Studying tau protein propagation and pathology in the mouse brain using adeno-associated viruses

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CHAPTER OUTLINE

Abstract

The progressive spread of pathological brain lesions containing aggregated tau protein is a hallmark of Alzheimer's disease and other neurodegenerative diseases. In AD, this process follows a distinct pattern along neuronal connections from the entorhinal cortex to

Methods in Cell Biology, ISSN 0091-679X, http://dx.doi.org/10.1016/bs.mcb.2017.06.014

hippocampal areas and further on through the limbic system. In other tauopathies, the spread of tau appears less hierarchical throughout the brain, and also nonpathological tau is reported to cross-synaptic connections in the brain. To be able to study the process of cell-to-cell transport of tau and the associated neurotoxicity in the brain in vivo, adeno-associated virus-mediated expression of tau can be used to express different forms of tau in distinct brain areas in rodent models. As an example, we describe how the expression of FTD-mutant human tauP301L in the entorhinal cortex of wild-type mice can be used to study the propagation of tau to connected neurons and to determine pathological consequences such as tau hyperphosphorylation, misfolding, and gliosis.

The approach described can easily be translated to study other aggregating and/or propagating proteins in the brain such as synuclein, Abeta, or SOD1.

1 INTRODUCTION

In Alzheimer's disease (AD), two hallmark pathologies become apparent and worsen with progression of the disease: the extracellular aggregation and deposition of amyloid-beta peptide leads to the formation of amyloid plaques throughout the brain, and the intraneuronal aggregation of tau protein in which form neurofibrillary tangles (NFTs), in which large amounts of misfolded and amyloid-like aggregated tau fill the neuronal cell body. NFTs can be stable over multiple days, weeks, and months (de Calignon et al., 2010; Gómez-Isla et al., 1997; Polydoro et al., 2013; Spires et al., 2006). During the course of disease, NFTs spread in a hierarchical pattern through the brain along synaptic connections: starting in the entorhinal cortex, tangles occur next in hippocampal and limbic areas, and finally in the association cortices (Braak & Braak, 1995; Hyman, Van Hoesen, Damasio, & Barnes, 1984; Fig. 1). The appearance of NFTs correlates spatially with neuronal loss and with cognitive decline of AD patients (Hyman, Van Hoesen, & Damasio, 1990), suggesting that tau acts as the neurotoxic entity in AD and other tauopathies.

The neuronal microtubule-associated protein tau (MAPT) is mainly expressed in the central nervous system (Cleveland, Hwo, & Kirschner, 1977) and is a highly soluble intrinsically disordered protein. In different neurodegenerative diseases—collectively termed "tauopathies" and including AD, frontotemporal dementia (FTD), Pick's disease, and supranuclear palsy, tau aggregates occur in distinct brain areas and are morphologically distinct. The reasons for aggregation-prone conformations of tau are not fully understood, but mutations (Von Bergen et al., 2001) and posttranslational modifications (Iqbal et al., 2005; Noble, Hanger, Miller, & Lovestone, 2013) appear to alter protein folding and increase the propensity for tau to aggregate. Accordingly, tau found in NFTs is hyperphosphorylated and can be immunolabeled with antibodies recognizing certain tau phospho-epitopes or protein conformations; "mature" tangles consist of amyloid-like tau beta-sheet aggregates that bind Thioflavin-S and Congo Red derivatives such as Thiazine Red. Although tangles are a well-described pathological change in AD, the cellular

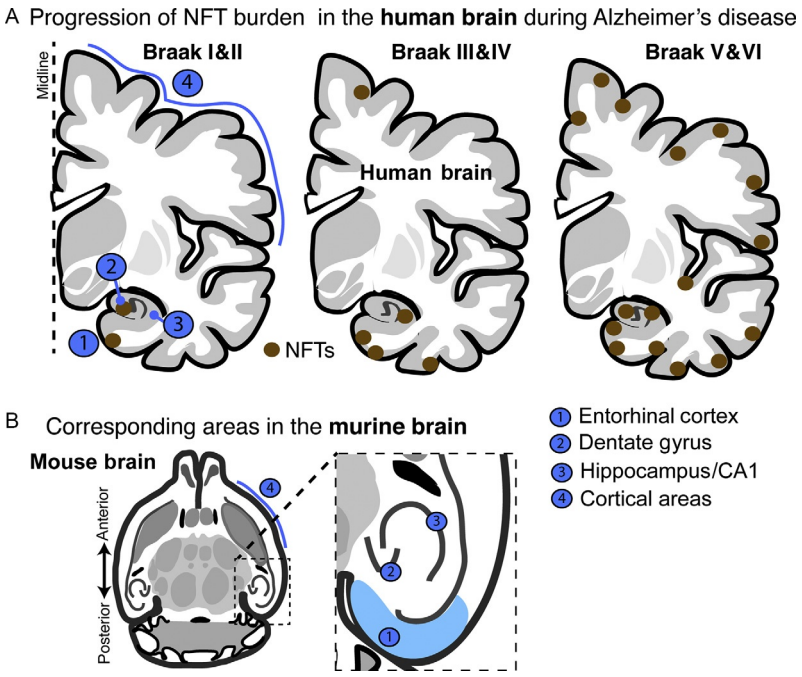

FIG. 1

Neurofibrillary tangle progression in Alzheimer's disease. (A) The severity of Alzheimer's disease in the human brain (shown as coronal section through of one brain hemisphere) is staged according to the occurrence of neurofibrillary tangles (NFTs) in different brain regions. NFTs develop first in the entorhinal cortex (EC, 1) from where they extend to the dentate gyrus (DG, 2, Braak I&II). During disease progression, NFTs progressively spread to the hippocampus (HPC, 3) and some cortical areas (4, Braak III&IV), and finally throughout the cortex of the brain (Braak V&VI). A coronal section through one brain hemisphere is shown. (B) In the rodent brain, the anatomical organization of EC, DG, HPC, and cortical areas are largely preserved, enabling the study of tau propagation in the brain and NFT spread along neural connections in murine (and rat) brain. A horizontal section through the whole mouse brain is shown. Regions corresponding to the brain areas with NFT burden in the AD brain (A) are indicated.

process of tau propagation through neural systems is still unclear; similar to other protein aggregation diseases, prion-like templated misfolding of tau has been suggested to be—at least in part—responsible for the progression of tau aggregates through the brain (Brettschneider, Del Tredici, Lee, & Trojanowski, 2015; Frost & Diamond, 2009; Sanders et al., 2014), although a major role for selective vulnerability of the recipient neuronal population also contributes. There is also strong evidence that non-aggregated tau can travel across synapses (Dujardin et al., 2014) in wild-type mice

and rats as well as in mouse tau knockout mice (Wegmann et al., 2015), in which the conversion of naïve endogenous mouse tau—a prerequisite of the templated protein misfolding paradigm—is not existent.

Several genetic mouse models have been developed to study the pathology, aggregation, and toxicity of tau in the brain. Most utilize the FTD-associated MAPT mutations P301L, P301S, and deltaK280 to trigger abnormal tau aggregation and NFT formation (Eckermann et al., 2007; Santacruz et al., 2005; Yoshiyama et al., 2007). More recently, mouse models expressing P301L tau largely restricted to the entorhinal cortex were used to visualize the travel of mutant misfolded tau across synaptic connections to the dentate gyrus (De Calignon et al., 2012; Liu et al., 2012) and the enhancement of tau travel and toxicity in the presence of Abeta (Pooler et al., 2015). However, in these mice, the pathological onset and progression is very slow (starts around 15 months of age) which contributes to difficulties in manipulating the phenotype experimentally. Further, genetic manipulation of these lines requires time-consuming and often complex breeding paradigms.

To overcome these technical issues, viral vector-based nongenetic models have been developed to study the progression and toxicity of tau in the brain. The most efficient and prominent viral vector systems used for heterologous protein expression in the brain are lentivirus- or adeno-associated virus (AAV) based, and the delivery of the viral particles is facilitated through intracranial injections in the target brain areas. However, the restriction of the target gene expression to a certain brain region and cell type depends on multiple factors (Choudhury et al., 2017; Hudry et al., 2016; Jackson, Dayton, Deverman, & Klein, 2016), such as the number of injected viral particles, the injection volume, the viral capsid serotype, the choice of a ubiquitous (e.g., chicken beta-actin (CBA) or cytomegalovirus (CMV)) vs a cell-type-specific promotor (e.g., synapsin-1 (syn-1) or CamK2a for excitatory neurons and glial fibril-associated protein (GFAP) for astrocytes), and the spread of viral particles along neuronal projections, and the site of injection, as well as the age of the animal. Reducing the number of viral particles and keeping the injection volume low are easy ways to restrict the region of target gene expression. AAVs have the advantage over lentiviruses in that they are not integrating into the genome of the host cell and, thus, are more suitable for translational use in human disease therapeutics. Current delivery methods depend on direct injection into the brain parenchyma, as peripheral injection of viral particles into the blood have proven to be largely inefficient given the blood–brain barrier (BBB) effectively blocks AAV particles from entering the brain. Novel AAV serotypes that can efficiently pass the BBB are currently developed (Deverman et al., 2016; Hudry et al., 2016; Merkel et al., 2016). However, although the administration of AAV particles through the blood stream by intravenous injection would be appreciated for a global transduction of brain cells, one major benefit of intracranial injections is the possibility for targeted expression in subregions of the brain and the restriction to cellular subpopulations, which can help to reduce side effects compared to a systemic treatment with drugs or antibodies. In fact, gene therapy based on intracranial AAV

injections is already used as therapeutic approach for some diseases (Katz et al., 2015; Sevin et al., 2006).

The progressive aggregation of certain proteins in the brain is a common feature of multiple neurodegenerative diseases, such as tau in AD; FTD, FUS, and TDP43 in amyotrophic lateral sclerosis (ALS); alpha-synuclein in Parkinson's disease (PD); and Huntingtin in Huntington disease. Targeted local protein expression in the brain allows for the investigation of the specific vulnerability of brain regions, as well as other important variables such as the effect of mouse genotype, age, stress, and therapeutic treatment.

Here, we report how to use AAV-mediated local expression of human tau protein to study spread along distinct anatomical connections and identify tau-induced pathological changes in the mouse brain. We used this method to test the hypothesis that human tau expressed in neurons in the entorhinal cortex can travel to neurons in the dentate gyrus and other connected areas (Fig. 1) and monitor changes in tau phosphorylation and glial activation induced by the overexpression of tau.

To achieve an unambiguous distinction between AAV-transduced neurons expressing human tau from neurons that received human tau through (synaptic or other) protein uptake, we created a viral AAV construct that encodes (i) the fluorescent reporter protein eGFP for identification of human tau expressing "donor neurons," (ii) 2a peptide sequence for the efficient self-cleavage upon translation (Szymczak et al., 2004), and (iii) untagged human full-length tau (2N4R, 441 amino acids) (Fig. 2). The expression of this construct is driven by the ubiquitous CBA promotor and enhanced by a woodchuck posttranslational regulatory element (WPRE). After stereotactic injection of AAV 8 particles encoding this viral construct into the entorhinal cortex or the hippocampus of wild-type mice (Fig. 3), we use postmortem immunohistology of fixed brain slices and stereology counting to analyze how many neurons (in relation to donor neurons) received human tau by cell-to-cell transfer (recipient neurons). Using immunofluorescence labeling, we are also able to detect the reaction of microglia and astrocytes to tau overexpression and injection injury, and can analyze tau hyperphosphorylation and aggregation in donor and recipient neurons. Brain extracts of injected animals allow for a biochemical characterization (e.g., by Western blot, ELISA, qPCR) of changes induced by AAV-mediated tau expression in different brain regions.

2 METHOD

2.1 AAV CLONING

AAV for in vivo transduction of brain cells (neurons and glia cells) need to fulfill some specific criteria in order to achieve effective target protein expression. For example, a high viral titer (concentration of infective particles; $\sim 10^{12}$–10^{13}), sterile

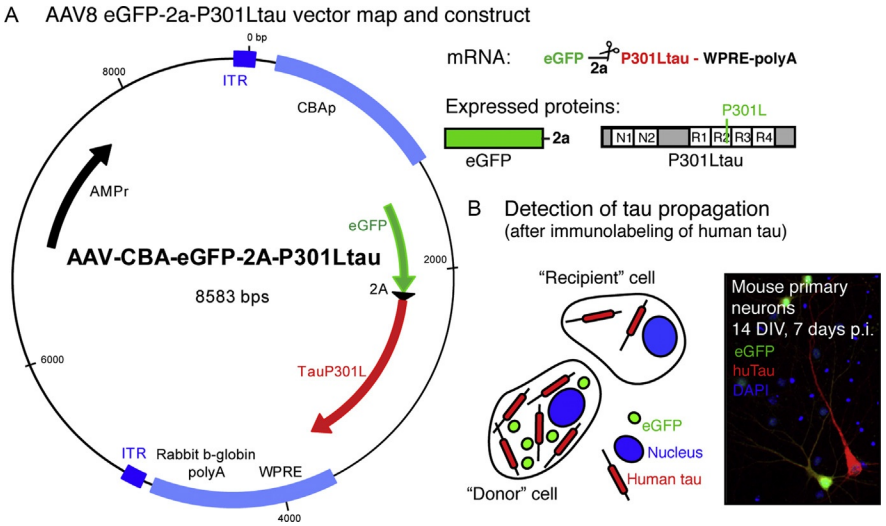

FIG. 2

Adeno-associated virus (AAV) construct to study human tau propagation in the brain. (A) In order to study tau propagation in the brain, we designed an AAV vector that leads to the expression of full-length human P301Ltau and eGFP as a cell transduction marker as two individual proteins. This is facilitated by a short peptide sequence, 2a, between the mRNA for eGFP and human tau. Upon translation, 2a leads to self-cleavage of the nascent peptide chain, which results in the expression foe GFP-2a and P301Ltau. *Note*: by putting eGFP upstream of tau, we avoid false-positive counting of tau recipient cells (GFP−, huTau+) in case of incomplete 2a self-cleavage (cleavage efficiency ~90%) of the construct.
(B) Expressing eGFP-2a-tau in cells leads to a labeling of construct expressing cell ("donor" cells) with eGFP (*green*). After fixation and immunohistochemical labeling for human tau (*red*), donor cells appear *green* and *red*, whereas "recipient" cells that received human tau through cell-to-cell transport appear *red* but not *green* (GFP−, huTau+). When expressed in primary cortical mouse neurons in vitro, transneuronal travel of human tau could be observed 7 days after transduction with AAV eGFP-2a-P301Ltau (AAV added at 7 DIV).

preparation, and adaptation of serotype (capsid protein) for efficient infection of specific cell types. Different AAV serotypes (e.g., AAV 1, 2, 5, 8, 9) can vary in their infection preferences for different brain cell types. Over the last years, AAV serotypes with more specific and novel infection characteristics have evolved (Choudhury et al., 2017; Hudry et al., 2016), and the development of new approaches is currently a lively field of research in itself. Information about AAV biology and approaches are reviewed in Daya and Berns (2008), Grieger and Samulski (2005), and Weitzman and Linden (2011).

For the production of the actual viral particles, many commercial and academic core facilities offer services that provide high-titer AAV preparations for in vivo studies upon delivery of AAV-compatible plasmid DNA containing the gene of

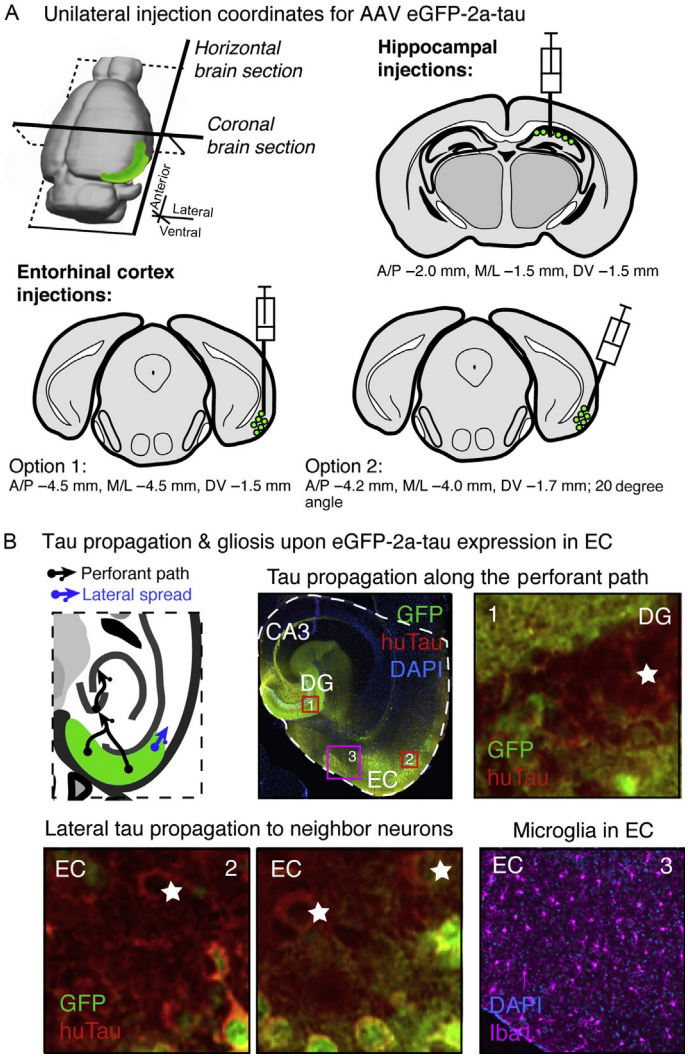

FIG. 3

AAV eGFP-2a-tau injection and au propagation in vivo. (A) To examine the propagation of human tau through the brain similar to AD, AAV eGFP-2a-tau is injected into the EC of adult wild-type mice as indicated; two possible options for injection coordinates targeting the EC are displayed. An example of injection coordinates to target the hippocampus is shown as well. (B) Human tau travel upon expression of AAV eGFP-2a-P301Ltau in the EC and DG (here 8 weeks *postinjection*) is best visualized in postmortem horizontal brain sections. Fixed brain sections were stained with antibodies against GFP (*green*; antibody chicken anti-GFP) and human tau (huTau, *red*; antibody mouse–anti-human tau Tau13, Biolegends), and for microglia (Iba1, *pink*; antibody rabbit–anti-Iba1, WAKO) to assess gliosis upon AAV transduction and human tau expression. Tau recipient neurons (GFP−, huTau+; *white asterisk*) can be observed in the DG (spread along the performant path) and in the EC in neurons adjacent to donor (GFP+, huTau+) neurons. Quantification of tau propagation and gliosis can be achieved, for example, by counting tau recipient cells and microglia in hippocampus and cortex near the injection site (ROI indicated by *white dashed line*).

interest; for the specific requirements, the producer facility has to be contacted; for methods and/or *in-house* production, please review Walker (2011). *Note*: AAV and other viral particles rapidly lose activity upon refreezing ($\sim 10^1$ per freeze–thaw cycle); to avoid the activity loss, aliquot your viral stock in volumes that can be used within a day and keep aliquots frozen at −80°C.

To produce target gene plasmid DNA that is compatible with AAV particle production, the gene of interest has to be cloned into a special ITR-harboring backbone containing other elements that are essential for the AAV-driven protein expression in the cells. In between the two ITR sites in the AAV plasmid backbone, which should not exceed a length of 5.0 kilobases (kb) to ensure gene expression in the transduced cells, different elements need to be chosen to customize gene expression in the brain: (i) a promotor for ubiquitous (all cell types) or cell-type-specific expression of the target gene. Common ubiquitous promotors are, for example, CBA, CAG, and CMV. For neuron-specific expression, common promotors are syn-1, CamK2alpha, and Gad67. For astrocytes, expression is mostly facilitated by the GFAP promotor, the transduction of microglia appears extremely difficult and inefficient, and, at the moment, no microglia-specific AAV-driven protein expression is available. (ii) The promotor is followed by the gene of interest, which in most cases should not exceed a length of 2.5 kb in order to fit the maximum length of the entire ITR cassette (~5.0 kb). Amplification of the AAV plasmid DNA is done in *Escherichia coli* following standard procedures for DNA production and purification. Because of the two ITR sites, autologous recombination can result in excision and loss of the ITR cassette, which results in a loss of the transgene in the vector. To avoid this, *E. coli* expression strains with low recombination characteristics (e.g., DH5alpha) can be chosen and a too high OD of the *E. coli* cultures should be avoided. Purification of the plasmid DNA has to be done using an endotoxin-free preparation (for example, using an Endo-free Maxi Prep Kit, Qiagen). Typical requirements for AAV production are 150–300 μg DNA at a concentration of ~1 mg/mL.

For the AAV vector presented here, which was designed to study tau propagation in the mouse brain and in vitro in cell culture, we chose the serotype AAV 8 for efficient targeting of neurons in the CNS; the target protein expression is driven by the ubiquitous CBA promotor. The target gene encodes—in one RNA transcript—the fluorescent protein eGFP (enhanced green fluorescent protein; transduction marker), followed by a translation interrupting 2a peptide (Szymczak et al., 2004) and full-length human mutant P301Ltau (AAV8-CBA-eGFP-2a-P301Ltau. The vector also contains a WPRE and polyA sequence downstream of the target gene (Fig. 2A; titer $\sim 6 \times 10^{12}$ infectious particles/mL; vector produced at Mass Eye & Ear (MEEI) vector core, Boston).

In this vector, eGFP-2a and P301Ltau are expressed as individual proteins, which allow the independent detection of eGFP, which labels the tau expressing "donor" cells, and the expressed P301Ltau, which occurs in both the tau expressing and tau receiving cells. Human tau is detected microscopically after labeling by immunohistochemistry (IHC) with antibodies that recognize human but not mouse tau (e.g., mouse–anti-human tau Tau13 by Biolegends, rabbit–anti-human tau TauY9

by Covance). Tau "Recipient" cells can be identified by the presence of human tau and the absence of eGFP (Fig. 2B and C).

Other versions of this vector, that were designed for imaging of tau propagation in the living brain and in live neurons, encode eGFP and tau tagged with red fluorescent proteins under the syn-1 promotor.

2.2 INTRACRANIAL AAV INJECTIONS

2.1. All procedures have to be performed according to ethical standards and national and institutional animal welfare rules; details of the entire procedure have to be approved by the responsible committee and be listed in an animal procedure protocol.

2.2. To prepare the mouse for the surgery, the animal is anesthetized (5% isoflurane for induction, 2% for maintenance) until full unconsciousness is reached. Fur on the head of the animal is trimmed close to the skin, and the animal is placed in a stereotax and secured using ear bars. The mouse's body temperature is controlled using a heating pad and a rectal probe thermometer. Depth of anesthesia is tested frequently throughout the surgery by toe pinch and monitoring of the breathing.

2.3. According to the animal protocol in place (e.g., after washing the skin with iodine/isopropanol and local anesthesia with subcutaneous injection of lidocaine), perform an anterior–posterior skin incision (~0.2–0.5 cm) above the skull and clean and dry the skull to remove hair- and gel-like aponeurosis with cotton swabs. Adjust the brain position so that Bregma and Lambda (A/P tilt) as well as left and right hemisphere (M/L tilt) are at the same height. Next, a burr hole (diameter ~ 1 mm) is drilled into the skull at the position of the desired injection coordinates (use mouse brain atlas to determine the precise anterior/posterior (A/P), medial/lateral (M/L), and dorsal/ventral (D/V) coordinates in relation to Bregma). For precision, determine the drill coordinates with a mounted syringe and needle. For example, entorhinal cortex injections can be done as followed (coordinates from Bregma): (i) vertical nontilted needle at A/P −4.5 mm, M/L + −4.5 mm, D/V from brain surface −1.5 mm. Or (ii) with a 20 degree tilted needle, A/P −4.2 mm, M/L + −4.0 mm, D/V from brain surface −1.7 mm. For hippocampal injections, you can use (coordinates from Bregma): vertical nontilted needle, A/P 2.0 mm, M/L ± 1.5 mm, D/V from brain surface −1.5 mm (Fig. 3A and B). *Note*: you may want to test the target coordinates and projection areas before pursuing to the use of AAV particles. This can be done by injecting similar volumes of methylene blue or fluorescently labeled dextran (~0.5–2.0 mL) for marking the target position in the brain, or by injecting anterogate (e.g., nontoxic Cholera toxin-b fragment, Ctb) or retrogate (e.g., *Phaseolus vulgaris*-leucoagglutinin, PHAL) transported substances to label respective projections and downstream/upstream connected cells; the brain of methylene blue injected animals can be harvested, fixed, and analyzed right after the injection, the tracer molecule

injected brains after ~24–96 h. Detection of the tracer molecules can be achieved through the use of directly fluorescence labeled tracers, or by immunohistochemical detection of the tracers.

2.4. Thaw the viral particles on ice, dilute to the desired concentration with sterile phosphate-buffered saline (PBS) (final viral particle number (vga) and volume (typically 0.1–3.0 μL)), store virus solution on ice, and load into a sterilized and PBS-rinsed 10 μL Hamilton glass syringe (series 701) equipped with a sterilized needle (gauge 30; sharpened tip). Make sure that there are absolutely no microair bubbles in the syringe since they will likely cause death of the animals when injected into the brain. After mounting the syringe in the stereotax mounted injector, position the needle at the precise injection coordinates with the needle tip gently touching the brain surface. Lower the needle into the brain to the desired depth, and start injecting the viral particles (typical flow rates are 0.1–0.2 μL/min). When the injection is finished, wait for another 2–5 min to let the viral solution diffuse in the brain; then slowly withdraw the needle from the brain.

2.5. Close the skin incision using suture or glue, remove the mouse from anesthesia, subcutaneously inject a systemic pain killer (e.g., Buprenex) and let the mouse recover from anesthesia on a heating pad. Injected mice may be housed under standard conditions until the experimental time point for harvesting the brain is reached. AAV-mediated protein expression in the brain can be detected after 1–2 weeks and can be relatively stable across the lifetime of the animal.

2.3 BRAIN HARVEST

2.3.1 Fixed brain sections for immunohistological staining of proteins

To prepare the brains for immunohistological staining, the brains are first fixed with 4% paraformaldehyde (PFA) in PBS and then cut into slices; this method preserves the structural details needed for stereological analysis of brain cell morphology and numbers (see later) and is, in this case, favored above cryostat thin sectioning of fresh frozen tissue, which sometimes can lead to the leakage of overexpressed proteins during the cutting process. At the desired time point, post-AAV injection (p.i.; protein expression starts ~1 week p.i.), the mice are euthanized and intracardially perfused with 10 mL PBS, followed by perfusion with 10 mL 4% PFA/PBS. Then the brain is carefully extracted from the skull, dropped into 2–3 mL 4% PFA/PBS in a 15-mL Falcon tube and kept at 4 degree overnight for postfixation. Note: If the brain needs to be used simultaneously for biochemistry, the extraction from the skull is done after PBS perfusion (no PFA), then the brain is dissected as desired, and the part that needs PFA fixation dropped into 4% PFA/PBS and kept at 4 degree for 2–3 days. After completed fixation, the storage solution is exchanged for 30% (w/v) sucrose/PBS or 15% (v/v) glycerol/PBS solution to cryoprotect the brain for slicing on a freezing microtome, and the brain is kept at 4°C until sunk to the bottom of the storage vial. Brain slices are cut on a freezing microtome (thickness 30–50 μm) or

cryostat (thickness 10–15 μm) as needed; for this, the fixed and cryoprotected brain is mounted on a frozen stage according to the instrument instructions and brain slices are cut in the orientation (coronal, horizontal, longitudinal) that is best to display and analyze the brain morphological features of interest; commonly used are horizontal sections for analysis of tau propagation from entorhinal cortex to dentate gyrus, coronal sections to display hippocampus. Brain sections are dropped into 1.5 mL Eppendorf tubes containing 15% glycerol solution and stored at −20°C until needed. The immunostaining of brain sections for GFP, human tau, and other markers is described later (see Section 2.4.1).

2.3.2 Brain extracts for biochemical analysis

To produce brain extracts from AAV injected brains (or brain regions), mice are euthanized and intracardially perfused with PBS, then the brain is carefully extracted and placed immediately on ice on a clean petri dish to minimize protein degradation. After dissecting out the brain regions of interest (for example, entorhinal cortex, hippocampus, etc.), the brain pieces are weighted on a fine balance and dropped into three times the weight-volume of ice cold extraction buffer. Common buffers are PBS or Tris-buffered saline (TBS) at pH 7.0–8.0 containing 50–200 mM NaCl and protease (Complete Mini, Roche) and phosphatase (phospho-stop, Roche) inhibitors; depending on the stability and activity of the proteins of interest, other ingredients can be added (for example, 1–2 mM DTT, 1–2 mM EDTA or EGTA, 1–5 mM $CaCl_2$ or $MgCl_2$, and detergents such as 1% Triton X-100, SDS, Sarkosyl, or Saponin). RIPA buffer (Sigma) is a commonly used commercially available buffer mix that already contains detergents and only needs the addition of protease/phosphatase inhibitors. Always keep the brain and solutions chilled. *Note*: Avoid the use of phosphatase inhibitors if brain extracts are used for subsequent treatment of cells in culture since they are not well tolerated.

Next, homogenize the brain in the extraction buffer using an electric or hand homogenizer (typically 30–50 strokes) until no large pieces are left. Then, centrifuge the brain lysate for 10–30 min at 4°C and a speed that leaves the protein and cell organelles of interest in the supernatant (typical centrifugation speeds: $3000 \times g$ for crude extracts that have most organelles and proteins in the supernatant including soluble and smaller high-molecular-weight aggregated/oligomeric tau (nuclei are in the pellet); $15{,}000 \times g$ for extracts containing soluble and most small oligomeric proteins (larger organelles like ER and mitochondria are in the pellet); $100{,}000 \times g$ for extracts containing only soluble proteins and no oligomers and organelles (all organelles, membranes, and vesicles are in the pellet; also see Harford & Bonifacino, 2011). After the centrifugation, the supernatant is collected and the total protein content is determined using a standard protein assay such as Bradford or bicinchoninic acid. Adjust the protein concentration in the brain extracts to a convenient level (e.g., 1, 5, or 10 mg/mg) using the extraction buffer and mix well. Aliquots of the produced extracts are stored at −80°C to avoid freeze–thaw cycles that can reduce protein integrity and activity. The Western blot analysis of brain extracts for tau and other markers is described later (see Section 2.5.1).

2.4 IMMUNOHISTOCHEMICAL ANALYSIS OF TAU PROPAGATION AND PATHOLOGY

2.4.1 IHC of floating brain sections

Brain sections floating in glycerol (see Section 2.3.1) are thawed at room temperature and washed with PBS or TBS. This can, for example, be done in a 24-well tissue culture dish. The use of strainers is optional. Using a brush, the brain sections are transferred into 0.2% TX-100 in PBS containing 5%–10% normal goat serum (NGS) for 20 min to assure permeabilization and blocking of the fixed tissue (The blocking of the unspecific binding sites can also be done after permeabilization or with 1% bovine serum albumin in PBS.) After washing the sections, three times with PBS, they are incubated with the primary antibody solution at 4°C overnight. Primary antibodies detecting human tau, GFP and other markers are diluted in 5% NGS/PBS.

The next day, after washing the sections three times for 10 min with PBS, fluorescently labeled secondary antibodies (for example, Alexa488-, Cy3-, and Alexa647-labeled anti-mouse or anti-rabbit IgGs) are diluted in 5% NGS/PBS and applied for 2 h at room temperature. Next, the brain sections are washed three times for 10 min in PBS and mounted on microscope slides. After drying in the dark, mounting medium containing DAPI (e.g., VectaShield) is applied and sections are covered with a cover glass slip. The images are recorded on a light microscope equipped with a epifluorescence lamp and appropriate filter sets, a camera, and imaging software, for example, a Zeiss AxioImager microscope equipped with a CoolSnap digital camera.

2.4.2 Example antibodies for IHC and Western blot analysis

For the IHC of brain sections and Western blot of brain extracts from AAV eGFP-2a-tau injected mice, the following antibodies can be used: rabbit–anti-human tau (N-terminus) TauY9 (1:500; Enzo Lifescience), mouse–anti-human tau (N-terminus) Tau13 (1:1000; Covance), rabbit anti-mouse and human tau (C-terminus; 1:1500; DAKO), mouse anti-mouse tau Tau/5 (Johnson&Johnson), mouse anti-phospho-tau CP13 (1:1000; pS202/sT205; all phosho-tau antibodies detect mouse and human tau), PHF1 (1:1000; pS396/pS404) (CP13 and PHF1 are courtesy of Peter Davies), 12e8 (pS262/pS356; Elan Pharmaceuticals), rabbit anti-"misfolded" tau Alz50 (1:500, Peter Davies), mouse anti-glial fibrillary acidic protein (1:1000; GFAP; Abcam), rabbit anti-Iba1 (1:500; for ICC or WB; WAKO), mouse anti-phosphoneurofilament proteins SMI312 (1:1000; Covance), and mouse anti-NeuN (1:1000; Millipore).

2.4.3 Data analysis for tau propagation and stereology

The propagation of tau can be assessed after immunostaining for human tau by determining the number of "recipient" cells (eGFP −, human tau +) in the mouse brain or in specific subregions. For example, when looking at the propagation of tau along the performant path from the entorhinal cortex to granule cells in the dentate gyrus (Fig. 3)—the neural connection first involved in tangle pathology in AD (Hyman

et al., 1984)—we injected AAV eGFP-2a-P301Ltau into layer 2/3 of medial entorhinal cortex and detected recipient neurons in the dentate gyrus and hippocampus, indicating the propagation of human tau across this synaptic connection. Lateral spread of human tau to neuronal cell bodies in close proximity to expressing neurons could be observed as well (Fig. 3). However, since the number of tau recipient neurons is generally very low, it is useful to determine the total number of recipient cells per brain sections. To account for the variability inherent to injection uncertainty, transduction efficiency, and animal-to-animal variability, one can normalize the number of recipient neurons the total number of transduced (eGFP+) cells in the analyzed brain sections or cell cultures.

If using this AAV approach with proteins other than tau that show more propagation, it might be useful to switch to a stereological counting method; in this method, a randomized fraction of the brain region of interest (usually following a superimposed grid) is analyzed for recipient cells, and the counts are extrapolated to the total size of the volume of interest. This approach is also useful when analyzing the total number of neurons or glial cells present in the brain or certain regions.

2.5 BIOCHEMICAL ANALYSIS OF BRAIN EXTRACTS

2.5.1 SDS-PAGE and Western blot

For the analysis of brain extracts (see Section 2.3.2) by Western blot, 10–30 µg total protein are loaded per lane onto a 4%–12% Bis-Tris SDS-polyacrylamide gel (Life Technologies). The separation of tau and other proteins is achieved by running the electrophoresis in either MOPS (better separation of larger proteins) or MES running buffer according to the manufacturer of the electrophoresis chamber device (for example, at 150 V for 60 min in a NuPage chamber, Life Technologies). *Note*: the acrylamide % of the gel and the running buffer system is chosen according to the proteins that have to be from each other with a good resolution.

After the SDS-PAGE is completed, the proteins are blotted onto a nitrocellulose membrane according to the manufacturer of the transfer chamber device (for example, at 90 V for 90–120 min in a BioRad transfer chamber). After the transfer to the membrane is completed and the membrane has been blocked for 1 h in blocking buffer (e.g., 1% (w/v) fat-free dry milk in PBS, 1% bovine serum albumin in PBS, or Odyssey blocking buffer (LI-COR)), the primary antibodies diluted in blocking buffer are applied overnight at 4°C (for example, choices of antibodies to detect tau and other markers in the mouse brain, see Section 2.4.2). The next day, wash the membrane three times for 10 min in 0.05% (v/v) Tween-20/TBS, then apply the secondary antibodies diluted in blocking buffer for 2 h at room temperature. To be able to visualize two proteins at the same time on the same membrane, we use fluorescently labeled secondary antibodies for the detection in an infrared reader (LI-COR; for example, goat anti-rabbit-IRDye680 and anti-mouse-IRDye800 (Rockland). Wash off the secondary antibodies with 0.05% (v/v) Tween-20/TBS blots and image the protein bands in an infrared reader. Alternatively, horseradish peroxidase coupled secondary antibodies can be used and the protein bands visualized by film exposure.

2.5.2 Protein band detection and data analysis in Western blots

The infrared-labeled protein bands on the nitrocellulose membranes were visualized using an Odyssey imaging system (LI-COR), which simultaneously can visualize emitted fluorescence of 700 nm (IRDye680) and 800 nm (IRDye800), and thus allows the detection of two proteins at a time. Protein bands are analyzed in terms of their position and fluorescence intensity, a measure for the amount of protein present, using ImageJ (http://rsb.info.NIH.gov/nih-image/) or other software.

3 CONCLUSIONS

AAV-mediated protein expression in the rodent brain became a standard procedure in the recent years. Intracranial injections of viruses—and more recently intravenous injection of viral particles that cross the BBB—proofed not only extremely useful and safe for research of brain anatomy and function in the living and postmortem brain, but it is also already used as a therapeutic approach for human disease (Sevin et al., 2006).

Studying protein propagation, templated prion-like seeding as well as toxicity are common goals in research of neurodegenerative diseases such as AD, FTD, ALS, PD, and Huntington's disease. By generating AAVs encoding GFP-2a-tau—or similar reporter-target protein constructs—we are now able to study the propagation of tau through neuronal systems in vivo and can test the impact of various genetic, physiological, and external factors on this process.

ACKNOWLEDGMENTS

We thank Eloise Hudry, Samantha Nicholls, and Zhanyun Fan for help with AAV construct design, and Molly J. Kirk, Louis Delorme, Danny MacKenzie, Ashley B. Robbins, and Nahel Tunio for conducting experiments that contributed to the successful use of the described AAV construct.

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