INTERACTION BETWEEN α-SYNUCLEIN AND TAU PATHOLOGIES IN A MOUSE MODEL OF LEWY BODY DEMENTIA

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ABSTRACT

Neuropathological and cell culture studies suggest that tau and α-synuclein (α-syn) pathologies may promote each other. In order to study the interaction between tau and α-syn more in detail we used adeno-associated virus (AAV)-vector mediated expression of human wild-type or mutated (P301S) tau in either wild-type or A30P mice. Green fluorescent protein (GFP) transduction was used as a control. Local infusions of AAV-vector were made bilaterally into the hippocampus. The mice were perfused for histology 11 weeks after the gene transduction. Neuronal loss and tau hyperphosphorylation analyses were done using a conventional light microscope. Co-localization analyses were done under a confocal microscope.

Tau transduction was not associated with marked neuronal loss, although a small decline in the pyramidal cell density was detected in A30P mice receiving wt tau injections. We also detected hyperphosphorylated tau in a subset of neuronal cell bodies in the CA3 area in mice with Tau13 positive cells. Of these tau-positive neurons, 18% in A30P mice but only 7% in wild-type mice receiving human wild-type tau transduction formed paired helical filament-1 (PHF-1) positive cytoplasmic densities, while only less than 5% of tau-positive neurons were also PHF-1 positive after transduction with P301S tau in mice of either genotype. Cytoplasmic α-syn positive inclusions were detected in pyramidal cell bodies in A30P mice transduced with either wild-type or P301S mutated human tau.

In conclusion, tau and α-syn proteins interact in vivo and their interaction promotes a-syn aggregation and tau hyperphosphorylation. However, this interaction inducing tau hyperphosphorylation is dependent on specific binding site on tau, and that mutation at the 301 site prevents this interaction.

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ABBREVIATIONS

α-syn	α-synuclein
AAV	Adeno-associated virus
AD	Alzheimer's disease
auto	Automatic counting of both sides
auto_L	Automatic counting of left side
auto_R	Automatic counting of right side
BSA	Bovine serum albumin
DAB-Ni	3,3'-Diaminobenzidine with nickel
DAPi	4',6-diamidino-2-phenylindole
DG	Dentate gyrus
GFP	Green fluorescent protein
GSK-3beta	Glycogen synthase kinase 3 beta
KPBS-T	Kallium phosphate buffered saline with triton
LBD	Lewy body dementia
MAP	Microtubule associated protein
man_1	First time manual counting of both sides
man_1L	First time manual counting of left side
man_1R	First time manual counting of right side
man_2	Second time manual counting of both sides
man_2L	Second time manual counting of left side
man_2R	Second time manual counting of right side
NFT	Neurofibrillary tangles
PB	Phosphate buffer

PBS	Phosphate buffered saline
PD	Parkinson’s disease
PHF	Paired helical filaments
TBS-T	Tris buffered saline with triton
tg-GFP	A30P α-syn transgenic mice injected with AAV vector carrying GFP
tg-wt tau	A30P α-syn transgenic mice injected with AAV vector carrying wild type tau
tg-m tau	A30P α-syn transgenic mice injected with AAV vector carrying mutated tau
wt-GFP	Wild type transgenic mice injected with AAV vector carrying GFP
wt-wt tau	Wild type transgenic mice injected with AAV vector carrying wild type tau
wt-m tau	Wild type transgenic mice injected with AAV vector carrying mutated tau

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1. INTRODUCTION

Parkinson's disease (PD) is a common neurodegenerative disorder, with the highest prevalence in people aged sixty or older (1). Lewy body dementia (LBD), also known as dementia with Lewy bodies, causes a progressive dementia with significant cognitive fluctuations and PD-like symptoms (2). α-Synuclein is an abundant presynaptic protein which is pathologically linked to PD (3). Tau is a microtubule-bound protein, which may become hyperphosphorylated by the imbalance of kinase and phosphatase activities in neurons, leading to formation of neurofibrillary tangles (4).

Both Parkinson's disease and Lewy body dementia are characterized by intracellular accumulation and aggregation of α-synuclein into Lewy bodies. Recent evidence suggests that besides α-synuclein, tau aggregates are also found in post-mortem brain examination of both diseases (5, 6). Furthermore, a recent study showed that *in vitro,* tau and α-syn induced each other's polymerization into fibrils, and *in vivo,* α-syn promoted the fibrillization of tau in brain sections from transgenic mice overexpressing human A53T mutated α-syn (7). These findings may provide a possible mechanistic explanation for the co-existing of α-synuclein and tau. In addition, phosphorylated tau in parallel with accumulation of aggregated α-syn were also found in mice overexpressing another α-syn mutation (A30P) (8). Another recent study provided additional evidence for the interaction of α-syn and tau by showing selective dose-dependent hyperphosphorylation of tau at Ser396/404 by the treatment of primary mesencephalic neurons or wild-type mice with the neurotoxin MPP(+)/MPTP. However, this hyperphosphorylated tau was not observed in transfected cells not expressing α-syn or in α-synuclein -/- mice (9). The kinase involved in this tau hyperphosphorylation , may be glycogen synthase kinase 3 beta (GSK-3beta),because GSK-3beta activation was observed in many MPP(+)/MPTP models of PD, but there is no evidence of GSK-3 beta activation in cells not expressing α-synuclein or in α-syn KO mice (10). In addition, it has also been demonstrated that tau overexpression increases α-syn aggregation in co-transfected cells (11).

To study the interaction between these two proteins more in detail we used AAV-vector mediated expression of human wild-type or mutated (P301S) tau in either wild-type mice or mice carrying familial PD-linked A30P α-syn mutation. Local infusion of AAV-vector were made bilaterally into the hippocampus, because the endogenous expression levels of α-syn in the A30P mouse (12) is the highest in mossy fibers connecting the dentate granule cells with CA3 pyramidal cells.

2. LITERATURE REVIEW

2.1 α-SYNUCLEIN

2.1.1 Brief introduction

α-Syn is a small (19 kDa) and conserved acidic protein, which is concentrated mainly in presynaptic axon terminals(13, 14). It appears physiologically in soluble membrane and lipid-bound fractions (15). α-Syn is encoded by its gene SNCA, also known as PARK1, which is located on chromosome 4q21 (16). It is a member of naturally unfolded proteins which do not contain a typical secondary structure (17). α-Syn has seven imperfect 11-residue repeat sequences, which are predicted to form five amphipathic helices on the amino-terminal half in its primary structure (18, 19, 20). α-Syn helices 1-4 are expected to be connected with lipid vesicles (21, 22), whilst helix 5 is likely to be related with protein-protein interactions (23). However, the secondary structure of α-syn is dependent on its environment, which makes α-syn very dynamic (22, 23).

α-Syn possibly works as a molecular chaperone, which has the ability of binding to other intracellular proteins due to its complicated biochemical structure (24).

Two findings strongly support this prediction. First, α-syn and the 14-3-3 family of molecular chaperone proteins share the same structural homology (24, 25). Second, α-syn expression is upregulated during periods of strengthened neuronal activity and plasticity (26, 27). Consistent with the chaperone prediction, α-syn interacts with numerous proteins and regulates dopamine homeostasis in nigrostratial dopamine neurons at axon terminals that may have the ability of regulating the monoaminergic vesicle content and the deposit of nerve terminal dopamine (28, 29). α-Syn may also play a role in terminating dopamine neurotransmission, because its expression changes dopamine transporter-mediated uptake of synaptic dopamine (30). The phosphorylation of α-syn regulates the catalytic activity of tyrosine hydroxylase, the rate-limiting enzyme in dopamine synthesis (31, 32). α-Syn takes a paramount role in the mediation of substrate-dependent increase of phosphorylated tyrosine hydroxylase and dopamine synthesis in nigrostratial dopamine neurons. It is directly related to dopamine replenishment and the differentiation of presynaptic dopamine (31, 32, 33). α-Syn is also capable of downregulating the p53 pathway to achieve an antiapoptotic function (34).

2.1.2 The role of α-synuclein in neurodegenerative diseases

Abnormal α-syn aggregation has been discovered in many neurodegenerative diseases. α-Syn aggregation and its antibodies have been identified in Down syndrome brains and in Alzheimer's disease (AD) (35). Pathological α-syn aggregates have also been found in PD and LBD patients (36). α-Syn was found to be the predominant protein in Lewy bodies in sporadic as well as in familial PD (37, 38, 39, 40), and the filamentous aggregation of α-syn was detected in LBD (39, 41). α-Syn was also found to be extensively nitrated in pathological lesions of LBD (42,43). Most of α-syn in Lewy bodies is serine (Ser)-phosphorylated and residue Ser-129 is the phosphorylation site of approximately 90% of the α-syn in Lewy bodies (44). In PD, Lewy body formation and neuronal death are mostly found in the substantia nigra, although α-syn is widely expressed throughout the central nervous system. This is possibly because dopamine metabolism and α-syn

conformations interact with each other , causing the selective dopamine loss in PD (45). There is evidence showing that α-syn could be a presynaptic regulator of dopamine secretion, storage or synthesis (46). This has been proved by showing that overexpression of α-syn, both *in vitro* and *in vivo*, decreases the activity of tyrosine hydroxylase and causes a corresponding reduction of dopamine levels that are not created by the increase in dopamine catabolism or efflux (47). Several studies indicate that the modification and aggregation of α-syn play a key role in neuronal cell death and Lewy body formation related with PD (48). Hyperphosphorylated α-syn is found in Lewy bodies and Lewy neurites, which contributes in important ways to PD process. The role of α-syn phosphorylation in neurodegenerative pathogenesis is starting to be revealed by studies of its phosphatases and kinases (49).

2.2 TAU

2.2.1 Brief introduction

Tau was found to be the first microtubule associated protein (MAP) that promotes microtubule assembly *in vitro* (50). Tau protein constitutes a group of closely connected isoforms which are partly created by alternative mRNA splicing (51). Tau is usually concentrated in axons (52, 53, 54), but it has been also identified in the somato-dendritic compartment in a pathological condition (55, 56). Tau proteins have been found to consist of three domains by different kinds of functional analysis including integrating sequence comparison. These three domains are a middle region full of proline that contains corresponding kinases recognizing sites, a highly acidic amino terminal domain that is accentuated from the microtubule surface on which it works with other cytoskeletal elements, and a carboxy terminal domain, which has microtubule binding activity due to its components of three or four duplicates of a highly conserved 18 amino acid sequence (57, 58, 59).

In vitro, tau has the ability to induce nucleation and growth of microtubules and cause their bundle formation (59). In addition, tau is able to decrease the dynamic instability of microtubuli (57, 60). Furthermore, *in vivo* studies demonstrated that microinjection of tau into fibroblast cells promoted tubulin polymerization and microtubuli stabilization (61), and that transfection of tau cDNA and the later expression of tau protein caused microtubule stabilization and bundling in nonneuronal cells (62, 63, 64). Tau protein is very important in mediating neuronal outgrowth, axonal transport and dynamic stability of microtubuli. It accomplishes these functions by the modulation of its site-specific phosphorylation (65).

2.2.2 The role of tau in neurodegenerative diseases

The aggregation of tau plays an important role in many neurodegenerative diseases, such as fronto- temporal dementia, AD, and PD. Tau aggregates usually exists in the form of neurofibrillary tangles (NFT), which contain hyperphosphorylated tau in the form of twisted or straight filaments or paired helical filaments (PHF) (66). It has been reported that in AD the number of NFT in the neocortex reflects the severity of the dementia (67). In frontotemporal dementia, formation of tau filaments is accelerated due to missense mutations in tau (68, 69, 70). It could be concluded that tau aggregation induces neuronal cell death, because many studies have revealed that without aggregation-inducer, tau over-expression does not cause toxicity (66). The increased ability of tau to aggregate into filaments in glia and neurons in various tauopathies possibly derives from the fact that the pool of tau which could form aggregation is promoted by the pathological status of neurons. Notably, tau fibrillization needs a high concentration of tau in many experimental models (66).

It is unlikely that the concentration of tau in different kinds of human tauopathies is as high as in cell culture or in transgenic mice in which tau overexpression was artificially induced, even though enhanced tau concentration have been shown in

the AD brain samples (66, 71). For instance, in transgenic mice expressing mutated human tau, tau aggregation displaced many cytoplasmic organelles from their normal site and reduced their number in neuronal cells (72).

Hyperphosphorylation of tau is thought to be the first pathological even leading to the development of NFTs in human tauopathies (73). In the AD brain, hyperphosphorylated tau appears in the form of straight filaments, paired helical filaments or NFTs (71, 74). Increased level of tau phosphorylation has also frequently been found in the cerebrospinal fluid of AD patients, which makes the increased level of hyperphosphorylated tau a clinically useful diagnostic marker (75, 76).

2.3 INTERACTION BETWEEN α-SYNUCLEIN AND TAU IN NEURODEGENERATIVE DISEASES

Neurodegenerative disorders, such as frontotemporal degeneration, prion diseases, Huntington's disease, PD and AD, belong to "proteinopathies", because they have common molecular and cellular mechanisms, which include the accumulation of proteins in the central nervous system (77, 78).

Tau and α-syn proteinaceous inclusions have been identified as hallmarks of AD and PD, respectively (79). α-Syn and tau may coaggregate in the same cell or same region of the brain in both transgenic mice and humans (80, 81, 6, 82). There is data suggesting that the oxidative modification of α-syn plays an important role in tau aggregation (83), and that the toxic interactions between tau and α-syn may cause tau hyperphosporylation and eventually lead to the deposition of both of these two proteins (84). Tau aggregates were found to be phosphorylated at Ser 262 and 396/404 in both A53T α-syn mutated mouse model of PD and PD patients in two recent studies (85, 86). α-Syn also induced GSK-3beta catalyzed tau phosphorylation in cellular MPP+ and *in vivo* MPTP models

(9,87,88). Tau phosphorylation at Ser 262 was found to be promoted by α-syn mutations (89). Genetic studies have shown interactions between tau and α-syn (90, 91), and A53T mutation was found to induce the fibrillization of tau and α-syn in familial PD (92). In addition, genetic variations of α-syn can modulate the extent of fibrillization of pathological tau (93).

3. AIMS

This study sets out to investigate whether the CNS expression of human tau (either wild-type or mutated) leads to neuronal loss and whether this neurotoxicity is dependent on the α-synuclein status of the neurons. The neuronal status of α-synuclein was varied by giving the injections to either transgenic mice carrying A30P α-synuclein mutation or to wild-type mice.

4. MATERIALS AND METHODS

4.1 ANIMALS

In total 37 male mice (7-8 months at the time of the AAV vector injections) were examined. Mice were divided into two groups with different genotypes in the study. The first group of mice consisted C57B16/J originating from Charles River (Laboratories Inc., Wilmington, MA, USA) with preserved mouse α-synuclein gene constituted the wild type (wt) group (n=18). The second group of mice (n= 19) carried transgenic human α-synuclein with the A30P mutation under the mouse prion protein promoter (94) constituted A30P group. This line was established as C57BL/6J ×DBA/2J hybrids, thereafter backcrossed for five generations into the original background C57BL/6J strain (Charles River

Laboratories Inc., Wilmington, MA, USA). The presence of mouse or human α-syn was verified with PCR as demonstrated earlier (94).

All the mice were housed individually and kept under controlled conditions (temperature: +21°C, light from 7:00 a.m. to 7:00 p.m., humidity 50-60%). Food and water were freely supplied. All experiments were conducted according to the Council of Europe and Finnish guidelines, and approved by the National Animal Experiment Board in Finland.

At the end of the study the mice were deeply anesthetized with a pentobarbital/chroralhydrate cocktail (60 mg/kg each) and perfused transcardially with ice-cold heparinized saline followed by 4% paraformaldehyde in PBS for 15 min. The brain was post-fixed for 4 h in the paraformaldehyde solution and then transferred to 30% sucrose in PBS overnight. Brains were frozen in a cryoprotectant and stored under – 20 ^{o}C until cut into 35 µm coronal brain sections in 1-in-6 series using a freezing sliding microtome and stored in a cryoprotectant at 4 °C.

4.2 INJECTION OF VIRAL VECTORS

The viral vector used in the study was provided by our Swedish collaborators. The vector used contains the AAV2 inverted terminal repeats pseudotyped in AAV5 capsid, since the AAV5 capsid yields higher transduction efficiency in the brain. Vector titers were determined by dot-blot analysis to be 3.5E12 and 1.0E12 vector genomes (vg)/ml for the wild-type and P301S mutant tau vectors, respectively, and 3.2E13 vg/ml for GFP vector.

Under isoflurane anesthesia (induction 4 %, maintenance 1%, in 30% O2 /70% N20) the mouse was placed in a mice stereotaxic frame (David Kopf, Tujunga, CA, USA). Before opening the scalp, the mouse received a local subcutaneous injection of 0.5 ml of lidocaine (Lidocain^{R} c. adrenalin 120 mg/ml, Orion Pharma,

Turku, Finland). Each animal received a 1 µl injection of virus suspension bilaterally into the dentate gyrus at the following coordinates: AP - 2.0 mm, ML 1.4 mm from bregma, DV 1.6 mm from the dura. The vector was injected at a rate of 0.1 µl per 30 s with a Hamilton syringe with a fitter glass capillary tip, after which the glass capillary was held in place for 5 min before withdrawal. Then the skin was sutured, and the mouse released from the stereotax. Mice received 5 mg/kg of carprofen (Rimadyl ®, Pfizer) as post-operative analgesic. The animals were kept in warm incubator (30° C) about 30 min before returning them to clean home cages.

4.3 CELL COUNTING

4.3.1 Pyramidal cell counting

From each mouse we took one unstained coronal section (Fig. 1B) at the same AP position (two even space distance from the reference section (Fig. 1A) in rostral hippocampus where both blades of dentate gyrus (DG) were visible) roughly corresponding to the level of AAV infusion.

First, all the sections of interest were picked out and let to stay in Phosphate buffer (PB buffer) overnight at room temperature. The next day, those sections were mounted on gelatinized slides, and then coverslipped by mounting medium with 4',6-diamidino-2-phenylindole (DAPi) (VECTASHIELD, Mounting Medium 1.5 mg/ml, Vector Laboratories Inc., Burlingame, CA, USA). The sections were stained with DAPi to visualize all nucleis with blue fluorescent color. After 24 hours, we started to take photos of the region of interest from both sides of hippocampus. In order to select the same region, an optical framework was placed to CA3 cell area in the dentate hilus and then moved to the border of CA2 area (Fig. 2A and B).

A digital photo was taken from the region of interest from the hippocampus on both sides (Fig. 2C and D). This was done under Olympus BX 40 microscope (No. 8B07416, Made in Japan). The Filter we chose was WU. The software we used was Viewfinder Lite (The values we set was like this: FL; Highest resolution: 2776 × 2074; Exposure Mode: manual; Exposure time: 1/ 1.5; Sensitivity: 100; Spot: 30 percent; Exposure Adjust: 0).

The final analysis was to count the number of DAPI+ nuclei in this reading frame on both sides. This has been done in two ways:

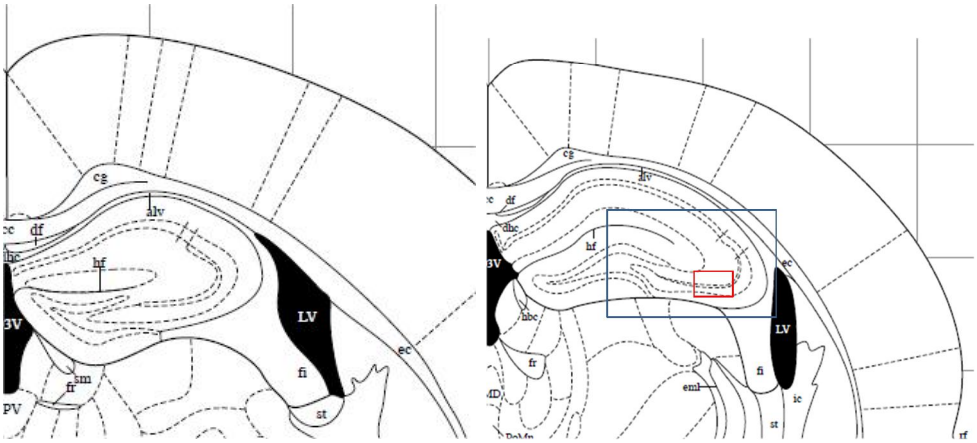
(1) Automatically using the analysis software

This was done by using Photoshop CS3 software. Because each nucleus consists of 3-4 intense spots on a more or less uniform background, granules and small spots were not counted as nucleuses. In this step, the stable values were set for the framework (Feather: 0 px, Style: fixed style, Width: 2500 p, Height: 800 px). When we started to choose the color, normal style was used. Left and right sides were counted separately. We always moved the frame to the right border of the region so that we were always counting the same area (Fig. 2E and F). After selecting the area, we started to choose the color from color ranging, and then began the analysis (in this step, there were huge number of chosen spots , because the software chose every same color spot as a nuclei, so we used “expand” and “contract” to decrease the number of spots to one in one cell.) In the end, we started to record the measurements.

(2) Direct visual observation on the computer screen with manual counting.

Left and right sides were counted separately. In this step, we first set the same stable values for the framework on Photoshop CS3 software as it was shown above, then we started to count the cells in the same area as we chose in counting by software. Granules and small spots were not counted as pyramidal cells.

Fig. 1



A **B**

Fig 1. Demonstration of the region of interest for cell counting. A: The reference section. **B:** Unstained coronal section. Blue frame area is the place which we took photos from. Red frame area is the place where we counted pyramidal cells.

Fig. 2

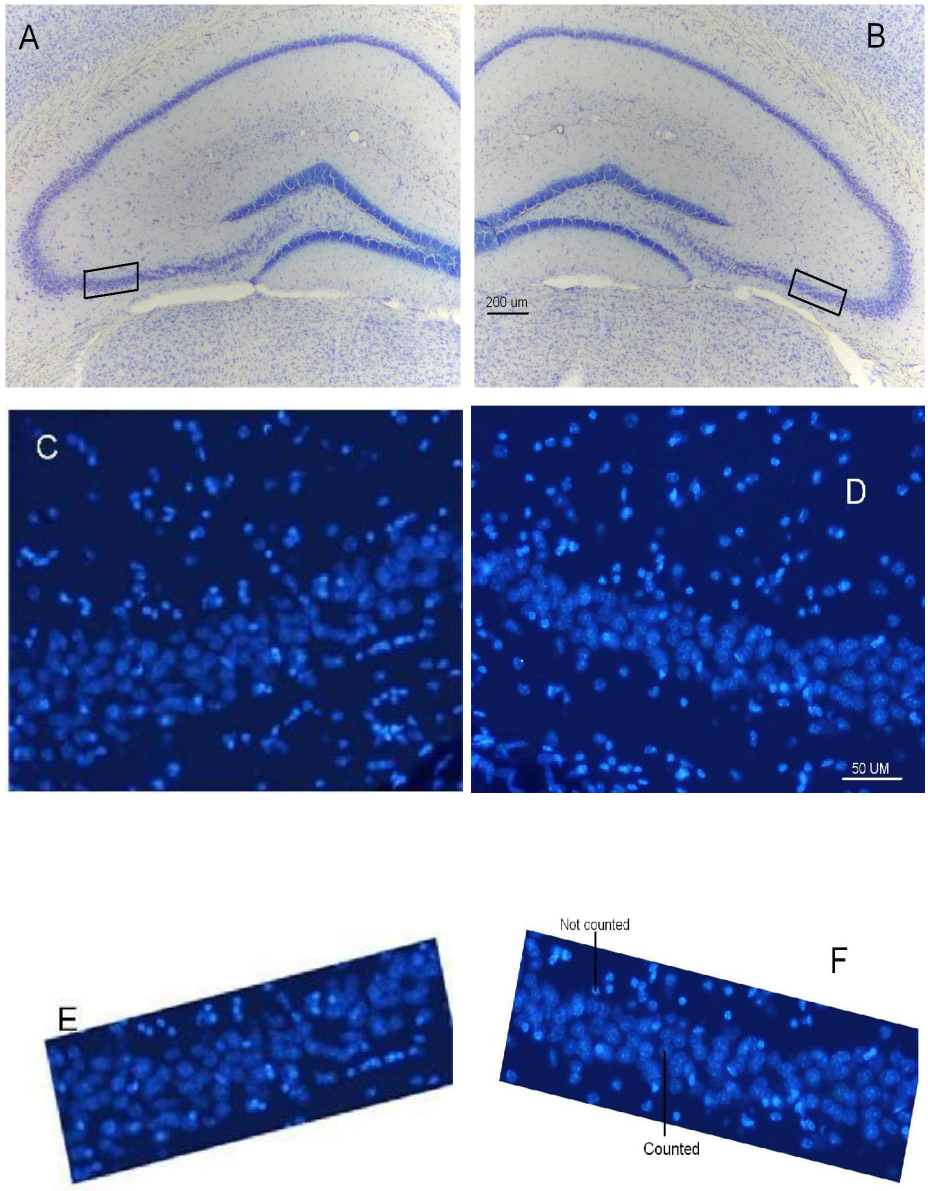


Fig 2. A and B: Cresyl violet staining of the region of interest (frame: left and right sides of hippocampus). Scale bar = 200 µm. **C, D, E and F:** DAPi staining of pyramidal cell nuclei. **C and D:** Amplified frame area of the region of interest (left and right sides of hippocampus). **E and F:** Counting area of the frame area (left and right sides of hippocampus). Scale bar = 50 µm in C, D, E and F.

4.3.2 Counting of cells expressing Tau 13 and PHF-1

In order to determine whether injected wild-type or mutated human tau protein becomes hyperphosphorylated, we stained brain sections from 28 tau transduced animals with the PHF-1 antibody. PHF-1 antibody has the ability of recognizing a posphorylated epitope of tau protein but displays little cross-reactivity with normal adult human tau (95). PHF-1 positivity is also considered to indicate an advanced state toward neurofibrillary tangle formation (96). Here we first counted the number of Tau-13 positive cells in CA3 area, then we counted the number of PHF-1 positive cells in the same area from the same section of hippocampus. We used the percentage of PHF-1 positive cells to Tau-13 positive cells to reflect the extent of hyperphosphorylation of wild-type or mutated human tau.

PHF-1 slides were made as follows. First, sections of interest were pretreated in hot citrate solution, and in a small (4 × 6) tray sections were rinsed 3 × 5 min each in TBS (Tris Buffered Saline; pH = 7.6). Approximately 20 ml / tray. Second, the sections were incubated with the primary antibody (mouse anti-tau at 1:500; Tau; MCL; Peter Davies Albert Einstein College of Medicine, New York, NY, USA for 2 weeks at + 4 °C in TBS-T, pH = 7.6 with azide on a shaker table in the dark (mouse anti-tau IgG at 1: 500 and 20 µl antibody in 10 ml tris buffered saline with triton (TBS-T) with azide). After that, sections were rinsed again in Phosphate buffer saline (PBS) and then incubated with the secondary antibody (Goat anti-mouse*biotin at 1 : 500; Sigma) for 2 hours. Third, sections were rinsed and put in Extravidin® (at 1 : 1000) for 2 hours. After that, sections were rinsed 3 × PBS (5 min each); approximately 20 ml. Visualization of PHF-1 tau was achieved by incubation with 3,3'-Diaminobenzidine with Nickel (DAB-Ni) solution. In the end, we mounted the sections on the slides. The method and process of making tau 13 slides is similar with PHF-1 slides, the only difference being that we made tau 13 slides by using mouse anti-tau IgG at 1:1000 instead of 1:500 in preparing PHF-1 slides.

Digital photos were taken from the region of interest of the hippocampus on both sides. This was done under Olympus BX 40 microscope (Japan). The software we used was Viewfinder Lite and the values were set like this: BF; Filter: Wu; Exposure Mode: Manual; Exposure time: 1/120; Resolution: 1392×1040; Sensitivity: 100; Spot: 30 percent; Exposure Adjust: 0.

PHF-1 positive cells were counted in 2-3 sections close to the injection site (same planes for all mice). A reading frame was first placed around the end of CA3c area in the dentate hilus and then moved systematically towards the CA2 border, so that each section was counted only once. All PHF-1and Tau 13 positive cells were counted from the selected CA3 region of interest in 28 tau injected animals. All analyzes were done bilaterally.

4.4 CO-LOCALIZATION ANALYSES

To visualize tau and α-syn co-localization in the hippocampus, we performed double fluorescent labeling using a rabbit monoclonal anti-α-syn antibody (1:1000, SA 3400, Biomol, United Kingdom) and the mouse monoclonal anti-human Tau13 antibody (Biosite). The specificity of the anti-α-syn antibody was confirmed by negative staining of sections from α-syn deficient mice. Before applying the primary antibody, endogenous peroxidase was quenched by incubation in 0.1 % H_2O_2 for 30 min and non-specific binding was blocked in 1% Bovine serum albumin (BSA) in Kallium phosphate buffer saline with triton (KPBS-T) for 30 min. The primary antibody was incubated overnight. Sections were incubated in fluorescent secondary antibodies Alexa 488 (1:250 molecular probe for tau in double staining) and Alexa 594 (1:250, molecular probe for α-syn in double staining) for 2 h at room temperature in darkness. After washing in TBS-T, the slides were coverslipped by mounting medium with DAPi (VECTASHIELD®, Mounting Medium 1.5 mg/ml, Vector Laboratories Inc., Burlingame, CA, USA).

Co-localization analyses was done under Zeiss LSM 700 confocal microscope. In order to know the extent of co-localization, we counted the percentage of α-syn tangles in each tau positive cell and its two neighboring α-syn positive cells in the CA3 area of hippocampus of mice from each group.

4.5 STATISTICAL ANALYSES

The neuronal cell counts and the area of α-syn inclusions were analyzed using two-way ANOVA with genotype and transduction as between-subject factors. All statistics were run with SPSS 11.0 for Windows software (SPSS Inc., Chicago, IL, USA).

5 RESULTS

5.1 TAU TRANSDUCTION IS NOT ASSOCIATED WITH MARKED NEURONAL LOSS

In order to assess the extent of neuronal loss in the hippocampus, we calculated pyramidal cell density in the CA3 subregion which was closest to the injection site. This was done both manually by a human observer blind to the treatment groups or with an automated image analysis. The correlation coefficient of pyramidal cell count was less than 0.9 from left and right sides of the hippocampus section in each counting method (Fig. 3), but the replicability of cell-difference counting results across analysis sessions was always higher than the left-right difference (Fig. 3 and 4). The correlation coefficient of the results between the second manual and auto counting was quite close to 1 (Pearson rho =

0.97, Fig. 4C), which shows high replicability, indicating that the results were methodologically right.

Although there was a small decline in the pyramidal cell density in A30P mice receiving wt tau injections (Fig. 5), there was no significant genotype [$F(1,30) = 2.7$, $p = 0.11$] or transgene [$F(2,30) = 2.7$, $p = 0.08$] main effect or their interaction [$F(2,30) = 0.2$, $p = 0.85$] (Fig. 5A). In addition, using the Fluoro-Jade B marker for degenerating cells, we found no evidence of apoptotic cells in the hippocampi of either wild-type of A30P transgenic mice that received tau transductions (data not shown).

Fig. 3

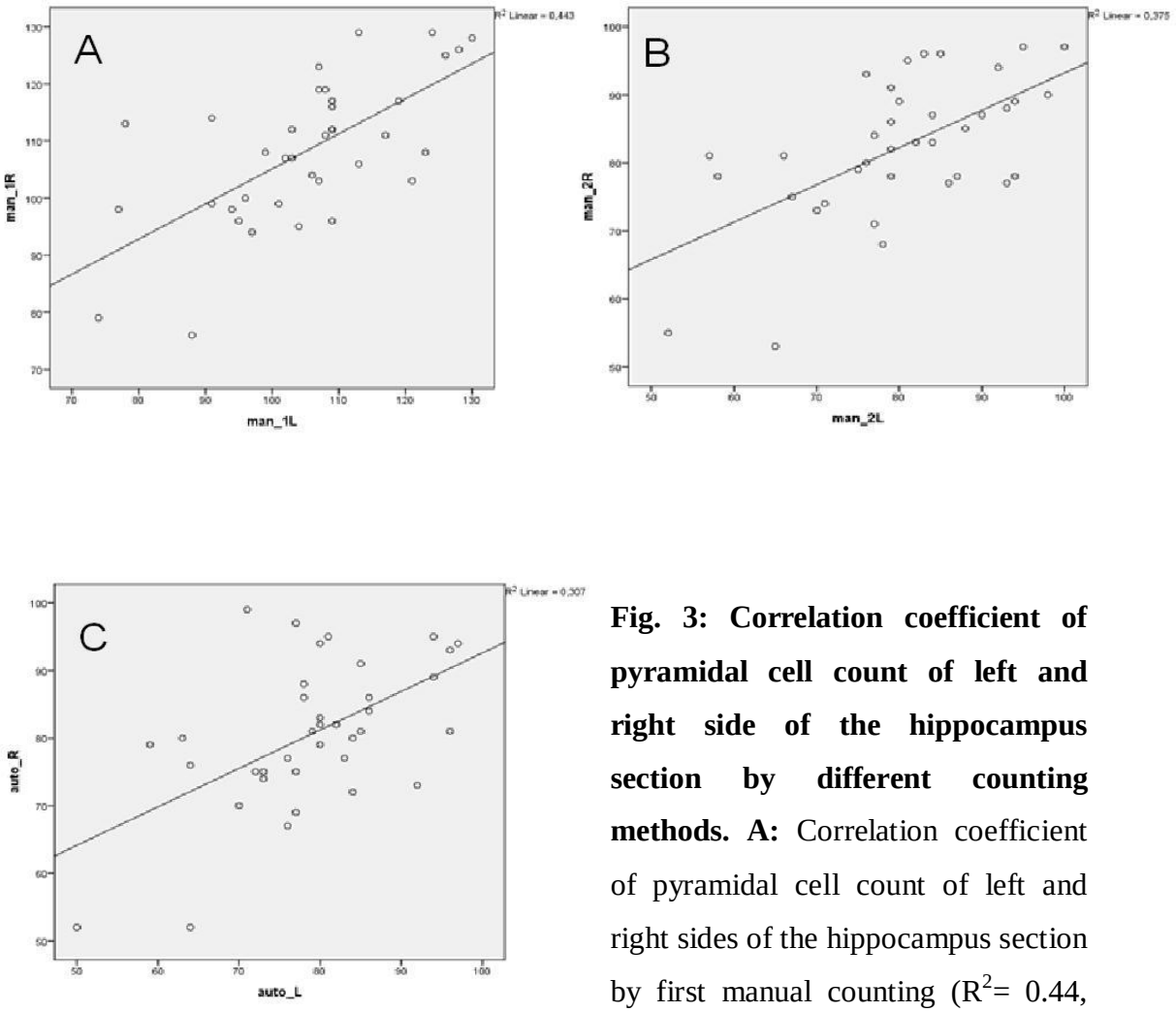


Fig. 3: Correlation coefficient of pyramidal cell count of left and right side of the hippocampus section by different counting methods. A: Correlation coefficient of pyramidal cell count of left and right sides of the hippocampus section by first manual counting (R^2= 0.44, correlation coefficient R=0.67). **B:** Correlation coefficient of pyramidal cell count of left and right sides of the hippocampus section by second manual counting (R^2 = 0.38, correlation coefficient R = 0.61). **C:** Correlation coefficient of pyramidal cell count of left and right sides of the hippocampus section by automatic counting (R^2 = 0.31, correlation coefficient R = 0.55).

Fig. 4

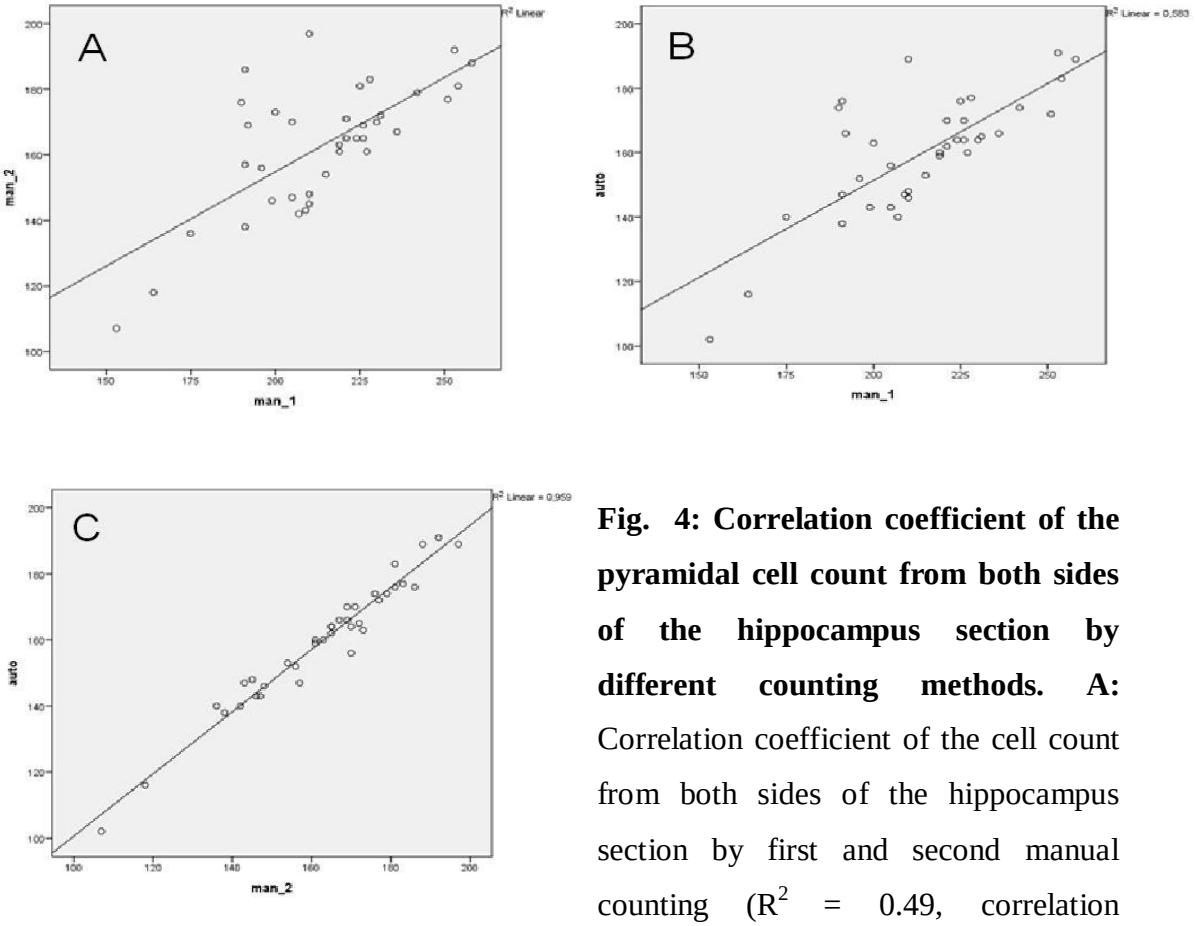
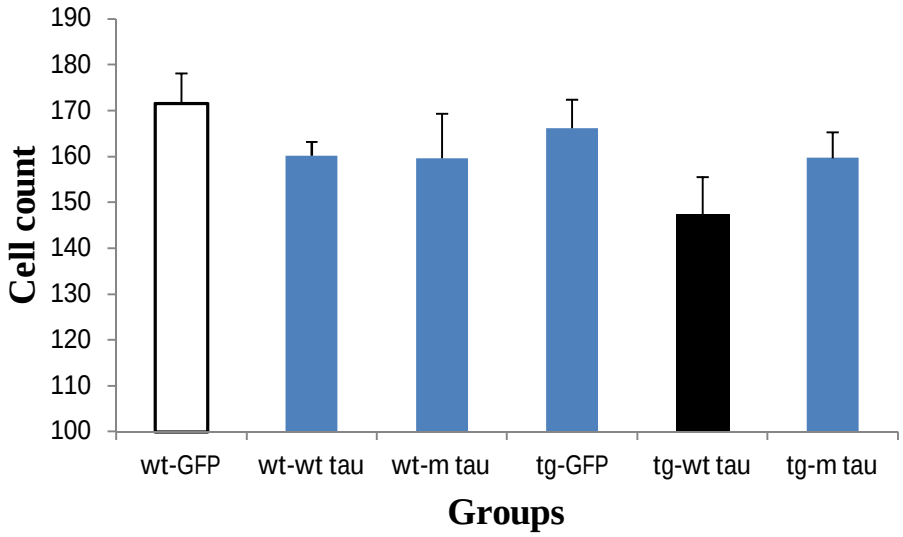


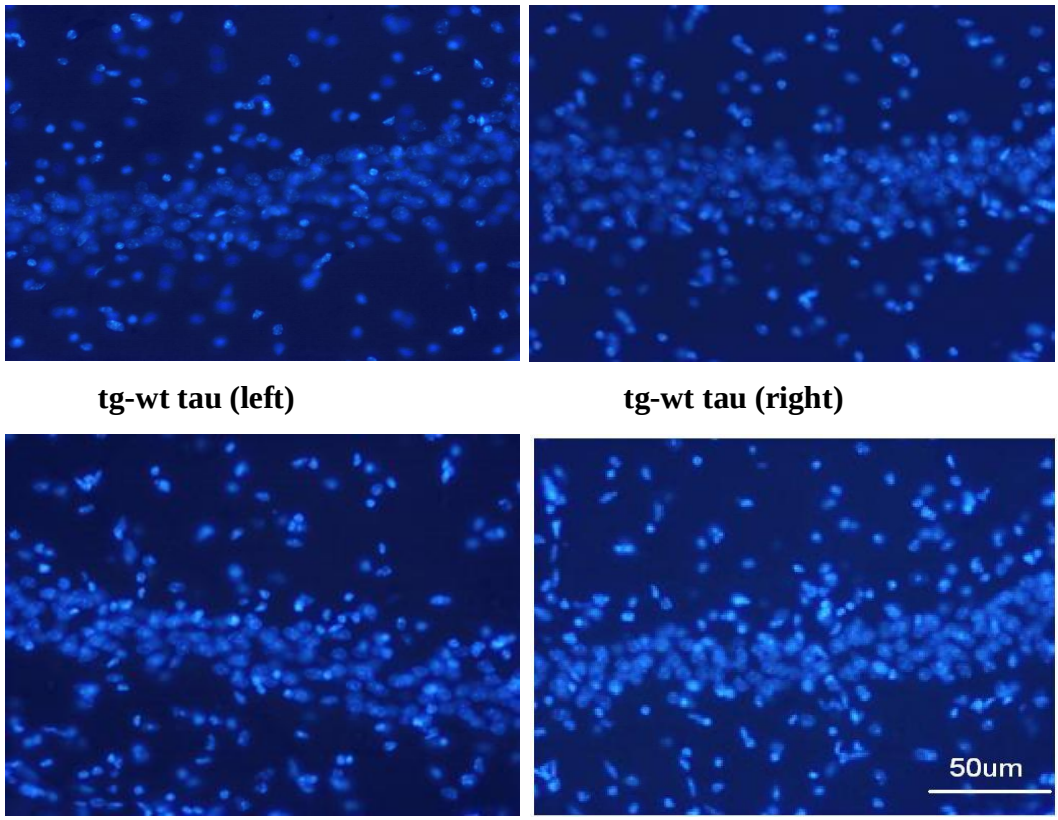
Fig. 4: Correlation coefficient of the pyramidal cell count from both sides of the hippocampus section by different counting methods. A: Correlation coefficient of the cell count from both sides of the hippocampus section by first and second manual counting (R^2 = 0.49, correlation coefficient R = 0.70). **B:** Correlation coefficient of the cell count from both sides of the hippocampus section by first manual and auto counting (R^2 = 0.58, correlation coefficient R = 0.76). **C:** Correlation coefficient of the cell count from both sides of the hippocampus section by second manual and automatic counting (R^2 = 0.96, correlation coefficient R = 0.98)

Fig. 5

A



B



tg-wt tau (left) **tg-wt tau (right)**

wt-GFP tau (left) **wt-GFP tau (right)**

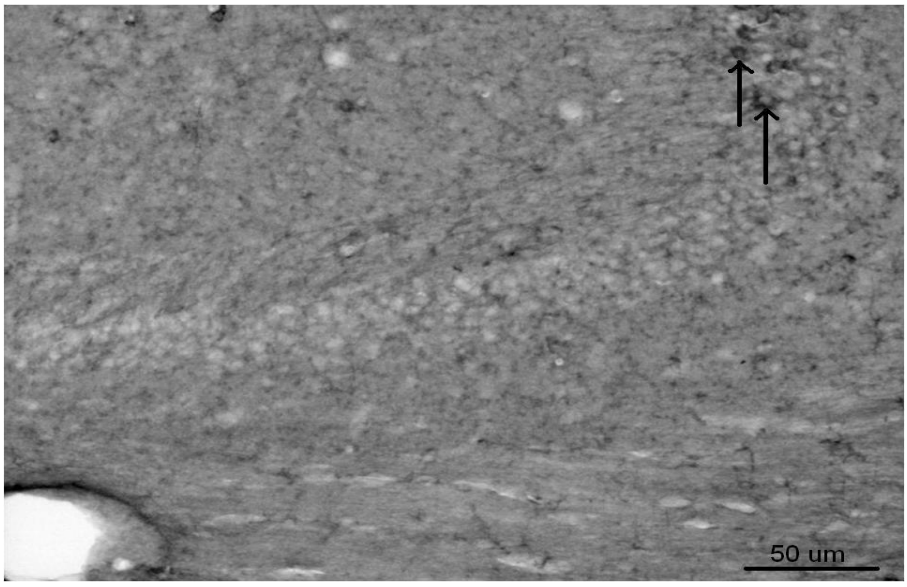
Fig 5. A: Comparison of different groups in CA3 pyramidal cell density (mean ± sem). Wt-GFP: 171.50 ± 6.614, wt-wt tau: 160.17 ± 3.103, wt-m tau: 159.63 ± 9.756, tg-GFP: 166.20 ± 6.200, tg-wt tau: 147.43 ± 8.112 and tg-m tau: 159.71 ± 5.630 (P>0.05, ANOVA). **B:** Comparison of pyramidal cell density of the CA3 layer from both right and left sides of the hippocampus section in tg-wt tau and wt-GFP groups. Scale bar = 50 µm.

5.2 HYPERPHOSPHORYLATION OF WILD-TYPE HUMAN TAU IN A30P α-SYN TRANSGENIC MICE

We indeed detected hyperphosphorylated tau in a subset of neuronal cell bodies in CA3 area in mice with Tau 13 positive cells (Fig. 6A). Since all PHF-1 positive neurons were also Tau 13 positive, we determined the average ratio of PHF-1 positive neurons of neurons with Tau 13 positive cytoplasm in each group of mice. Surprisingly, less than 5% of tau-positive neurons transduced with the P301S mutated tau stained positively for PHF-1 in either wild-type or A30P mutant mice. In contrast, transduction with wild-type human tau resulted in PHF-1 positivity in 7% of wild-type and as many as 18% of A30P mice (Fig. 6B). The ANOVA showed that there was no significant effect of the genotype [$F(1,24) = 3.9$, $p = 0.061$] and a significant effect of the injection type [$F = 21.0$, $p = 0.000$], as well as a significant effect of both genotype and injection type [$F = 7.4$, $p = 0.012$].

Fig. 6

A



B

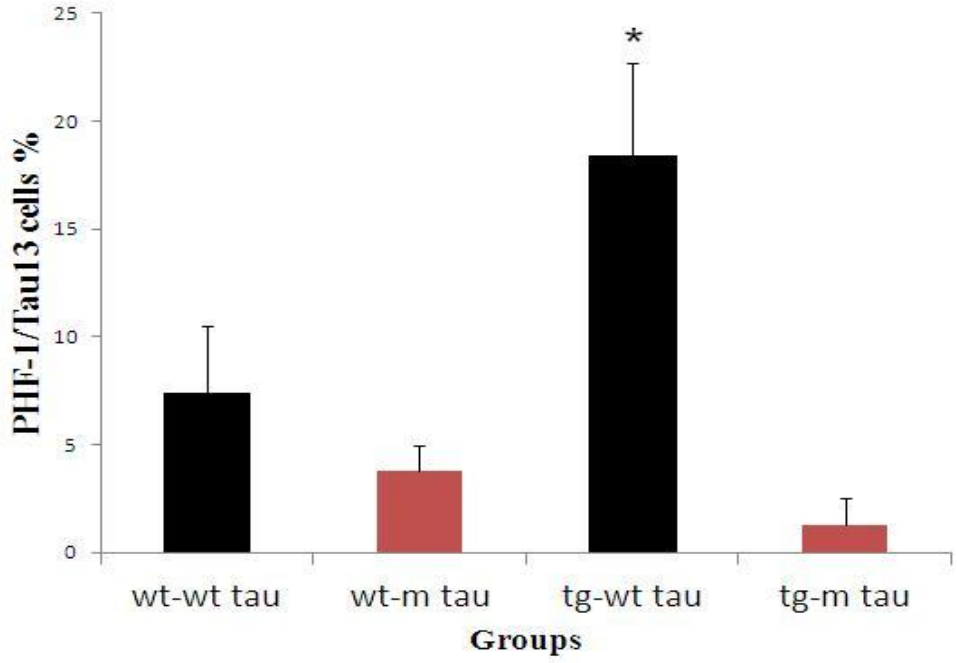


Fig 6. A: Example of PHF positive cells in the CA3 pyramidal cell layer (arrows) in an A30P transgenic mouse with wild-type human tau transduction. Scale bar = 50 µm. **B:** Proportion of PHF-positive neurons of Tau 13 positive neurons in the CA3 area of hippocampus in each group (mean ± sem). * Significantly different from all other groups (p = 0.012, ANOVA).

5.3 PRESENCE OF INTRACELLULAR α-SYN INCLUSIONS

α-Syn in the A30P mouse hippocampus is mainly expressed in axon terminals. In addition, we also detected cytoplasmic α-syn positive inclusions in pyramidal cell bodies (Fig. 7). We found the size of these cytoplasmic inclusions in randomly selected 20 Tau13-positive CA3 pyramidal cells and their two neighboring α-syn positive cells in A30P mice receiving either wild-type or P301S mutated human tau transduction by using confocal microscope. By counting the percentage of the size of α-syn inclusions of the whole cell body, overall, the tau transduced neurons did not differ from their neighbouring α-syn positive neurons [$F(1,71) = 2.1$, $p = 0.15$]. However, when the two outliers with extremely large difference were removed, we found that only in the group of A30P mice that received P301S mutated tau transduction do we see more a-syn inclusions in the cytoplasm in Tau13-positive cells than in neighboring α-syn positive cells [$F(1,69) = 6.7$, $p = 0.012$].

Fig. 7

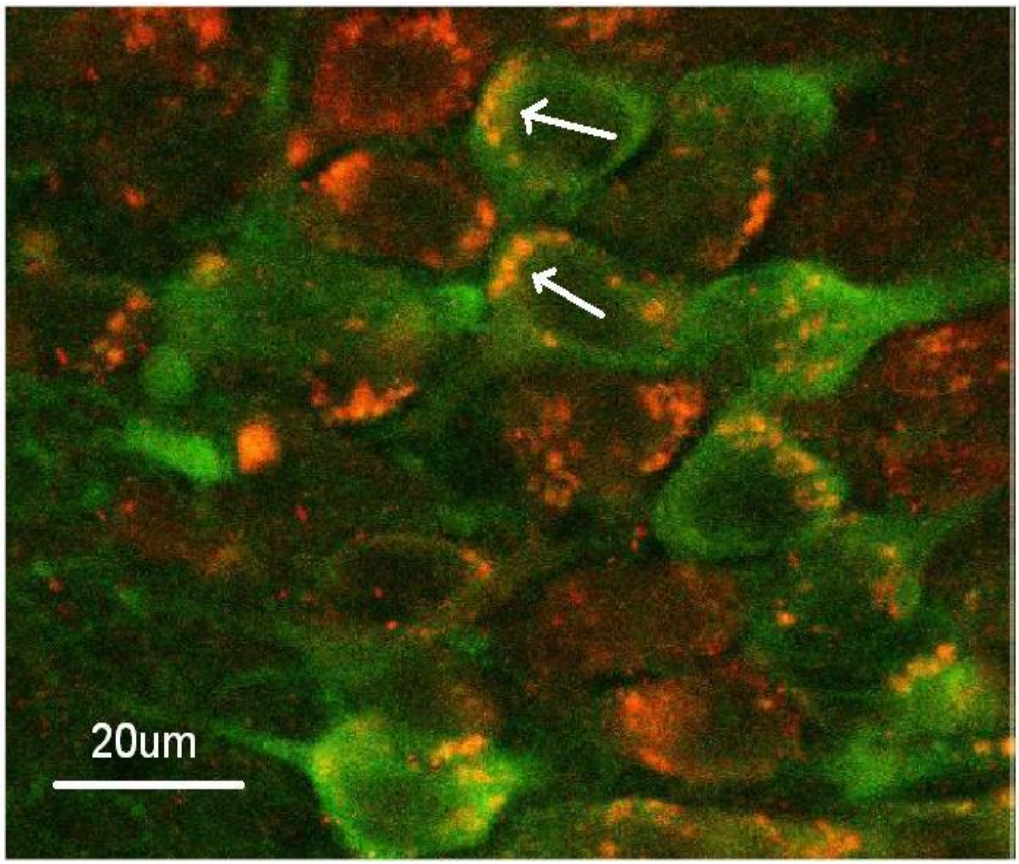


Fig 7. Influence of tau transduction on cytoplasmic α-synuclein inclusions. Confocal image of double-labeled CA3 pyramidal cells from an A30P transgenic mouse with P301S tau transduction. Red = anti- α-synuclein, green = anti-Tau 13. α-Synuclein forms small cytoplasmic inclusions (arrows). Scale bar = 20 µm.

6 DISCUSSION

In this study we have successfully transferred a target gene of interest selectively into one desired brain region with an AAV vector. The expression of transduced gene was detected mostly in axons of the hippocampus, which matches the normal functions of tau as microtubulus associated protein (97). In addition, we have demonstrated that this transduction does not induce micro- or astrogliosis. Therefore, when assessing the possible neuronal loss we could take the total cell count in a hippocampal CA3 pyramidal cell layer as directly reflecting the number of neurons. The cell counting results appear reliable, since we found that the replicability of cell-difference counting results across analysis sessions was extremely high. In fact, this replicability was even higher than the left-right difference, which could be explained by the biological variation in the number of the neurons in different sides of the hippocampus.

Frasier and coworkers (8) found that the A30P transgenic mice had clear motor symptoms, and showed a four-fold increase in PHF-1 reactivity compared to their nontransgenic controls, but without any difference in the total levels of tau. Some researchers have also detected the highest total tau level and the proportion of neurons which stained positively with phosphorylation specific tau antibodies in the spinal cord, in which most severe neuron loss and neurodegeneration was also found in P301S tau transgenic mice (98). However, the appearance of motor symptoms, high level of tau inclusion, neuron loss and neurodegeneration were only found in the advanced age of those tau transgenic mice mentioned above. For example, the cytoplasmic inclusion was showed at 5 months age of P301S transgenic mice (98). Therefore, one possible reason for no significant neuron loss and neurodegenerative signs in our wild-type or human A30P transgenic mice with substantial amounts of human wild-type or P301S mutated tau is that tau protein did not have enough time to be exposed to tau-kinases to be hyperphosphorylated and aggregated into filaments. Nonetheless, one still could see the trend that those groups with the highest expression of PHF-1 positive

neurons (for example, tg-wt tau) also have the lowest neuronal counts in the CA3 subregion of the hippocampus.

We did not detect significant tau hyperphosphorylation in either wild-type or A30P α-syn transgenic mice transduced with P301S mutated vector, but a lot of tau hyperphosporylation was shown in A30P mice receiving wild-type human tau vector. This result is contrary to the idea that the ability of tau to bind to microtubuli will be reduced by the familial frontotemporal dementia linked mutations at the sites of P301L and P301S, which induces tau polymerization into aggregation (99). However, there is also evidence indicating that the P301L mutation prevented tau binding to α-syn (100). It is possible that the P301S mutation also has a similar effect. One possible mechanism of α-syn induced tau aggregation is that it may act as a chaperone protein to facilitate tau hyperphosphorylation at Ser396/404 residues (the PHF-1 binding site) by altering the presentation of these serine residues to the related reactive kinases such as Cdk5, PKA, GSK3β and MAPK(9). So tau hyperphosphorylation would be down-regulated by these enzymes when the binding of tau with α-syn is impaired.

We have detected α-syn inclusions in tau positive pyramidal cells. Conversely, the presence of α-syn could also promote tau aggregation (101), which indicates that tau and α-syn would have the ability of inducing fibrillization of each other. However, we only detected significantly more α-syn inclusions in tau positive cells than their neighbouring α-syn positive cells in A30P mice that tranduced with P301S mutated tau. One possible explanation is that tau reacts with α-syn, so that the α-syn epitope gets masked from the antibody, but the cross-reaction does not happen between P301S tau and A30P a-syn, which is consistent with the PHF-1 staining result showing that P301S tau does not show hyperphospholyration in A30P mice, but wild-type tau does.

In conclusion, the present study demonstrates that tau and α-syn proteins interact *in vivo* and that their interaction promotes α-syn aggregation and tau hyperphosphorylation. However, this interaction inducing tau hyperphosphorylation is dependent on specific binding site on tau, and that

mutation at the 301 site prevents this interaction. It would be interesting in future studies to specifically compare α-syn and tau co-occurrence in the brains of P301L or P301S mutant carriers and those with other tauopathies.

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