβ2-mediated cell death via APP, as shown in Fig. 1A–C. These results may exclude the first and the third possibilities. In accordance, treatment with a γ-secretase inhibitor did not antagonize TAG-1-induced inhibition of TGFβ2-induced neuronal cell death via APP (Supplementary Fig. 1).

3.4. TAG-1 reduces the binding of TGFβ2 to APP competitively

To test the second possibility, we purified recombinant APP-ED/Fc secreted from conditioned media of COS-7 cells transfected with an APP-ED/Fc-encoding vector. We mixed APP-ED/Fc from conditioned media and recombinant TGFβ2 in the presence or the absence of recombinant TAG-1, pulled down APP-ED/Fc by protein G-conjugated Sepharose, and examined the levels of TGFβ2 and TAG-1 that co-precipitated with APP-EP/Fc. As shown in Fig. 3, the levels of co-precipitated TGFβ2 was attenuated in the presence of recombinant TAG-1 in a TAG-1-dose–responsive fashion. This result suggests that the TAG-1 binding to APP inhibits the binding of TGFβ2 to APP in a competitive manner.

As compared with inhibition of TGFβ2-induced neuronal cell death via APP by recombinant TAG-1 shown in Fig. 1D, greater concentrations of TAG-1 were needed for inhibition by recombinant TAG-1 of the TGFβ2 binding to APP shown in Fig. 3. This difference was explained by the difference in the amounts of the APP-ED/Fc or the APP molecule, contained in these two assays. The concentration of the APP-ED/Fc molecule in the mixture was approximately 20–30 nM in Fig. 3. This concentration was estimated to be approximately five times larger than that of wt-APP, expressed on the cell surface of F11 cells, transfected with the wt-APP-encoding vector shown in Fig. 1D (data not shown).

3.5. Hippocampal expression of TAG-1 is downregulated in an age-dependent manner

TAG-1 is expressed in neurons and astrocytes in the whole embryonic brain [14,15,22]. This result is in agreement with the fact that TAG-1 is involved in neurogenesis. The expression of TAG-1 is downregulated in the adult brain, with the exception of certain areas exhibiting structural plasticity, such as the spinal cord and dorsal root ganglia [23]. In this study, we found that TAG-1 is expressed in hippocampal neurons in mice at 8 weeks of age (Fig. 4) and younger mice. The expression levels of TAG-1 in the hippocampus were reduced in mice at 8 and 20 months (Fig. 4). These results suggest that TAG-1-mediated inhibition of TGFβ2-induced neuronal cell death via APP in the hippocampus is attenuated in aged mice. Neurons may become more sensitive to TGFβ2-induced death via APP in the brains in the middle-aged and older mice than in young mice.

4. Discussion

It was reported that TGFβ2 levels were upregulated in astrocytes and neurons of brains with familial AD-linked mutant PS1 [12]. Recently, we have found that TGFβ2 levels are upregulated in neurons of hippocampi and cerebral cortices of solitary AD cases [unpublished observation]. Aβ appears to induce the expression of TGFβ2 transcriptionally [13]. TGFβ2, but not TGFβ1 or TGFβ3, activates APP-mediated death signal pathways, by binding to APP [8]. Collectively, this AD-linked upregulation of TGFβ2 in brains may contribute to the onset and the progression of AD-related neuronal cell death [8,11], although this notion should be tested *in vivo* death models relevant to AD. We also demonstrated that TGFβ2-induced death of primary cortical neurons, in which the V642I-APP mutation was knocked in, without overexpression of APP [8].

In this study, we used the TGFβ2-induced death of F11 cells overexpressing wt-APP as the TGFβ2-induced neuronal cell death via APP model to show that TAG-1 antagonized TGFβ2-induced neuronal cell death via APP by inhibiting the binding of TGFβ2 to APP competitively (Fig. 3B). In addition, we found that TAG-1 expression levels are reduced in an age-dependent manner (Fig. 4). Together, these results suggest that the age-related reduction in TAG-1 expression may contribute to the onset and the progression of AD-linked neuronal cell death, triggered by the upregulated binding of TGFβ2 to APP.

It is possible that there is an endogenous defense mechanism inhibiting the onset and the progression of AD. Accumulating evidence has indicated that Humanin appears to be an endogenous defense factor against AD [24]. Humanin or its relative may inhibit AD-relevant neuronal death [25], including TGFβ2-induced neuro-

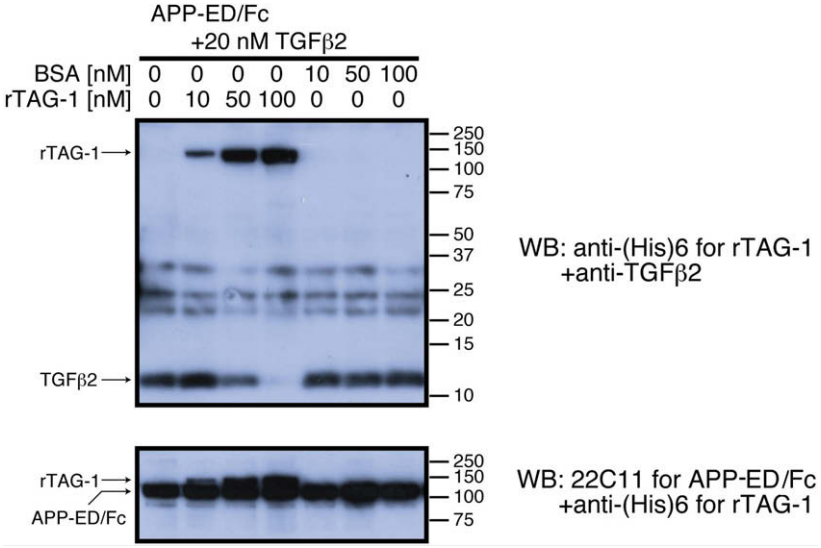

Fig. 3. TAG-1 inhibits the binding of TGFβ2 to APP in a competitive fashion. Ten picomoles recombinant TGFβ2 (final concentration in PBS; 20 nM) was mixed with APP-ED/Fc or Fc protein in the presence or absence of indicated concentrations of recombinant TAG-1 (rTAG-1) or BSA for co-immunoprecipitation analysis. Fractionated immunoprecipitates were immunoblotted with antibodies to TGFβ2 and 6× histidine (for the detection of recombinant 6× histidine-tagged TAG-1) (top panel). The same membrane was sequentially used for immunoblot analysis with antibody to APP (for the detection of APP-ED/Fc) without stripping of the previously used antibodies (bottom panel).

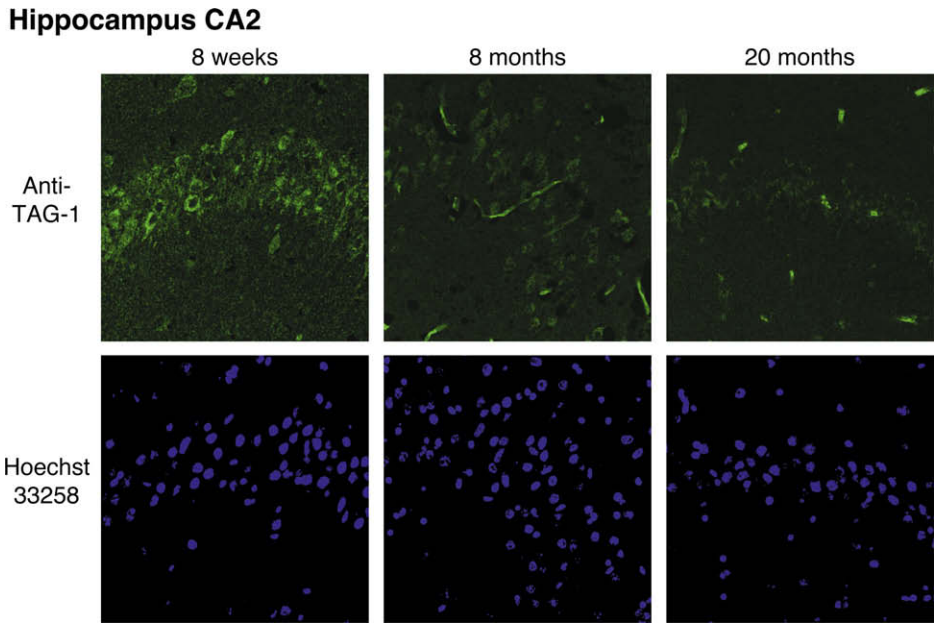

Fig. 4. TAG-1 levels in hippocampal neurons are reduced in an age-dependent manner. Hippocampal sections (pyramidal layers of the CA2 regions from mice at ages of 8 weeks, 8 months, and 20 months were immunostained with antibody to mouse TAG-1.

nal cell death via APP (unpublished observation). This inhibition is by the Humanin binding to its specific receptor on the cell surface, composed of gp130, ciliary neurotrophic factor receptor α, and WSX-1 [20] and by activating the JAK2/STAT3 pro-survival pathway [26]. Humanin also improves AD-relevant neuronal dysfunction in AD mouse models, including FAD gene-overexpressing transgenic mice, by activating the JAK2/STAT3 pathway [26]. Other than Humanin, this study suggests that TAG-1 is another endogenous defense factor against AD in the hippocampus with a death-suppressing mechanism quite different from that by Humanin.

Aging is the major risk factor for AD. Few human AD cases were younger than thirty years. However, it remains unknown how aging contributes to the onset and the progression of AD. A recent study showed that the levels of phosphorylated STAT3 levels, an activated form of STAT3, were reduced in hippocampal neurons of both AD mouse models in an age-dependent manner and in solitary human AD cases [26]. This AD-related reduction in the pSTAT3 level, caused by both the AD-related and possibly age-related reduction of the Humanin signal [26], contributes to the onset and the progression of AD. Our results suggest that TAG-1 also contributes to the age-related onset of AD. From a standpoint of AD treatment, increasing the TAG-1 level in brains of AD patients may be a new promising strategy. Future investigation is required to confirm this strategy.

Conflict of interest

There is no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.02.127.

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