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(54) Title: INHIBITION OF TAU-MEDIATED EARLY SYNAPTIC DYSFUNCTION

(57) Abstract: The present invention relates to the field of neurodegenerative diseases, including Tauopathies, and the prevention and/or treatment of the early synaptic dysfunction arising in patients thereof. In particular, the present invention relates to screening methods for producing compounds which can prevent the early synaptic dysfunction by inhibiting the reduction of synaptic vesicle mobilization and neurotransmitter release and which can prevent early pathology in various Tauopathies.



Inhibition of Tau-mediated early synaptic dysfunction

FIELD OF THE INVENTION

The present invention relates to the field of disorders of the central nervous system, in particular Tauopathies, and the prevention and/or treatment thereof. In particular, the present invention relates to the finding that pathological Tau protein is mislocalized, which allows its binding to synaptic vesicles in the presynapse, thereby causing presynaptic dysfunction. The invention reveals that inhibition of N-terminal Tau protein domain binding to synaptic vesicles reduces the presynaptic dysfunction and restores neurotransmitter release. Accordingly, the invention provides methods and (high content) screening assays to produce compounds able to interfere with the pathological Tau association with synaptic vesicles and as such with the presynaptic dysfunction observed in Tauopathies.

BACKGROUND

Tau protein function is implicated in more than twenty neurodegenerative diseases including Alzheimer's disease (AD), Parkinson disease (PD), and frontotemporal dementia with Parkinsonism-17 (FTDP-17) (reviewed in Ballatore et al., 2007; Morris et al., 2011; Wang and Mandelkow, 2016). Synaptic dysfunction is thought to be an early pathological manifestation in AD and other Tauopathies (Bellucci et al., 2012; Masliah et al., 2001; Milnerwood and Raymond, 2010; Scheff et al., 2006). Tau is mainly expressed in neurons and is highly concentrated in the axon where it associates with microtubules. However, under disease conditions, Tau falls-off microtubules as abnormal phosphorylation or FTDP-17 pathogenic mutations impair its binding affinity to microtubules (Wang and Mandelkow, 2016). Pathological Tau protein has been detected in colocalization with both pre- and postsynaptic markers in the staining of isolated synaptosomes from AD patient brains (Fein et al., 2008; Henkins et al., 2012; Sokolow et al., 2015; Tai et al., 2012). Immunohistological analyses of transgenic mouse brains made similar observations, that FTDP-17 pathogenic mutations drive mislocalization of Tau to both presynaptic terminals and dendritic spines (Harris et al., 2012; Sahara et al., 2014). These data together implicate that Tau mislocalizes to both pre- and post-synapses under pathological conditions.

Patients suffer from early synaptic dysfunction prior to Tau aggregate formation, but the underlying mechanism is unclear. Recent studies suggest that soluble Tau, rather than the neurofibrillary tangle-associated aggregated form, is the main toxic element that is able to induce early synaptic deficits preceding synapse and neuronal loss (Crimins et al., 2012; Erez et al., 2014; Hoover et al., 2010; Menkes-Caspi et al., 2015; Polydoro et al., 2014; Sydow et al., 2011; Yoshiyama et al., 2007). However, pathogenic mechanisms of non-aggregated Tau in neurons remain enigmatic. Several studies have suggested a pathophysiological role of Tau at dendritic spines in affecting the trafficking of postsynaptic receptors (Hoover et al., 2010; Ittner et al., 2010). In contrast, the role for mislocalized Tau at presynaptic terminals remains unclear.

SUMMARY OF THE INVENTION

The present invention relates to the finding that pathogenic Tau mislocalizes to presynaptic terminals in fly neurons, where it binds synaptic vesicles to promote presynaptic dysfunction. The underlying mechanism involves a dual action of Tau in binding synaptic vesicles and simultaneously promoting presynaptic actin polymerization to restrict vesicle mobilization. Pathogenic Tau thus reduces synaptic transmission during prolonged neuronal activity in both fly neurons, and similar defects are observed in rat neurons. In brief, Tau surprisingly associates with synaptic vesicles via its N-terminal domain and interferes with presynaptic functions, including synaptic vesicle mobility and release rate, lowering neurotransmission in fly and rat neurons. Pathological Tau mutants lacking the vesicle binding domain still localize to the presynaptic compartment but do not impair synaptic function in fly neurons. Moreover, an exogenously added membrane-permeable peptide that competes for Tau-vesicle binding, suppresses Tau-induced synaptic toxicity in rat neurons. Hence, disruption of the interaction between Tau and synaptic vesicles is sufficient to rescue these defects, suggesting that the mechanism we identified is a key mechanism for Tau-induced presynaptic pathology.

Overall, the invention uncovers a novel and unexpected presynaptic function for the N-terminal Tau protein domain, with relevance demonstrated in Tauopathies, in particular in Alzheimer's Disease. In particular, the present invention relates to methods and (high content) screening assays to interfere with Tau association to synaptic vesicles, which rescues presynaptic pathology. Compounds produced from this screening approach are candidates for developing therapeutics for the treatment of Tauopathies.

A first aspect of the invention relates to a method for producing a compound that prevents the Tau-mediated early synaptic dysfunction comprising the steps of:

- a) providing an *in vitro* system comprising N-terminal Tau protein domain as depicted in SEQ ID NO: 1 or a homologue with at least 65 % amino acid identity thereof, and synaptic vesicles,
- b) administering a test compound to said *in vitro* system,
- c) monitoring said N-terminal Tau protein domain binding to synaptic vesicles in said *in vitro* system, wherein, as compared to the binding under the same test conditions in the same system without the test compound, a reduction in the N-terminal Tau protein binding to synaptic vesicles identifies said test compound as a compound that prevents the Tau-mediated early synaptic dysfunction.

In one embodiment, the *in vitro* system in step a) of the above method comprises immobilized synaptic vesicles using a molecule with specificity for protein present on said synaptic vesicle and a buffer condition suitable to perform an immune-based assay. In another embodiment, said *in vitro* system comprises a buffer condition suitable to perform a vesicle sedimentation assay. And in a further embodiment, said *in vitro* system is a neuronal cell.

In another embodiment, the monitoring of said N-terminal Tau protein domain binding to the synaptic vesicles in step c) of the above method comprises a vesicle sedimentation assay followed by immune-based detection. And in further embodiments, said monitoring of the N-terminal Tau protein domain binding to the synaptic vesicles comprises an immune-based assay.

- 5 Another embodiment relates to a method wherein the produced compound of the method, is a candidate for Lead optimization preventing or rescuing early synaptic dysfunction in neuronal cells.

In a further aspect, the invention also relates to a polypeptide inhibiting the binding of N-terminal Tau protein domain to synaptic vesicles, as can be determined with the above monitoring method, when a reduction in binding is observed. In one embodiment, said polypeptide comprises SEQ ID NO: 1 or a
 10 homologue with at least 65 % amino acid identity thereof and a cell penetrant carrier. In another embodiment, said protein comprising SEQ ID NO: 1 or a homologue with at least 65 % amino acid identity thereof and a cell penetrant carrier is useful as a medicament. More specifically, said protein comprising SEQ ID NO: 1 or a homologue with at least 65 % amino acid identity thereof and a cell penetrant carrier
 15 can be used for treatment of a Tauopathy, wherein said treatment prevents or reduces early synaptic pathology. In another alternative embodiment, said Tauopathy is Alzheimer's Disease. Finally, one embodiment provides an antibody as an inhibiting protein of the binding of N-terminal Tau protein domain to synaptic vesicles, and more specifically, an antibody specifically binding an epitope in SEQ ID NO:1 or a homologue with at least 65 % amino acid identity thereof.

20 DESCRIPTION OF THE FIGURES

Figure 1. Tau localizes to presynaptic terminals and binds to synaptic vesicles via its N-terminal domain.

(A) Tau and Cysteine string protein (CSP) immunolabeling at neuromuscular junctions (NMJs) of *Drosophila* larvae expressing wild type (WT) or FTDP-17 pathogenic mutant Tau (R406W, V337M and
 25 P301L) under the D42-Gal4 motor neuron driver. Axons (arrowheads) and synaptic boutons (SBs; arrows) are indicated. Scale bar, 50 μ m.

(B) Quantification of fluorescence intensity of Tau within SBs as ratio to the intensity of axonal Tau. **P<0.01, Student's *t* test, n=10-12 NMJs ($\geq$ 5 animals).

(C-D) Super-resolution structured illumination microscopy analysis of Tau and CSP immunolabeling within
 30 SBs under non-treated condition (C) or after depletion of synaptic vesicles in *Shi^{ts1}* mutant background by KCl stimulation at the non-permissive temperature (D). Scale bar, 10 μ m.

(E-F) Immunoblots of Tau (anti-His tag) and synaptic vesicle (SV) proteins Synaptobrevin (Syb), Synaptotagmin (Syt) and Synapsin (Syn) from sedimentation assay (E) and co-immunoprecipitation (co-
 IP) using anti-His antibodies (F) assessing recombinant human Tau binding to crude synaptic vesicles.

35 (G) Electron microscopy imaging of recombinant Tau (probed by Ni-NTA-Nanogold) bound to ultra-pure synaptic vesicles *in vitro*. Scale bar, 50 nm.

(H-J) Mapping of the vesicle-binding domain of Tau *in vitro* by co-IP assay. Truncations of the N-terminal (NT), proline-rich (PRD), microtubule-binding (MTB) or C-terminal (CT) domains of Tau were generated

as indicated in the schematic (H). Immunoblots of recombinant Tau domain-truncations and Syb (SV marker) from co-IP using anti-His antibodies (I) and quantification of relative Syb intensity (J). *** $P < 0.005$, Student's *t*-test, $n=3$ independent experiments.

5 Figure 2. Pathogenic mutant Tau reduces synaptic transmission and vesicle cycling/release during sustained high-frequency stimulation.

Drosophila larvae used in these assays express UAS-Tau (WT, R406W, V337M or P301L) under the D42-Gal4 motor neuron driver.

(A and B) Electrophysiological recordings of synaptic transmission during 10 Hz stimulation for 10 minutes.

10 Plot of evoked junction potential (EJP) amplitudes (A) and representative traces (B). Two-way ANOVA, $n=7-9$ NMJs (one NMJ per animal).

(C and D) FM1-43 dye loading with stimulation at 3 Hz (recycling vesicle pool) or 10 Hz (reserve vesicle pool) for 10 min. Representative images of FM1-43 dye loading (A) and plot of FM1-43 dye loading intensity (B). Student's *t*-test, $n=14-20$ NMJs (≥ 7 animals).

15 (E-G) Synapto-pHluorin (SpH) responses to stimulation at 10 Hz with the presence of bafilomycin. Representative images of SpH responses (E) and plot of fluorescence change ΔF at ratio to maximal ΔF (NH_4Cl dequenching) (F). Two-way ANOVA, $n=7-11$ NMJs (animals). Plot of maximal ΔF (NH_4Cl dequenching) calibrated to control levels.

(G) Student's *t*-test, $n=7-11$ NMJs (one NMJ per animal). ** $P < 0.01$, *** $P < 0.005$, ns, not significant.

20 Figure 3. Pathogenic mutant Tau increases F-actin levels and reduces synaptic vesicle mobility at presynaptic terminals.

Drosophila larvae used in these assays express UAS-Tau (WT, R406W, V337M or P301L) under the D42-Gal4 motor neuron driver.

25 (A and B) Immunolabeling of LifeAct-GFP probing for F-actin within synaptic boutons. Representative images of immunolabeling (A) and quantification of Life Act-GFP intensity (B). Student's *t*-test, $n=12-14$ NMJs (≥ 7 animals).

(C-F) FRAP measurement of vesicle mobility within synaptic boutons. Representative images acquired immediately before photobleaching (pre-bleach) and immediately after bleaching at 0 sec to 60 sec post-

30 bleaching time points (C). Plot of fluorescence recovery (% of initial fluorescence) over time and fit with double-exponential curve (D). Plots of fast and slow recovery rates calculated from fluorescence recovery curve (E and F). Student's *t*-test, $n=20-25$ boutons (≥ 5 animals). ** $P < 0.05$, *** $P < 0.001$, ns, not significant.

(G and H) Proposed model for Tau clustering synaptic vesicles to F-actin to restrict reserve pool vesicle mobilization and release.

35 Figure 4. Interfering with Tau N-terminal-dependent vesicle-binding reverts Tau-induced presynaptic deficits in fly and rat neurons.

Drosophila larvae used in (A-C) express UAS-Tau ΔN (R406W, V337M or P301L) under the D42-Gal4 motor neuron driver.

(A) FRAP measurements of vesicle mobility within SBs. Fluorescence recovery (% of initial fluorescence) was plotted over time and fit with double-exponential curves. n=20-25 boutons (≥ 5 animals).

(B) Synapto-pHluorin responses to stimulation at 10 Hz with the presence of bafilomycin. Fluorescence change ΔF at ratio to maximal ΔF (NH_4Cl dequenching) was plotted over time during the stimulation. Two-way ANOVA, n=7-12 NMJs (one NMJ per animal).

(C) Electrophysiological recordings of EJP amplitudes during stimulation at 10 Hz. Two-way ANOVA, n=7-9 NMJs (animals).

Autaptic rat hippocampal neuronal cultures used in (D-H) were transduced with AAV vectors expressing GFP or Tau variants.

(D) Autaptic neuronal culture transduced with AAV-EGFP is visualized by bright-field (upper) and GFP (lower) fluorescence.

(E) Colloidal Coomassie staining of purified Tat-NT^{Tau} peptide; NT^{Tau} corresponds to the N-terminal domain (aa 1-113) of Tau.

(F-H) Electrophysiological recordings using patch clamp in response to 10 consecutive high frequency stimulation trains (10 Hz for 10 s with 30 s interval). The representative traces are shown in (F) and the relative first EPSCs were plotted to train numbers (G-H). In (H), TauP301L expressing neurons were acutely treated with 5 μM Tat-NT^{Tau} or control Tat-mCherry peptides before patch clamp recordings.

Figure 5. Pathologically phosphorylated Tau is localized to the presynaptic terminals of Alzheimer's disease patient brains.

Ultrathin (70 nm) sections of healthy control or AD patient brains were analysed by array tomography. Presynaptic terminals were labeled with anti-Synapsin I antibody and phospho-Tau was labeled with AT8 antibody.

(A-E) Immunostainings show AT8-positive Tau at presynaptic terminal (arrows) of AD patient brain (A-C), but not in control patient brain (D), while negative control staining showed no background staining from secondary antibodies (E).

Presynaptic phospho-Tau was observed in all three AD cases examined but not in any of the four healthy control cases examined. Scale bar: 10 μm , 2 μm (inset).

Figure 6. Tau protein characterization and isolation, and synaptic vesicle isolation.

(A-B) Relative Tau expression levels in the brains of UAS-Tau transgenic fly lines.

(A) Representative immunoblot detects total Tau expression under the control of the D42-Gal4 motor neuronspecific driver (with DAKO antibody); Tubulin was used as a loading control.

(B) Quantification of Tau expression from immunoblots shows similar expression levels of Tau variants in the brains of tested transgenic lines (n= 3 independent experiments).

(C-D) Isolation of crude synaptic vesicles from mouse brains. (C) Schematic summarization of the procedure of preparing crude synaptic vesicles from mouse brains. (D) Representative immunoblot detects synaptic vesicle marker proteins in the fractions obtained during the purification of the crude synaptic vesicles (LP2 faction). The LP2 fraction is enriched with the synaptic vesicle marker proteins Synaptophysin and Synaptotagmin and largely depleted of the post-synaptic marker protein PSD-95.

(E-G) Purification of recombinant human Tau from bacterial cultures. (E) Schematic depicting purification of GST/His double-tagged Tau from bacterial cultures, and proteolytic removal of the N-terminal GST tag by PreScission protease to result in the final product Tau-His. (F-G) Colloidal Coomassie staining of purified full-length WT and R406W Tau-His1-383 (F), N-terminally truncated Tau113-383-His (Δ N) and N-terminal fragment (Tau1-112-His) recombinant proteins used for *in vitro* synaptic vesicle binding assays. (H) The N-terminal domain of Tau is sufficient to bind synaptic vesicles. Representative immunoblot detects Tau1-112-His (N-Term) and the synaptic vesicle marker Synaptobrevin from co-IP using anti-His antibodies.

Figure 7. N-terminally truncated Tau characterization.

(A-D) N-terminally truncated pathogenic mutant Tau localizes to presynaptic terminals of fly NMJs, but exhibits reduced association with synaptic vesicles *in vivo*.

(A-C) *Drosophila* larvae used in these assays express UAS-Tau or UAS-Tau Δ N (R406W, V337M or P301L) under control of the D42-Gal4 motor neuron driver. (A) Immunolabeling of Tau and CSP at NMJ synapses analysed by confocal microscopy imaging. Scale bar, 10 μ m. Quantification shows that full-length Tau and N-terminally truncated Tau are present at similar levels within SBs (B). However, N-terminally truncated Tau, as compared to full-length Tau, displayed reduced co-localization with the synaptic vesicle marker CSP as quantified using the Pearson's co-localization coefficient (C). Student *t*-test, ****P*<0.005, n=20-30 NMJs (≥ 7 animals).

(D) Super-resolution structured illumination microscopy analysis of Tau and CSP immunolabeling within SBs after depletion of synaptic vesicles in the *Shits1* mutant background. Unlike full-length Tau which largely follows the re-localization of CSP to presynaptic membrane, the N-terminally truncated Tau remains within SBs to some extent, suggesting that NS7 terminally truncated Tau exhibits reduced association with synaptic vesicles *in vivo*. Scale bar, 10 μ m.

(E) N-terminally truncated Tau increases presynaptic F-actin levels. *Drosophila* larvae used in this assay co-express UAS-LifeAct-GFP and UAS-Tau or UAS-Tau Δ N (R406W or V337M) under control of the D42-Gal4 motor neuron driver. Relative intensities of bouton LifeAct-GFP probing for F-actin were plotted, showing that N-terminally truncated pathogenic mutant Tau (R406W or V337M) increases presynaptic F-actin levels similar to their full length counterparts. Student's *t*-test, n= 12 NMJs (≥ 6 animals).

(F) The Tat-NT^{Tau} peptide competes with full-length Tau for vesicle binding *in vitro*. Intact synaptic vesicles (SVs) were immobilized to Dynabeads using anti-Synaptobrevin 2 (Syb) antibodies. Following immobilization, SVs were incubated with recombinant full-length Tau (40 nM) with or without the presence of 0.1, 1.0 or 4.0 μ M purified Tat-NT^{Tau} peptide. Immunoblots were probed for full-length Tau, Tat-NT^{Tau} and the synaptic vesicle marker Syb. Note that Tat-NT^{Tau} reduced the amount of full-length Tau bound to SVs in a dose-dependent manner.

Figure 8. Pathological Tau species accumulate on synaptic vesicles in AD patient brain.

(A) Schematic depicting purification scheme of SVs from human brain tissue samples. (B) Immunoblotting of fractions taken during SV isolation from a representative control patient show enrichment of the SV markers Synaptotagmin (Syt) and Synapsin (Syn), and depletion of the post-synaptic marker PSD-95 and

the mitochondrial marker TOM20. (C) Immunoblots detecting phospho-Tau (pTau) using PHF-1 antibody or total Tau (DAKO antibody) of representative subcellular fractions collected during SV isolation from three representative AD patient brains in comparison to two representative control patients. Immunoblotting of Syt verifies enrichment of SVs in the SV fractions. Note the presence of both monomeric (migrating around 50 kDa) as well as oligomeric (100 kDa and larger) pTau species. (D) Representative immunoblots directly comparing the amount of pTau detected with PHF-1 (phospho-Ser396/Ser404 epitope) or AT8 (phospho-Ser202/Thr205 epitope) antibodies or total Tau (DAKO antibody) present in total homogenates or in SV fractions from AD patients and non-demented controls. GAPDH and Syt are used as loading controls for total homogenates and SV fractions, respectively. (E) Quantifications of immunoblots comparing pTau or Tau levels in control and AD patient brain in either total homogenate or SV fractions from n = 13 non-demented control patients and n = 15 AD patients. Note that we also observe enrichment of Tau on SVs from control patients, but this amount is 2-4-fold increased on SVs from AD patients. Graphs depict mean $\pm$ SEM; ** p<0.01, ***p<0.001, n.s. not significant in Student's t-test.

DETAILED DESCRIPTION TO THE INVENTION

Scope of the invention

The invention relates to the discovery that Tau protein fundamentally alters presynaptic properties under pathogenic conditions, which complements the generally accepted idea that pathological Tau affects postsynaptic functions. In pathogenic conditions, localization of Tau is surprisingly increased to presynaptic terminals. In addition, abnormal phosphorylation of Tau in AD results in the dissociation of Tau from microtubules. Both wild type and pathogenic mutant Tau can bind synaptic vesicles *in vitro*, but only pathological Tau is mislocalized to the presynapse. Therefore, a novel 'unifying' mechanism could be common to various Tauopathies. The presence of Tau presynaptically, where it associates with synaptic vesicles via its N-terminal domain on the one hand, and polymerizes presynaptic actin using its proline-rich and microtubule-binding domains on the other hand, results in crosslinking of synaptic vesicles and slows their mobilization, lowering synaptic transmission during intense stimulation. This multi-step mode of action where disease triggers the dissociation of Tau from microtubules, followed by an early soluble phase where the protein induces pathological synaptic dysfunction at the presynapse was shown to be purely dependent on the N-terminal Tau protein domain. This finding advocated that interfering with Tau-N-terminal domain-dependent vesicle-binding could be exploited therapeutically to prevent these aspects of presynaptic pathology.

Definitions

The present invention will be described with respect to particular embodiments and with reference to certain drawings but the invention is not limited thereto but only by the claims. Any reference signs in the claims shall not be construed as limiting the scope. Of course, it is to be understood that not necessarily all aspects or advantages may be achieved in accordance with any particular embodiment of the invention. Thus, for example those skilled in the art will recognize that the invention may be embodied or carried out

in a manner that achieves or optimizes one advantage or group of advantages as taught herein without necessarily achieving other aspects or advantages as may be taught or suggested herein.

The invention, both as to organization and method of operation, together with features and advantages thereof, may best be understood by reference to the following detailed description when read in conjunction with the accompanying drawings. The aspects and advantages of the invention will be apparent from and elucidated with reference to the embodiment(s) described hereinafter. Reference throughout this specification to "one embodiment" or "an embodiment" means that a particular feature, structure or characteristic described in connection with the embodiment is included in at least one embodiment of the present invention. Thus, appearances of the phrases "in one embodiment" or "in an embodiment" in various places throughout this specification are not necessarily all referring to the same embodiment, but may. Similarly, it should be appreciated that in the description of exemplary embodiments of the invention, various features of the invention are sometimes grouped together in a single embodiment, figure, or description thereof for the purpose of streamlining the disclosure and aiding in the understanding of one or more of the various inventive aspects. This method of disclosure, however, is not to be interpreted as reflecting an intention that the claimed invention requires more features than are expressly recited in each claim. Rather, as the following claims reflect, inventive aspects lie in less than all features of a single foregoing disclosed embodiment.

Where an indefinite or definite article is used when referring to a singular noun e.g. "a" or "an", "the", this includes a plural of that noun unless something else is specifically stated. Where the term "comprising" is used in the present description and claims, it does not exclude other elements or steps. Furthermore, the terms first, second, third and the like in the description and in the claims, are used for distinguishing between similar elements and not necessarily for describing a sequential or chronological order. It is to be understood that the terms so used are interchangeable under appropriate circumstances and that the embodiments, of the invention described herein are capable of operation in other sequences than described or illustrated herein. The following terms or definitions are provided solely to aid in the understanding of the invention. Unless specifically defined herein, all terms used herein have the same meaning as they would to one skilled in the art of the present invention. Practitioners are particularly directed to Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 4th ed., Cold Spring Harbor Press, Plainview, New York (2012); and Ausubel et al., *Current Protocols in Molecular Biology* (Supplement 114), John Wiley & Sons, New York (2016), for definitions and terms of the art. The definitions provided herein should not be construed to have a scope less than understood by a person of ordinary skill in the art.

"About" as used herein when referring to a measurable value such as an amount, a temporal duration, and the like, is meant to encompass variations of $\pm 20\%$ or $\pm 10\%$, more preferably $\pm 5\%$, even more preferably $\pm 1\%$, and still more preferably $\pm 0.1\%$ from the specified value, as such variations are appropriate to perform the disclosed methods. The term "abnormal" when used in the context of organisms, tissues, cells or components thereof, refers to those organisms, tissues, cells or components thereof that differ in at least one observable or detectable characteristic (e.g., treatment, processing, function, etc.) from those organisms, tissues, cells or components thereof that display the "normal"

(expected or healthy) respective characteristic. Characteristics which are normal or expected for one cell or component thereof, might be abnormal for a different cell or component thereof.

As used herein, the term "wild-type" refers to a gene or gene product isolated from a naturally occurring source. A wild-type gene is that which is most frequently observed in a population and is thus arbitrarily
5 designed the "normal" or "wild-type" form of the gene. In contrast, the term "modified", "mutant" or "variant" refers to a gene or gene product that displays modifications in sequence, post-translational modifications and/or functional properties (i.e., altered characteristics) when compared to the wild-type gene or gene product. It is noted that naturally occurring mutants can be isolated; these are identified by the fact that they have altered characteristics when compared to the wild-type gene or gene product.

10 The term "compound" or "test compound" is used herein in the context of a "drug candidate compound" or a "candidate compound for Lead optimization" in therapeutics, described in connection with the methods of the present invention. As such, these compounds comprise organic or inorganic compounds, derived synthetically or from natural resources. The compounds include polynucleotides, lipids or hormone analogs that are characterized by low molecular weights. Other biopolymeric organic test compounds
15 include small peptides or peptide-like molecules (peptidomimetics) comprising from about 2 to about 40 amino acids and larger polypeptides comprising from about 40 to about 500 amino acids, such as antibodies or antibody conjugates.

The terms "protein", "polypeptide", "peptide" are interchangeably used further herein to refer to a polymer of amino acid residues and to variants and synthetic analogues of the same. Thus, these terms apply to
20 amino acid polymers in which one or more amino acid residues is a synthetic non-naturally occurring amino acid, such as a chemical analogue of a corresponding naturally occurring amino acid, as well as to naturally-occurring amino acid polymers. A "protein domain" is a distinct functional and/or structural unit in a protein. Usually a protein domain is responsible for a particular function or interaction, contributing to the overall role of a protein. Domains may exist in a variety of biological contexts, where similar domains
25 can be found in proteins with different functions. By "recombinant polypeptide" is meant a polypeptide made using recombinant techniques, i.e., through the expression of a recombinant or synthetic polynucleotide. When the chimeric polypeptide or biologically active portion thereof is recombinantly produced, it is also preferably substantially free of culture medium, i.e., culture medium represents less than about 20 %, more preferably less than about 10 %, and most preferably less than about 5 % of the
30 volume of the protein preparation.

The term "antibody" as used herein, refers to an immunoglobulin molecule which specifically binds with an antigen. Antibodies can be intact immunoglobulins derived from natural sources or from recombinant sources and can be immunoreactive portions or fragments of intact immunoglobulins. Antibodies are typically tetramers of immunoglobulin molecules. The antibodies in the present invention may exist in a
35 variety of forms including, for example, polyclonal antibodies, monoclonal antibodies, Fv, Fab and F(ab)₂, as well as single chain antibodies and humanized antibodies (Harlow et al., 1999, In: Using Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, NY; Harlow et al., 1989, In: Antibodies: A Laboratory Manual, Cold Spring Harbor, NY; Wörn and Plückthun, 2001; Koerber et al., 2015.). By the term "specifically binds," as used herein with respect to an antibody, is meant an antibody which
40 recognizes a specific antigen, but does not substantially recognize or bind other molecules in a sample.

For example, an antibody that specifically binds to an antigen from one species may also bind to that antigen from one or more species. But, such cross-species reactivity does not itself alter the classification of an antibody as specific. In some instances, the terms "specific binding" or "specifically binding," can be used in reference to the interaction of an antibody, a protein, or a peptide with a second chemical species, to mean that the interaction is dependent upon the presence of a particular structure (e.g., an antigenic determinant or epitope) on the chemical species; for example, an antibody recognizes and binds to a specific protein structure rather than to proteins generally. If an antibody is specific for epitope "A", the presence of a molecule containing epitope A (or free, unlabeled A), in a reaction containing labeled "A" and the antibody, will reduce the amount of labeled A bound to the antibody. Collectively, the six Complementarity-determining regions (CDRs) confer antigen-binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three CDRs specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

An immunoglobulin single variable domain that can specifically bind to and/or that has affinity for a specific antigen or antigenic determinant (e.g. epitope) is said to be "against" or "directed against" said antigen or antigenic determinant. An immunoglobulin single variable domain is said to be "cross-reactive" for two different antigens or antigenic determinants if it is specific for both these different antigens or antigenic determinants.

By "isolated" is meant material that is substantially or essentially free from components that normally accompany it in its native state. For example, an "isolated polypeptide", as used herein, refers to a polypeptide, which has been purified from the molecules which flank it in a naturally-occurring state, e.g., a Tau protein which has been removed from the molecules present in the production host that are adjacent to said polypeptide. An isolated Tau protein (optionally an N-terminal protein domain) can be generated by amino acid chemical synthesis or can be generated by recombinant production. Another example concerns an isolated neuronal cell, which refers to a neuronal cell which has been extracted and purified from the naturally-occurring state, involving tissue. An isolated antibody is one that has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials that would interfere with diagnostic or therapeutic uses for the antibody, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. Isolated antibody includes the antibody in situ within recombinant cells since at least one component of the antibody's natural environment will not be present. In some instances, isolated antibody will be prepared by at least one purification step. An isolated neuronal cell preparation can be obtained from several neuronal tissue types using for example specialized commercial kits that make use of proteases to digest intercellular protein junctions followed by gentle mechanical disruption to liberate individual cells, or for instance but not limited to the exemplified method.

The term "inhibitor" or "inhibiting polypeptide" or "inhibitory antibody" of the binding of N-terminal Tau protein domain to synaptic vesicles as used herein, refers to the fact that the protein can inhibit the binding or interaction between said N-Tau and SVs. "Inhibitory" can mean full inhibition (no binding at all) or may mean partial inhibition. For instance, inhibition can mean 10% reduction of binding, 20% reduction, 25% reduction, 30% reduction, 40% reduction or more. Particularly, inhibition will be at least 50%, e.g. 50%

inhibition, 60% inhibition, 70% inhibition, 75% inhibition, 80% inhibition, 90% inhibition, 95% inhibition or more. % inhibition typically will be evaluated against a suitable control, as will be readily chosen by the skilled person.

The terms "prevents", "inhibits", or "reduces" presynaptic dysfunction are used interchangeably and mean the observed degree of the presynaptic dysfunction is significantly lower in comparison to the presynaptic malfunctioning observed when pathological Tau is present. The term "early synaptic dysfunction", "presynaptic dysfunction", "early synaptic pathology" refer to abnormal functioning of the synapse as a measure of reduced synaptic vesicle mobility, slower neurotransmitter release rate, and hence reduced synaptic neurotransmission. Consistently, a defect to maintain normal levels of neurotransmitter release was observed for instance in fly and rat neurons expressing pathogenic Tau. This transmitter release defect is very similar to that seen upon overexpression of Synapsin, for which it has been demonstrated in different model synapses, to result in a decline of neurotransmission in response to strong stimulation trains (Fioravante et al., 2007; Vasileva et al., 2013). Consistent with the finding that expression of Tau lowers neurotransmitter release efficiency, more recent studies in mice overexpressing Tau P301L indicated early stage defects and show a loss of synaptic input prior to synapse loss in intact neocortical pyramidal cells as well as alterations in the probability of neurotransmitter release in entorhinal cortex (Menkes-Caspi et al., 2015; Polydoro et al., 2014).

The terms "subject", "individual" or "patient", used interchangeably herein, refer to any subject, particularly a vertebrate subject, and even more particularly a mammalian subject, for whom therapy or prophylaxis is desired. Suitable vertebrate animals that fall within the scope of the invention include, but are not restricted to, primates, avians, fish, reptiles, livestock animals (e.g., sheep, cows, horses, donkeys, pigs), laboratory test animals (e.g., rabbits, mice, rats, guinea pigs, hamsters), companion animals (e.g., cats, dogs) and captive wild animals (e.g., foxes, deer, dingoes). However, it will be understood that the aforementioned terms do not imply that symptoms are present.

Detailed description

In a first aspect the present invention relates to a method for producing a compound that prevents Tau-mediated early synaptic dysfunction. The method is performed in an *in vitro* system comprising N-terminal Tau protein domain as depicted in SEQ ID NO: 1 or a homologue with at least 65 % amino acid identity thereof, and synaptic vesicles. Accordingly, the invention relates to the use of N-terminal Tau protein defined by SEQ ID NO: 1 or by amino acid sequences with 65% identity to said SEQ ID NO:1, in association with synaptic vesicle proteins, thereby providing a suitable system for the test compounds to identify inhibitors of said N-terminal Tau vesicle binding.

SEQ ID NO: 1 depicts the amino acid sequence of the human N-terminal Tau domain (aa 1-112).

SEQ ID NO: 1: *Human* N-terminal Tau domain (aa1-112)(derived from P10636-6; 0N4R isoform-D):

MAEPRQEFVMEHDAGTYGLGDRKDQGGYTMHQDQEGDTDAGLKAEEAGIGDTPSLEDEAAGHVTQ
ARMVSKSKDGTGSDDKKAKGADGKTKIATPRGAAPPQGQKQANATR

Derived from: >sp|P10636-6|TAU_HUMAN Isoform Tau-D of Microtubule-associated protein tau
OS=Homo sapiens GN=MAPT:

MAEPRQEFVEMEDHAGTYGLGDRKDQGGYTMHQDQEGDTDAGLKAEAEAGIGDTPSLEDEA
AGHVTQARMVSKSKDGTGSDDKKAKGADGKTKIATPRGAAPPGQKGQANATRIPAKTPPAP
5 KTPPSSGEPPKSGDRSGYSSPGSPGTPGSRSRTPSLPTPPTREPKKVAVVRTPPKSPSSAKS
RLQTAPVPMPLDKNVKSKIGSTENLKHQPGGGKVQIINKLDLSNVQSKCGSKDNIKHVPGGG
SVQIVYKPVDLSKVTSCGSLGNIHHKPGGGQVEVKSEKLDKDRVQSKIGSLDNITHVPGGG
NKKIETHKLTFRENAKAKTDHGAEIVYKSPVVSGDTSRHLNSVSSTGSIDMVDSPQLATLAD
EVSASLAKQGL

- 10 In a first embodiment, the invention relates to the use of SEQ ID NO: 1 or a homologue with at least 65 % amino acid identity thereof to be contained in the *in vitro* system. The use as meant here is any use of the protein, and may be, as a non-limiting example, the recombinant protein. Methods for quantification of protein (e.g. via antibodies recognizing the protein) are known to the person skilled in the art. "Sequence similarity" indicates the percentage of amino acids that either are identical or that represent conservative amino acid substitutions. "Sequence identity" between two amino acid sequences indicates the percentage of amino acids that are identical between the sequences. The alignment for determining sequence similarity, preferably sequence identity, can be done with art known tools, preferably using the best sequence alignment, for example, using CLC main Workbench (CLC bio) or Align, using standard settings, preferably EMBOSS::needle, Matrix: Blosum62, Gap Open 10.0, Gap Extend 0.5. The term
- 15 "amino acid identity" as used herein refers to the extent that sequences are identical on an amino acid-by-amino acid basis over a window of comparison. Thus, a "percentage of sequence identity" is calculated by comparing two optimally aligned sequences over the window of comparison, determining the number of positions at which the identical amino acid residue (e.g., Ala, Pro, Ser, Thr, Gly, Val, Leu, Ile, Phe, Tyr, Trp, Lys, Arg, His, Asp, Glu, Asn, Gln, Cys and Met) occurs in both sequences to yield the number of
- 20 matched positions, dividing the number of matched positions by the total number of positions in the window of comparison (i.e., the window size), and multiplying the result by 100 to yield the percentage of sequence identity.

- In another embodiment, the invention relates to a method wherein the *in vitro* system comprises an N-terminal Tau protein domain as depicted in SEQ ID NO: 2 or a homologue with at least 65 % amino acid identity thereof.
- 30

SEQ ID NO: 2 depicts the amino acid sequence of the human N-terminal Tau domain (aa 1-170).

SEQ ID NO: 2: Human N-terminal Tau domain (aa1-170)(derived from P10636-8; 2N4R isoform):

- MAEPRQEFVEMEDHAGTYGLGDRKDQGGYTMHQDQEGDTDAGLKESPLQTP TEDGSEEP
35 GSETSDAKSTPTAEDVTAPLVDEGAPGKQAAAQPHTEIPEGTTAEAEAGIGDTPSLEDEAAAGHV
TQARMVSKSKDGTGSDDKKAKGADGKTKIATPRGAAPPGQKGQANATR

In alternative embodiments, said *in vitro* system comprises an N-terminal Tau protein domain as depicted in SEQ ID NO:1 or a homologue with at least 75 % amino acid identity thereof, or at least 85 % amino acid identity thereof, or at least 90 % amino acid identity thereof, or at least 95 % amino acid identity thereof. Finally, other embodiments provide an *in vitro* system comprising an N-terminal Tau protein domain as depicted in SEQ ID NO:2 or a homologue with at least 75 % amino acid identity thereof, or at least 85 % amino acid identity thereof, or at least 90 % amino acid identity thereof, or at least 95 % amino acid identity thereof.

In one embodiment, the present invention provides a method to produce compounds that inhibit Tau-mediated early synaptic dysfunction, wherein said "Tau-mediated" involves an active role for the Tau protein. More specifically, N-terminal Tau protein domain association to synaptic vesicles results in Tau-mediated early synaptic dysfunction in pathological conditions of Tauopathies. Inhibition of this early synaptic dysfunction can be obtained by producing a compound which can interfere on the association of Tau with synaptic vesicles. The discovery of a function for the N-terminal domain dissociates the vesicle binding and actin polymerization role of pathogenic Tau at synapses. The N-terminal domain of Tau is dispensable for actin polymerization because Δ N-pathogenic Tau mutants localize to presynaptic terminals and still support the formation of excess F-actin at synapses. These results also indicate that the mere presence of F-actin is not sufficient to measurably hinder vesicle mobilization, but that these vesicles also need to be crosslinked, e.g. to cytoskeletal elements. Noteworthy, Tau also dimerizes and multimerizes ahead of tangle formation, which could potentially also add to the ability of Tau to impede with vesicle mobility. Interestingly, a similar pathogenic function, the direct crosslinking of vesicles by multimerization, has been suggested for α -synuclein (Diao et al., 2013; Wang et al., 2014), a protein that aggregates in Lewy body disease and Parkinson's disease, suggesting that Tau and α -synuclein harbor overlapping effects on presynaptic function. These findings suggest that Tau employs a similar mode of vesicle immobilization as Synapsins, which also bind vesicles and F-actin to keep vesicles in a reserve pool (De Camilli et al., 1990; Shupliakov et al., 2011; Yoshiyama et al., 2007).

The "*in vitro* system" refers to a system comprising at least the necessary components and environment to execute said method, and makes use of biological molecules, organisms, a cell (or part of a cell) outside of their normal naturally-occurring environment, permitting a more detailed, more convenient, or more efficient analysis than can be done with whole organisms. "Synaptic vesicles" provided in said *in vitro* system are defined as small secretory vesicles that contain a neurotransmitter, and are naturally abundantly present organelles of a uniform size inside an axon near the presynaptic membrane. The vesicles have a diameter of about 40 nm and accommodate a number of proteins and phospholipids. In physiological context, when a nerve impulse moves down the axon of a neuron and arrives at an axon terminal, it stimulates synaptic vesicles in the terminal to discharge neurotransmitters. After fusion with the membrane, a synaptic vesicle releases its contents into the synaptic cleft (Dillon and Goda, 2005).

The invention relates to said method comprising a step c) in which the binding of said N-terminal Tau protein domain to synaptic vesicles is monitored, wherein a compound that prevents Tau-mediated early

synaptic dysfunction is identified when for said compound as compound to the binding under the same test conditions in the same system without the test compound, a reduction in the N-terminal Tau protein binding to synaptic vesicles is observed. Said reduction is obtained when binding of N-terminal Tau protein domain to the synaptic vesicles is at least 20 %, at least 30 %, at least 40 %, at least 50 %, at least 60 %, at least 70 %, at least 80 %, at least 90 %, at least 95 % or even 100 % weaker as compared to the normal (100 %) binding of the N-terminal Tau protein domain to the synaptic vesicles.

In one embodiment, the method comprises a high content screening (HCS) of suitable test compounds. In some instances, HCS is a screening method that uses an *in vitro* system to perform a series of experiments as the basis for high throughput compound discovery. Typically, HCS is an automated system to enhance the throughput of the screening process. However, the present invention is not limited to the speed or automation of the screening process. In another embodiment of the invention, the HCS assay provides for a high throughput assay. Preferably, the assay provides automated screening of thousands of test compounds.

Compounds tested in the screening method of the present invention are not limited to the specific type of the compound. In one embodiment, entire compound libraries are screened. Compound libraries are a large collection of stored compounds utilized for high throughput screening. Compounds in a compound library can have no relation to one another, or alternatively have a common characteristic. For example, a hypothetical compound library may contain all known compounds known to bind to a specific binding region. As would be understood by one skilled in the art, the methods of the invention are not limited to the types of compound libraries screened. For high-content screening, compound libraries may be used. Examples include, but are not limited to, natural compound libraries, allosteric compound libraries, peptide libraries, antibody fragment libraries, synthetic compound libraries, etc. In one embodiment, high throughput screening methods involve providing a library containing a large number of compounds (candidate compounds) potentially having the desired activity. Such "combinatorial chemical libraries" are then screened in one or more assays, as described herein, to identify those library members (particular chemical species or subclasses) that display a desired characteristic activity. The compounds thus identified can serve as conventional "hit compounds" or can themselves be used as potential or actual therapeutics.

In another embodiment, the said *in vitro* system comprises immobilized synaptic vesicles using molecules with specificity for proteins present on said synaptic vesicles, and a buffer condition suitable to perform an immune-based assay. Said molecules with specificity for synaptic vesicle proteins could be but are not limited to for example antibodies, which recognize and bind to other molecules based on their shape and physicochemical properties and can for instance be immobilized by covalent linkage to primary amines or sulfhydryl groups, or via protein G binding covalently attached to a blocked magnetic bead surface. A skilled person should be able to provide an *in vitro* system with immobilized synaptic vesicles, being conscious of the existence of tools as exemplified here. Furthermore, particular embodiments require a buffer condition suitable for immune-based assays, in which "a buffer condition" refers to the composition of the solution in which the immune-based assay is performed. The said composition includes buffered

solutions and/or solutes such as pH buffering substances, water, saline, physiological salt solutions, glycerol, preservatives, etc. for which a person skilled in the art is aware of the suitability to obtain optimal immune-based assay performance.

In specific embodiments, immune-based assays are comprised in step c) of the claimed method, more specifically “immune-based assays” or “immune-based detection” for monitoring N-terminal Tau protein domain binding to synaptic vesicles in a method for producing a compounds that prevents said binding. In said embodiments, “immune-based assays” comprise the most broadly used bio-detection technologies that are based on the use of antibodies, and are well known in the art. Antibodies are highly suited for detecting small quantities of target proteins in the presence of complex mixtures of proteins. As used herein, an “immune-based assay”, “immunoassay” or “immune-based detection” (each of these terms can be used interchangeably) refers to a biochemical binding assay involving binding between antibodies and antigen, which measures the presence or concentration of a substance in a sample, such as a biological sample, using the reaction of an antibody to its cognate antigen, for example the specific binding of an antibody to a protein. Both the presence of the antigen or the amount of the antigen present can be measured.

Examples of immunoassays are enzyme linked immunosorbent assays (ELISAs), enzyme linked immunospot assay (ELISPOT), immunobead capture assays, Western blotting, gel-shift assays, protein arrays, multiplexed bead arrays, magnetic capture, fluorescence resonance energy transfer (FRET), a sandwich assay, a competitive assay, an immunoassay using a biosensor, an immunoprecipitation assay etc. Examples of assay detection methods for producing compounds in the context of the present invention are described in the Example section, without the purpose of being limitative. It should be clear to the skilled artisan that the present screening methods might be based on a combination or a series of measurements, particularly when establishing the link between the reduction of association of N-terminal Tau protein domain to synaptic vesicles and inhibition of this interaction by specific test compounds. Also, it should be clear that there is no specific order in performing these measurements while practicing the present invention.

In general, immune-based assays involve contacting a sample suspected of containing a molecule of interest (such as the test compound) with an antibody to the molecule of interest or contacting an antibody to a molecule of interest (such as antibodies to N-terminal Tau) with a molecule that can be bound by the antibody, as the case may be, under conditions effective to allow the formation of immunocomplexes. Contacting a sample with the antibody to the molecule of interest or with the molecule (e.g. synaptic vesicle protein) that can be bound by an antibody to the molecule of interest under conditions effective and for a period of time sufficient to allow the formation of immune complexes (primary immune complexes such as N-terminal Tau bound to synaptic vesicle protein) is generally a matter of simply bringing into contact the molecule or antibody and the sample and incubating the mixture for a period of time long enough for the antibodies to form immune complexes with, i.e., to bind to, any molecules (e.g., antigens) present to which the antibodies can bind. In many forms of immunoassay, the sample-antibody composition, such as a tissue section, ELISA plate, dot blot or Western blot, can then be washed to remove any non-specifically

bound antibody species, allowing only those antibodies specifically bound within the primary immune complexes to be detected.

Immunoassays can include methods for detecting or quantifying the amount of a molecule of interest (such as N-terminal Tau protein after competition of the test compound for binding to synaptic vesicle proteins) in a sample, which methods generally involve the detection or quantitation of any immune complexes formed during the binding process. In general, the immune-based detection is well known in the art and can be achieved through the application of numerous approaches. These methods are generally based upon the detection of a label or marker, such as any radioactive, fluorescent, biological or enzymatic tags or any other known label. See, for example, U.S. Pat. Nos. 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149 and 4,366,241, each of which is incorporated herein by reference in its entirety and specifically for teachings regarding immune-based detection methods and labels. As used herein, a label can include a fluorescent dye, a member of a binding pair, such as biotin/streptavidin, a metal (e.g., gold), or an epitope tag that can specifically interact with a molecule that can be detected, such as by producing a coloured substrate or fluorescence. Substances suitable for detectably labelling proteins include fluorescent dyes (also known herein as fluorochromes and fluorophores) and enzymes that react with colorimetric substrates (e.g., horseradish peroxidase). The use of fluorescent dyes is generally preferred in the practice of the invention as they can be detected at very low amounts. Furthermore, in the case where multiple antigens are reacted with a single array, each antigen can be labelled with a distinct fluorescent compound for simultaneous detection. Labelled spots on the array are detected using a fluorimeter, the presence of a signal indicating an antigen bound to a specific antibody. Fluorophores are compounds or molecules that luminesce. Typically, fluorophores absorb electromagnetic energy at one wavelength and emit electromagnetic energy at a second wavelength.

A variety of immunoassays can be used to detect one or more of the proteins disclosed or incorporated by reference herein. ELISA is a heterogeneous immunoassay, which can be used in the methods disclosed herein. The assay can be used to detect protein antigens in various formats. In the "sandwich" format the antigen being assayed is held between two different antibodies. In this method, a solid surface is first coated with a solid phase antibody. The *in vitro* system composition containing the antigen, such as N-terminal Tau and/or synaptic vesicle proteins, and then added the test compound, allowed the test compound to compete with the N-terminal Tau binding to the synaptic vesicle proteins, and therefore reduce the detection of N-terminal Tau and or synaptic vesicle protein via reaction with the bound antibody. Any unbound antigen is washed away. A known amount of enzyme-labelled antibody is then allowed to react with the bound antigen. Any excess unbound enzyme-linked antibody is washed away after the reaction. The substrate for the enzyme used in the assay is then added and the reaction between the substrate and the enzyme produces a colour change. The amount of visual colour change is a direct measurement of specific enzyme-conjugated bound antibody, and consequently the antigen present in the sample tested. ELISA can also be used as a competitive assay. In the competitive assay format, the test specimen containing the antigen to be determined is mixed with a precise amount of enzyme-labelled antigen and both compete for binding to an anti-antigen antibody attached to a solid surface. Excess free enzyme-labelled antigen is washed off before the substrate for the enzyme is added. The amount of colour intensity resulting from the enzyme-substrate interaction is a measure of the amount of antigen in the

sample tested. A heterogeneous immunoassay, such as an ELISA, can be used to detect any of the proteins disclosed or incorporated by reference herein. In many immunoassays, as described elsewhere herein, detection of antigen is made with the use of antigens specific antibodies as detector molecules. However, immunoassays and the systems and methods of the present invention are not limited to the use of antibodies as detector molecules. Any substance that can bind or capture the antigen within a given sample may be used. Aside from antibodies, suitable substances that can also be used as detector molecules include but are not limited to enzymes, peptides, proteins, and nucleic acids. Further, there are many detection methods known in the art in which the captured antigen may be detected. In some assays, enzyme-linked antibodies produce a colour change. In other assays, detection of the captured antigen is made through detecting fluorescent, luminescent, chemiluminescent, or radioactive signals. The system and methods of the current invention is not limited to the particular types of detectable signals produced in an immunoassay.

Assays can be performed in an *in vitro* system, with in a particular embodiment, said *in vitro* system comprising neuronal cells. Neuronal cells or neurons are a type of cell in the central nervous system, which receive, integrate, and pass along information by releasing neurotransmitters. Said neurotransmitters are chemicals that cross-over from the terminal button at the end of an axon over the synapse to the neighbouring neuron. Non-limiting examples of neuron cells are primary cortical neurons, primary basal forebrain cholinergic neurons, primary neural stem cells, sensory neurons (e.g. retinal cells, olfactory epithelium cells), motoneurons (e.g. spinal motor neurons, pyramidal neurons, Purkinje cells) and interneurons (e.g. dorsal root ganglia cells).

In yet an alternative embodiment, the said *in vitro* system comprises a buffer conditions suitable to perform a vesicle sedimentation assay, wherein said vesicle sedimentation assay comprises a method to fractionate synaptic vesicles from a mixture, as for example provided in, but not limited to, the method described in the Example section (12. Synaptic Vesicle sedimentation assay). Hence the buffer condition refers to the composition of the solution in which the vesicle sedimentation assay is performed. The composition includes buffered solutions and/or solutes such as pH buffering substances, water, saline, physiological salt solutions, glycerol, preservatives, etc. for which a person skilled in the art is aware of the suitability to obtain satisfying synaptic vesicle fractionation and reliable results.

We found that expression of mutant Tau proteins lacking the N-terminal protein domain do not elicit presynaptic defects anymore, and reasoned that tools that compete with Tau for synaptic vesicle binding would be beneficial. Treatment of rat neurons with a membrane permeable N-terminal Tau domain (Tat-NT^{Tau} peptide) showed that this molecule could compete for synaptic vesicle binding, and thereby rescued the pathological Tau-induced inability to maintain neurotransmitter release. These results provided the first proof-of principle that it is possible to target the ability of Tau to interact with synaptic vesicles to rescue presynaptic defects, thereby paving the way for the development of more specific tools with higher affinity and specificity, using the above claimed method.

In a second aspect, the invention relates to a polypeptide inhibiting the binding of N-terminal Tau protein domain to synaptic vesicles, more specifically, said polypeptide comprising SEQ ID NO: 1 or a homologue with at least 65 % amino acid identity thereof and a cell penetrant carrier, wherein said cell penetrant carrier can enter a cell through a sequence which mediates cell penetration (or cell translocation). In the latter case said protein molecule is modified through the recombinant or synthetic attachment of a cell penetration sequence. Thus, the molecule (or polypeptide) may be further fused or chemically coupled to a sequence facilitating transduction of the fusion or chemical coupled proteins into prokaryotic or eukaryotic cells. Sequences facilitating protein transduction are known to the person skilled in the art and include, but are not limited to Protein Transduction Domains. It has been shown that a series of small protein domains, termed protein transduction domains (PTDs), cross biological membranes efficiently and independently of transporters or specific receptors, and promote the delivery of peptides and proteins into cells. Preferably, said sequence is selected from the group comprising TAT protein from human immunodeficiency virus (HIV-1), a polyarginine sequence, penetratin and a short amphipathic peptide carrier, Pep-1. Still other commonly used cell-permeable peptides (both natural and artificial peptides) are disclosed in Joliot A. and Prochiantz A. (2004) *Nature Cell Biol.* 6 (3) 189-193.

In a preferred embodiment, the protein comprising SEQ ID NO: 1 or a homologue with at least 65 % amino acid identity thereof and a cell penetrant carrier is used as a medicament. Said medicament is needed in a therapeutically effective amount. One of ordinary skill in the art will recognize that the potency and, therefore, an "effective amount" can vary for the inhibitory agents of the present invention. One skilled in the art can readily assess the potency of the inhibitory agent. A medicament to prevent and/or to treat an individual with a neurological disorder, in particular a Tauopathy, relates to a composition comprising agents as described above and a pharmaceutically acceptable carrier or excipient (both terms can be used interchangeably) to treat or to prevent Tauopathies, such as Alzheimer's disease, as described herein. While a connection between vesicle mobilization defects and neuronal death awaits further investigation, it is conceivable that a defect to maintain normal release contributes to the early synaptic defects seen in neurodegenerative disease. In humans, long lasting high-frequency activity has been observed during cognitive tasks (Crone et al., 2011; Herrmann et al., 2004; Lachaux et al., 2012), suggesting that Tau-induced vesicle mobilization defects and the inability to maintain normal levels of neurotransmitter release could potentially contribute to cognitive decline in dementia. In an alternative embodiment said polypeptide or protein comprises SEQ ID NO:1, or in another embodiment, said polypeptide comprises SEQ ID NO:2. Alternatively, said polypeptide inhibiting the binding of N-terminal Tau to synaptic vesicles comprises a homologue of SEQ ID NO:1 with at least 65 % amino acid identity thereof, or at least 75 % amino acid identity thereof, or at least 85 % amino acid identity thereof, or at least 90 % amino acid identity thereof, or at least 95 % amino acid identity thereof. Alternatively, said polypeptide inhibiting the binding of N-terminal Tau to synaptic vesicles comprises a homologue of SEQ ID NO:2 with at least 65 % amino acid identity thereof, or at least 75 % amino acid identity thereof, or at least 85 % amino acid identity thereof, or at least 90 % amino acid identity thereof, or at least 95 % amino acid identity thereof.

Another embodiment relates to a polypeptide inhibiting said binding of N-terminal Tau protein domain with synaptic vesicles, wherein said polypeptide comprises an antibody. More particular, said polypeptide

comprises an antibody specifically binding an epitope within SEQ ID NO:1 or a homologue with at least 65 % amino acid identity thereof. In another embodiment, said polypeptide comprises SEQ ID NO:2. Alternatively, said antibody inhibiting the binding of N-terminal Tau to synaptic vesicles is specifically binding an epitope within a homologue of SEQ ID NO:1 with at least 75 % amino acid identity thereof, or at least 85 % amino acid identity thereof, or at least 90 % amino acid identity thereof, or at least 95 % amino acid identity thereof. Alternatively, said antibody inhibiting the binding of N-terminal Tau to synaptic vesicles is specifically binding an epitope within a homologue of SEQ ID NO:2 with at least 65 % amino acid identity thereof, or at least 75 % amino acid identity thereof, or at least 85 % amino acid identity thereof, or at least 90 % amino acid identity thereof, or at least 95 % amino acid identity thereof.

Further embodiments involve isolated antibodies inhibiting the binding between N-terminal Tau protein domain and synaptic vesicles, with an epitope within said N-terminal Tau protein domain as for instance but not limited to the sequences provided by SEQ ID NO:1 and SEQ ID NO:2. In some cases, said isolated antibodies are provided in a pharmaceutical composition. A pharmaceutical composition comprises in that case said polypeptide comprising an antibody and a pharmaceutically acceptable excipient. Said pharmaceutical composition in some cases comprises said polypeptide comprising an antibody linked to a cell penetrant carrier, and a pharmaceutically acceptable excipient suitable for use in humans. Pharmaceutically acceptable excipients have been amply described in a variety of publications, including, for example, A. Gennaro (2000) "Remington: The Science and Practice of Pharmacy", 20th edition, Lippincott, Williams, & Wilkins; Pharmaceutical Dosage Forms and Drug Delivery Systems (1999) H. C. Ansel et al., eds 7th ed., Lippincott, Williams, & Wilkins; and Handbook of Pharmaceutical Excipients (2000) A. H. Kibbe et al., eds., 3rd ed. Amer. Pharmaceutical Assoc. The pharmaceutically acceptable excipients, such as vehicles, adjuvants, carriers or diluents, are readily available to the public. Moreover, pharmaceutically acceptable auxiliary substances, such as pH adjusting and buffering agents, tonicity adjusting agents, stabilizers, wetting agents and the like, are readily available to the public.

To this point, it will be interesting to assess if Tau-dependent vesicle binding constitutes an essential aspect of the endogenous Tau function, as such a function would be inhibited by the produced competition compounds. In relation to this, while short-term exposure to the current N-terminal peptide itself did not elicit obvious neuronal toxicity, it will also be essential to assess if longer exposure of produced compounds that inhibit Tau vesicle binding affect aspects of presynaptic or other neuronal function for their therapeutic potential. Nonetheless, the invention relates to a new aspect of Tau biology and suggests that a window to tackle the presynaptic defects induced by pathological Tau exists.

The following examples are intended to promote a further understanding of the present invention. While the present invention is described herein with reference to illustrated embodiments, it should be understood that the invention is not limited hereto. Those having ordinary skill in the art and access to the teachings herein will recognize additional modifications and embodiments within the scope thereof. Therefore, the present invention is limited only by the claims attached herein.

EXAMPLES

1. Pathogenic Tau associates with vesicles at presynaptic terminals

Pathological Tau was previously detected at both pre- and post-synaptic compartments in isolated synaptosomes from AD patient brains (Fein et al., 2008; Henkins et al., 2012; Sokolow et al., 2015; Tai et al., 2012). Array tomography (Kay et al., 2013) was applied to determine the localization of pathological Tau *in situ* in the brain of AD patients. Ultrathin (70 nm) brain sections were prepared and labeled with antibodies against pathological phospho-Tau (AT8) and the presynaptic marker protein Synapsin. Pathological phospho-Tau was abundantly present at the presynaptic terminals of AD patient brains but was absent from the presynaptic terminals of healthy controls (Figure 5). Together with previous studies, this observation argues for the relevance of exploring the role of presynaptic Tau in eliciting synaptic pathology. Given that the mechanisms of presynaptic function are well conserved across species (Hadley et al., 2006; Lloyd et al., 2000), we resorted to *Drosophila* larval neuromuscular junctions (NMJs) to study

the presynaptic role of Tau. Presynaptic terminals at the NMJ are large allowing for imaging and they can be genetically separated from the post-synaptic muscle. We inserted expression constructs of human wild type or FTDP-17 pathogenic mutant Tau (R406W, V337M and P301L) at identical genomic loci in the fly genome, resulting in very similar expression levels in motor neurons when using the D42-Gal4 driver (Figures 6A and 6B). At the NMJ, both wild type and pathogenic mutant Tau were localized to axons, but compared to wild type Tau, the pathogenic mutants showed increased localization to presynaptic terminals labeled by the synaptic vesicle marker Cysteine string protein (CSP) (Figures 1A and 1B). The increased

presynaptic localization of the pathogenic mutant Tau was likely attributed to their lower affinity for binding microtubules as previously reported (Wang and Mandelkow, 2016). We next assessed the sub-boutonic localization and distribution of synaptic-localized Tau using super-resolution structured illumination microscopy. We found that pathogenic Tau at presynaptic boutons was distributed in a 'doughnut-like' pattern that closely resembles that of synaptic vesicles marked by anti-CSP labeling (Figure 1C). We therefore questioned whether Tau associates with synaptic vesicles and performed a vesicle depletion assay using a temperature sensitive Dynamin mutant (*Shi^{ts1}*) that blocks endocytosis at restrictive temperature (Kawasaki et al., 2000). When stimulated with KCl, synaptic vesicles fused with the membrane but were not retrieved by endocytosis, as shown by the relocation of the synaptic vesicle marker CSP to the plasma membrane (Figure 1D). Similar to CSP, Tau relocated to the plasma membrane (Figure 1D), suggesting that mislocalized pathogenic Tau at presynaptic terminals associated with synaptic vesicles *in vivo*. To further analyze the vesicle-binding properties of Tau we incubated synaptic vesicles isolated from mouse brain (Figures 6C and 6D) with purified recombinant human Tau (Figures 6E-G). Recombinant Tau co-sedimented with synaptic vesicles in a vesicle sedimentation assay (Figure 1E). Synaptic vesicles were also co-immunoprecipitated using recombinant Tau as bait, evidenced by the recovery of synaptic vesicle marker proteins (Figure 1F). Finally, electron microscopy of recombinant Tau incubated with isolated synaptic vesicles *in vitro* showed the protein binding to vesicle membranes (Figure 1G). Note that both wild type and pathogenic mutant Tau were able to bind to synaptic vesicles *in vitro* (Figures 1E and 1F); however, only mutant Tau was prominently present at presynaptic terminals, allowing

it to bind to synaptic vesicles *in vivo* (Figure 1A). To determine the mode of vesicle binding we generated and purified Tau-deletion proteins and assessed if they could co-immunoprecipitate synaptic vesicles. Only truncation of the N-terminus (aa1-112) of Tau, but not the truncation of any other domain, strongly impeded with the binding of Tau to synaptic vesicles (Figures 1H-J). In agreement, the Tau N-terminal
5 fragment alone co-immunoprecipitated vesicles (Figure 6H). Therefore, the N-terminus of Tau was necessary and sufficient for synaptic vesicle binding.

2. Pathogenic Tau restricts synaptic vesicle mobility impeding neurotransmitter release

Next, we wondered if vesicle-associated Tau elicited functional defects at presynaptic terminals. To measure neurotransmitter release we stimulated motor neurons and recorded excitatory junctional
10 potentials (EJPs). At low-frequency stimulation (0.2 Hz), the EJP amplitudes measured in controls and mutant Tau-expressing animals were similar. However, during sustained high frequency stimulation (10 Hz), pathogenic Tau mutant-expressing animals failed to maintain normal levels of release (Figures 2A and 2B). Such a defect could reflect slowed synaptic vesicle reformation by endocytosis (Saheki and De Camilli, 2012; Sara et al., 2002; Verstreken et al., 2002), a reduced number of synaptic vesicles that
15 participate in release, and/or slower vesicle mobilization (Alabi and Tsien, 2012; Fernández-Alfonso and Ryan, 2004; Gaffield et al., 2006; Verstreken et al., 2005). We first assessed synaptic vesicle endocytosis using Synaptobrevin (SpH), a pH sensitive GFP fused to the luminal domain of the synaptic vesicle protein Synaptobrevin (Miesenböck et al., 1998). The fusion of synaptic vesicles with the presynaptic membrane caused SpH fluorescence to increase, while vesicle endocytosis and acidification resulted in
20 a decrease in fluorescence. We did not observe significant differences in the rise and decay of SpH fluorescence in response to short stimulation paradigms in controls and mutant Tau-expressing animals. In an alternative approach, we also used an exogenously added fluorescent dye (FM 1-43) that is endocytosed into newly formed synaptic vesicles. During mild 3 Hz stimulation, we did not observe a difference in FM 1-43 uptake in the pathogenic Tau-expressing animals and in controls (Figures 2C and
25 2D). Hence, under these conditions, endocytosis is not affected. We next assessed synaptic vesicle mobilization during sustained stimulation. We measured SpH fluorescence during 10 min of 10 Hz stimulation in the presence of bafilomycin, a drug that blocks reacidification of newly endocytosed vesicles. Thus, vesicles that fused at least once during the stimulation paradigm remain fluorescent, revealing the cumulative signals from vesicle fusion over time. Expression of pathogenic Tau mutants slowed the vesicle
30 release rate significantly, indicating a smaller active vesicle pool compared to controls or animals expressing wild type Tau (Figures 2E and 2F). This effect was not due to change in total synaptic vesicle pool size, as NH₄Cl unquenching of all SpH fluorescence at the synapse did not show significant differences (Figure 2G). We further confirmed the smaller cycling vesicle pool upon expression of pathogenic Tau by labeling the entire cycling vesicle pool with FM 1-43 dye using prolonged high
35 frequency stimulation (10 min at 10 Hz). We observed significantly less dye uptake in pathogenic Tau-, but not wild type Tau-expressing NMJs as compared to controls (Figures 2C and 2D), indicating fewer labeled vesicles. Note that only mutant Tau, not wild type Tau elicited vesicle cycling defects, again correlating with the increased presynaptic localization of the mutant proteins (Figure 1A). While our data revealed that the N-terminal domain of Tau binds vesicles, it was also previously described that the
40 proline-rich and microtubule-binding domains of Tau promote actin polymerization (Fulga et al., 2007; He

et al., 2009). We therefore hypothesized that vesicle-binding and simultaneous F-actin formation by Tau may restrict synaptic vesicle mobility (De Camilli et al., 1990; Shupliakov et al., 2011). Accordingly, we found that the presence of Tau increased presynaptic F-actin levels using the genetic probe LifeAct-GFP (Figures 3A and 3B). We then performed fluorescence recovery after photobleaching (FRAP) experiments to measure the mobility of synaptic vesicles labeled by Synaptotagmin-GFP within presynaptic boutons. Fluorescence recovery, and thus vesicle mobility, was significantly slower in animals expressing pathogenic mutant Tau compared to controls or animals expressing wild type Tau (Figures 3C-F). We therefore propose a model in which pathogenic Tau behaves as a vesicle clustering molecule by interacting with vesicles using its N-terminal domain, and crosslinking them to F-actin to restrict vesicle mobility and release (Figures 3G and 3H).

3. Interference with synaptic vesicle binding rescues Tau-induced presynaptic pathology

To test our proposed model, we generated transgenic flies that express N-terminally truncated pathogenic mutant Tau (ΔN_{R406W} , ΔN_{V337M} and ΔN_{P301L}) and assessed vesicle mobility and presynaptic function. These N-terminal truncation mutants all localized to presynaptic boutons (Figures 7A and 7B) and increased presynaptic F-actin levels (Figure 7E) to similar extents as their full-length counterparts. However, ΔN -pathogenic Tau mutants showed a more diffuse localization, distinct from the CSP-labeled “doughnut-like” synaptic vesicle localization pattern (Figures 7A and 7C). These data corroborated the inability of ΔN -Tau to bind vesicles. We confirmed this observation using the *sh^{ts1}* vesicle depletion assay (see Figure 1D). In stimulated *sh^{ts1}* mutants, CSP relocated to the plasma membrane but the ΔN pathogenic Tau mutants remained diffusely present in the boutons (Figure 7D). Together, these data indicated reduced association of ΔN -Tau with synaptic vesicles *in vivo*. To assess vesicle mobility we used the Synaptotagmin-GFP FRAP assay. In contrast to synapses expressing full length pathogenic Tau, synapses expressing ΔN -pathogenic Tau mutants showed Synaptotagmin-GFP fluorescence recovery rates that were very similar to those measured in controls (Figure 4A). Furthermore, animals expressing N-terminally truncated pathogenic Tau mutants also did not show defects in the rate of synaptic vesicle mobilization and in the size of the active synaptic vesicle pool as assessed by SpH in the presence of bafilomycin (Figure 4B). Finally, expression of N-terminally truncated Tau mutants did not cause defects in maintaining neurotransmitter release during 10 Hz stimulation (Figure 4C). Thus, the N-terminal domain of Tau mediated synaptic vesicle mobilization and the presynaptic defects upon expression of pathogenic Tau *in vivo*.

We further confirmed our findings in rat hippocampal autaptic cultures (Bekkers and Stevens, 1991) (Figure 4D). We performed whole cell voltage clamp and measured neurotransmitter release in response to 10 consecutive high frequency stimulation trains assessing the ability of vesicle pools to mobilize in order to sustain release (10 Hz for 10 s with 30 s interval). Neuronal cultures transduced with AAV expressing wild type Tau maintained release efficacy similar to cells transduced with control AAV expressing GFP (Figures 4F and 4G). In contrast, neurons expressing pathogenic P301L Tau were unable to maintain release (Figures 4F and 4G), in agreement with our observations in *Drosophila*. Next, we designed and purified a peptide corresponding to the human Tau N-terminal domain (NT^{Tau}, aa1-113) fused to a minimal HIV-derived cell-penetrating Tat peptide (Figure 4E). Tat-NT^{Tau} was able to compete

for synaptic vesicle binding with full-length Tau *in vitro* (Figure 7F). Acutely treating neurons expressing P301L Tau with Tat-NT^{Tau} significantly restored neurotransmitter release, while adding a control TatmCherry peptide had no effect (Figures 4F and 4H). Hence, both fly and rat neurons displayed comparable presynaptic defects upon expression of pathogenic Tau, but not wild type Tau, and blocking the ability of the Tau N-terminal domain to interact with synaptic vesicles rescued the presynaptic defects induced by pathogenic Tau.

4. Pathogenic Tau species accumulate on synaptic vesicles in Alzheimer's Disease

We described the presynaptic localization of pathological hyperphosphorylated Tau in Alzheimer's disease (Tai et al., 2012; Example 1), and we showed that Tau is able to bind presynaptic vesicles (SVs) *in vitro* and *in vivo* in fly and rodent models of Tauopathy. To validate the association of Tau with presynaptic vesicles as a potential disease mechanism, we biochemically fractionated post-mortem human brain samples from Alzheimer's Disease (AD) patients or non-demented control patients to isolate synaptic vesicles (Figure 8A and 8B). We then immunoblotted subcellular fractions and checked for the presence of pathological Tau species using antibodies detecting hyperphosphorylated Tau. Comparing subcellular fractions, we detected robust enrichment of phospho-Tau (pTau) in the synaptic vesicle fractions from AD patient brain, which was absent in controls (Figure 8C). We observed a similar enrichment pattern using a pan-Tau antibody, which also revealed enrichment of Tau in vesicle fractions from control patients (Figure 8C and 8D). In agreement with previous studies, investigating a larger cohort of patient samples we found increased levels of phospho-Tau in total homogenates from AD patients, whereas total Tau levels remained unchanged (Figures 8D and 8E). In the synaptic vesicle fractions, both total Tau levels and phospho-Tau levels were significantly increased in vesicle fractions from AD patients in comparison to controls (Figures 8B, 8D and 8E). Notably, we observe the presence of monomeric Tau species (migrating around 50 kDa) as well as oligomeric Tau species (around 100 kDa and larger) enriched on synaptic vesicles. These data verify the presence of pathological Tau species on synaptic vesicles in AD and validate this pathway as warranting further exploration as a potential therapeutic avenue to target synaptic dysfunction induced by Tau.

Materials and Methods

1. Array Tomography of Human Brain Tissue.

Human brain samples from superior temporal gyrus were provided by collaborating neuropathologists Prof. Colin Smith (University of Edinburgh Sudden Death Brain Bank) and Prof. Matthew Frosch (Massachusetts ADRC brain bank). Use of human tissue conformed to national and institutional ethics guidelines and was approved by the Edinburgh Brain Bank Ethics Committee and the Academic and Clinical Central Office for Research and Development medical research ethics committee. Brain tissue was prepared for array tomography as described previously (Kay et al., 2013). Briefly, tissue was collected at autopsy, small tissue blocks of approximately 1 mm x 1 mm x 5 mm containing all 6 cortical layers from the pia to white matter were cut with a razor blade, and samples were fixed in 4% paraformaldehyde with 2.5% sucrose for 2-3 hours. Tissue was then dehydrated and embedded in LR White resin. Embedded

blocs were cut with an ultramicrotome (Leica Ultracut) into ribbons of serial ultrathin (70 nm) sections, which were mounted on gelatin subbed coverslips (Fisher #1.5). Sections were stained on the first staining day by blocking in 0.05% Tween and 0.1% BSA in TBS for 30 minutes followed by overnight incubation in the following primary antibodies diluted in blocking buffer: Synapsin I (1:100, rabbit, Millipore AB1543) and AT8 (1:50, mouse, Thermo Scientific MN1020). The next day, sections were stained with for 30 min in the following secondary antibody solutions diluted 1:50 in blocking buffer: Alexa594-conjugated donkey anti rabbit antibodies (Invitrogen A21207) and Alexa488-conjugated donkey anti mouse antibodies (Invitrogen A21202). To stain nuclei, 0.005 mg/mL DAPI was included in the secondary antibody solution. Images were acquired on a Zeiss axio Imager Z2 epifluorescence scope. A tilescan image was acquired with a 10x objective of DAPI staining on the entire ribbon. High resolution images were acquired in the same location on every section on the ribbon using a 63x oil 1.4 NA objective. All images were taken with the same exposure times between cases (chosen on an AD case with tau pathology).

2. Drosophila genetics.

All *Drosophila* stocks and experimental crosses were kept on standard corn meal and molasses food. Stocks were kept at room temperature. Experimental crosses involving UAS/GAL4 bipartite expression were kept at 25 °C. All UAS-human Tau (0N4R) flies used in this work were generated by insertion of transgene into a same genomic locus (68A4) and were expressed under the motor neuron-specific D42-Gal4 driver. The UASTau_WT and UAS-Tau_R406W fly lines were obtained as gifts from lab of Dr. Guy Tear (Povellato et al., 2014). The UAS-Tau_V337M and UAS-Tau_P301L fly lines were generated in this current work. Additional stocks used in this study include UAS-Synapto-pHluorin (Miesenböck et al., 1998), UAS-LifeAct-GFP (BSC 57326) and UASsynaptotagmin-eGFP (BSC 6925) from Bloomington Stock Center.

3. Synapto-pHluorin imaging at fly NMJs.

Third-instar *Drosophila* larvae were dissected in Ca²⁺-free HL3 saline (110 mM NaCl, 5 mM KCl, 10 mM NaHCO₃, 5 mM HEPES, 30 mM sucrose, 5 mM trehalose, 10 mM MgCl₂, pH 7.2) and then incubated in HL3 supplemented with 1 mM calcium and 500 μM 1-Naphthylacetyl spermine trihydrochloride (Sigma) to prevent muscle contractions (Uytterhoeven et al., 2011). Motor nerves were stimulated at 2x threshold using an Axoclamp 900A amplifier, and images were captured through a water-dipping objective (40x, 0.8 N.A.) using a cooled CCD camera (Andor Clara DR-328G-CO1-SIL) mounted on a Nikon ECLIPSE FN1 microscope. Images were analyzed using NIS-Elements AR3.2 software. To assess vesicle exocytosis and endocytosis, motor nerves were stimulated at 10 Hz for 5 s and images were captured at 900 ms intervals before, during and after stimulation. The fluorescence changes (ΔF spH) reflect the intensities of bouton fluorescence at corresponding time points subtracting the basal fluorescence intensities before stimulation. To assess endocytosis, ΔF was normalized by ratio to peak ΔF and the fluorescence decays were fitted in two-phase curves for fast and slow time constants to assess the rates of endocytosis. To assess vesicle release during sustained stimulation, 0.5 μM bafilomycin (Merck Millipore) were added into the incubation bath of dissected larva. Motor nerves were stimulated at 10 Hz for 10 min and images were

captured at 15 s intervals. After stimulation, 50 mM NH_4Cl (pH7.4) was added to dequench spH fluorescence signals. The ratios of $\Delta F / \Delta F_{\text{max}}$ (NH_4Cl) were plotted to corresponding time points. In all experiments the NMJs at muscles 12 and 13 in segments A2–A4 were selected for imaging. Data are expressed as average $\pm$ standard error of the mean.

5 4. FM1-43 dye labeling.

Third-instar larvae were dissected in Ca^{2+} -free HL3 saline on Sylgard-coated plates. Motor nerves were stimulated at 2x threshold using an Axoclamp 900A amplifier to load FM1-43 dye (4 μM ; Molecular Probes) in HL3 solution supplemented with 1 mM Ca^{2+} . FM dye loading of NMJs at muscle 12/13 in segments A2-A4 was imaged using a 60 $\times$ 1.0 N.A water immersion lens on a Nikon fluorescent microscope. The microscope filter was set for FM1-43 emission and excitation.

5. Electrophysiological Recording at fly NMJs.

Intracellular voltage recordings from third-instar larval muscle 12 in segment A2 or A3 were performed as described (Uytterhoeven et al., 2011) using ~20 M Ω sharp electrodes and stimulation at 2x threshold. Excitatory junction potentials (EJPs) were recorded with an Axoclamp 900A amplifier digitized using a Digi-data 1440A and stored using pClamp 10.2 software (Molecular Devices). For quantification of 10 Hz recordings, EJP amplitudes were binned per 30 s and averages are normalized to the amplitude measured in the first 15 stimuli.

6. Fluorescence recovery after photo-bleaching (FRAP) assay.

Experiments were performed on third-instar larval fillets dissected in HL3 saline on sylgard-coated plates essentially as previously described (Seabrooke et al., 2010). For FRAP analysis, a Nikon A1R confocal laser microscope equipped with a 60 $\times$ 1.0 N.A. water immersion objective were used. All images were acquired within one hour from the time of dissection. Recordings of vesicle dynamics were taken from type Ib boutons on NMJs at muscles 12 and 13, with a maximum of 5 boutons from the same larvae used. Images were collected at 1.12 μs / pixel with a pinhole of 1 airy unit and a resolution of 512 $\times$ 512. To select the area for bleaching, a region of interest (ROI) 24 $\times$ 30 pixels was selected on the digital image. Four baseline scans were acquired using 5% of full laser power. Before the fifth scan, the laser increased to 95 % of maximal and rapidly iterated the ROI 9 times, after which, returning to 5 % of maximal power to complete the remaining scans. Fluorescence intensity was analyzed using NIS-Elements AR3.2 software for the bleached region, the background and the reference bouton region using the Time Series Analyzer plug-in. Differences between recovery curves were assessed by fitting the data with a double exponential curve. A double exponential curve was used to account for the two phases in fluorescence recovery observed with respect to vesicle mobility, the initial more rapid recovery immediately post bleaching and the later plateau (Seabrooke et al., 2010).

7. Vesicle depletion in shi^{ts1} mutant background.

Experiments were performed with third-instar larval fillets essentially as described previously (Costello and Salkoff, 1986; Kawasaki et al., 2000; Kosaka and Ikeda, 1983; Poodry and Edgar, 1979; Ramaswami et al., 1994; Salkoff and Kelly, 1978). Briefly, third-instar larvae were dissected in Ca²⁺-free HL3 buffer on a sylgard dish at room temperature. The sylgard dish with the dissected preparation in Ca²⁺-free HL3 buffer was placed in a 34 °C oven, and the buffer was replaced by pre-warmed Ca²⁺-free HL3 buffer (34 °C). After allowing 3-5 min for temperature to equilibrate, the preparation was stimulated in pre-warmed high K⁺ (HL3 buffer supplemented with 60 mM KCl and 1.5 mM Ca²⁺) at 34 °C for 10 min. This stimulation was followed by 5 min incubation in pre-warmed Ca²⁺-free HL3 at 34 °C. The samples were then fixed in pre-warmed 4 % PFA at 34 °C for 10-15 min and proceed with washing and immunofluorescence staining. Images were obtained with a Zeiss ELYRA super resolution microscope.

8. Immunohistochemistry.

Third-instar *Drosophila* larvae were dissected in fresh HL-3 solution and fixed in 3.7 % formaldehyde for 20 min at room temperature. After washing with PBS, tissue was permeabilized with PBS containing 0.4 % TritonX-100 (PBT) for 1 h, followed by blocking in 1 % BSA for 1 h at room temperature. Immunostaining was performed with the following primary antibodies: DAKO anti-total tau rabbit pAb (DAKO, A0024), anti-CSP2 mouse mAb (DSHB, 6D6) and anti-GFP rabbit pAb (Invitrogen, A-11122) for probing LifeAct-GFP. The secondary antibodies used were: goat anti-rabbit Alexa 488 (Invitrogen) and goat anti-mouse Alexa 555 (Invitrogen). Following antibody labeling, the preparations were washed in PBT and mounted in Vectashield (Vector Laboratories). Samples were visualized on a Nikon confocal microscope or a Zeiss ELYRA super resolution microscope.

9. Immunoblotting.

All protein samples were reduced in 1x lithium dodecyl sulfate (LDS) sample buffer (Invitrogen) supplemented with 1% β-mercaptoethanol for 10 min at 70 °C. Proteins were separated on NuPAGE Novex 4-12 % Bis-Tris polyacrylamide gels (Invitrogen) in MOPS buffer and then transferred to nitrocellulose membrane using the Trans-Blot Turbo transfer system (BioRad). For immunoblotting, membranes were blocked in TBST (Tris-buffered saline + 0.05 % Tween-20) with 5 % milk powder for 1 hour at room temperature before incubation with primary antibodies diluted in blocking buffer. The following primary antibodies were used in this study: DAKO against total Tau (DAKO, A0024), anti-pTau pSer396/pSer404 (PHF-1, custom antibody, Greenberg et al., 1992; Otvos et al., 1994), anti-pTau pSer202/pThr205 (AT8, Thermo Fisher, MN1020), anti-Synaptobrevin 2 (Synaptic Systems Clone 69.1), anti-Synaptotagmin (DSHB, Asv48), anti-Synapsin (Merck Millipore AB1543P), anti-Synaptophysin (Synaptic Systems Clone 7.2) and anti-Tubulin (DSHB, E7). After incubation with primary antibodies, HRP-conjugated species-specific secondary antibodies were added at a concentration of 1:10,000 for 1 hour at room-temperature. Signal was detected using the Western Lightning Plus ECL kit (Perkin Elmer) and imaged on a Fuji-Film digital imaging system.

10. Purification of recombinant human Tau.

The cDNAs encoding full-length or domain-truncated human Tau (0N4R isoform) were cloned into the *Bam*HI and *Eco*RI restriction sites of pGEX-6P-1 (GE Healthcare) using a reverse primer encoding a 8x-His tag. The resulting fusion proteins contained an N-terminal GST tag followed by a PreScission Protease cleavage site, Tau, and a C-terminal His tag. The plasmid was transformed into Rosetta bacteria (Merck Millipore) for recombinant expression. For expression, bacteria were inoculated in LB medium containing

ampicillin and chloramphenicol and grown overnight at 37 °C. The next day, bacteria were diluted to an OD₆₀₀ of 0.2, and let grow at 37 °C until the culture reached an OD₆₀₀ of 1.0, at which point IPTG (Thermo Scientific) was added to a final concentration of 0.4 mM to induce recombinant protein expression.

Bacteria were incubated for 2 hours at 37 °C following induction, then were immediately pelleted and stored at -80 °C until lysis. For cell lysis, frozen bacterial cell pellets were resuspended in bacterial lysis buffer (PBS supplemented with 10% glycerol, 1% Triton-X-100, 1 mM PMSF, Complete protease inhibitors (Roche) and Benzonase (Sigma) and incubated at 4 °C for 30 min with rotation, followed by centrifugation at 16,000 x g for 20 minutes. Cleared cell lysates were loaded onto pre-washed Glutathione Sepharose 4B (GE Healthcare) and incubated for 2 hours at 4 °C with gentle rotation. After the incubation, the resin was washed twice with PBS supplemented with 250 mM NaCl, and then twice with PreScission Protease cleavage buffer (20 mM Tris-HCl pH 7.0, 50 mM NaCl, 0.5 mM EDTA, 1 mM DTT, 0.01% Tween-20). Proteolytic cleavage of the N-terminal GST tag was performed by incubation with PreScission Protease (GE Healthcare) overnight at 4 °C. The following day, the supernatant of the cleavage reaction was collected and incubated with Glutathione Sepharose for 1 hour at 4 °C to remove residual GST-tagged protease and uncleaved protein. Proteins were further purified against the C-terminal His tag by applying the supernatant onto washed Ni-NTA Profinity Resin (BioRad) and bound for 45 min at 4 °C. The Ni-NTA resin was subsequently washed three times in buffer containing 50 mM NaH₂PO₄ pH 8.0, 300 mM NaCl, 20 mM imidazole before eluting bound His-tagged buffer in the same buffer containing 250 mM imidazole. Protein eluates were concentrated using 0.5 mL centrifugal filter units (Merck Millipore) with a 10 kDa MWCO. Protein was quantified using Quick Start Bradford Reagent (BioRad). Protein purity was evaluated with Page Blue colloidal Coomassie staining solution (Thermo Scientific). All *in vitro* binding experiments utilized freshly purified Tau protein immediately following purification.

11. Isolation of crude synaptic vesicles from mouse brain and human brain.

All animal experiments were performed with ethical permission from and under the guidelines of the KU Leuven animal ethics committee. Preparation of crude synaptic vesicles essentially followed the protocols described previously (Ahmed et al., 2013; Huttner et al., 1983). Brains of six-week old wild type C57BL/6J mouse brain were pooled and homogenized in ice-cold homogenization buffer (320 mM sucrose, 4 mM HEPES pH 7.4, Complete protease inhibitors; 2 mL buffer per brain) with a Potter-Elvehjem Teflon glass homogenizer by 10 strokes at 600 rpm. Homogenates were centrifuged at 800 x g for 10 min to remove nucleus and intact cells, and the supernatants were recovered and further centrifuged at 9,200 x g for 15 min. The pellet was washed once with homogenization buffer and collected again by centrifugation at 10,

200 μ g for 15 min. The pellet fraction (P2') was then resuspended in homogenization buffer (100 μ l for sample from each hemisphere), and mixed with 9 volumes of ice-cold water (supplemented with 5 mM HEPES-KOH, pH 7.4, protease inhibitors) to induce osmotic shock, the mixture was incubated with gentle rotation at 4 °C for 30 min and then subjected to centrifugation at 25,000 $\times$ g for 30 min. The pellet fraction (LP1) was collected, and the supernatant (LS1) was further centrifuged at 165,000 $\times$ g for 2 hours to collect the crude synaptic vesicle fraction (LP2). All manipulation and centrifugation procedures were performed at 4 °C, and buffers were supplemented with fresh Complete protease inhibitor cocktail. The crude synaptic vesicle fraction (LP2) was re-suspended in buffer containing 5 mM HEPES pH 7.4, 300 mM glycine and stored at -80 °C. To check vesicle enrichment, fractions collected during the vesicle preparation were lysed in RIPA buffer (Sigma) with protease inhibitors. Protein concentrations were measured and equal amounts of protein were reduced in LDS sample buffer and probed for presynaptic and postsynaptic membrane markers by immunoblotting.

For isolation of synaptic vesicles from human brain, 1 g of human brain tissue was collected from the posterior hippocampus of brain from Alzheimer's disease patients or non-demented healthy control patients. The age range of the control group was 37-94 years with a mean of 60 ± 17 years, and the age range of the Alzheimer patient group was 67-86 years with a mean of 82 ± 5 years. Frozen tissue samples were thawed, cut into 5 mm² cubes, resuspended in ice-cold homogenization buffer, and homogenized and fractionated to obtain synaptic vesicles using an identical procedure as was used for mouse brain above. The use of human tissue samples conformed to national and institutional ethics guidelines and was approved by the KU Leuven institutional ethics committee.

12. Synaptic vesicle sedimentation assay.

The co-sedimentation assay was performed essentially as previously described (Mills et al., 2012). Briefly, 200 ng of freshly purified recombinant human Tau-His was incubated with an excess amount of crude synaptic vesicles (equivalent to 20 μ g of protein) prepared from mouse brains in synaptic vesicle binding buffer (4 mM HEPES pH 7.4, 5 mM Tris-HCl pH 7.4, 220 mM glycine, 220 μ M NaN₃, 100 μ g/ml BSA, protease inhibitors) at 4 °C for 2 hours in a volume of 100 μ l. After 2 hours of incubation, vesicles were pelleted by ultra-centrifugation at 400,000 $\times$ g for 30 min. Pellets were then re-suspended and reduced in LDS sample buffer. The presence of recombinant human Tau-His in the synaptic vesicle pellet was assessed by immunoblot.

13. Co-Immunoprecipitation of recombinant Tau and synaptic vesicles.

In this co-IP assay, recombinant Tau-His was used as bait to pull-down intact synaptic vesicles. Briefly, 1 μ g of freshly purified recombinant Tau-His protein was immobilized to Protein G Magnetic Dynabeads (Invitrogen) using anti-His antibodies (Invitrogen) in vesicle binding buffer supplemented with 1% BSA for 2 hours at 4 °C. The Tau-bound Dynabeads were then washed and incubated with mouse crude synaptic vesicles (equivalent to 20 μ g of protein) in synaptic vesicle binding buffer supplemented with 1% BSA, 0.1% NP-40, 0.1% Tween-20, and protease inhibitors in a 500 μ l reaction mixture for 2 hours at 4 °C.

Note that this low concentration of detergents increases specificity of binding without lysing synaptic vesicles. The Dynabeads were thoroughly washed after incubation, and the bound proteins were retrieved by boiling in LDS containing 1 % β -mercaptoethanol and assessed for the presence of vesicle marker proteins by immunoblotting. For the competition experiment with full-length Tau and Tat-NT^{Tau}, intact synaptic vesicles (equivalent to 20 μ g of protein) were immobilized on Dynabeads using 1 μ g anti-Synaptobrevin 2 antibodies for 4 hours at 4 °C. After 4 hours, SV-bound Dynabeads were washed and then resuspended in vesicle binding buffer containing 1 μ g (40 nM) of freshly purified full-length Tau-His with varying concentrations of freshly purified Tat-NT^{Tau}-His. Reactions were left mixing at 4 °C for 2 hours, after which beads were thoroughly washed and then boiled in LDS and assessed for the amount of full-length Tau bound to synaptic vesicles by immunoblotting.

14. EM analysis of recombinant Tau binding to synaptic vesicle *in vitro*.

Ultrapure synaptic vesicle fractions from rat brain were obtained by size exclusion chromatography on Sephacryl S-1000 chromatography medium as previously described (Ahmed et al., 2013). To assess the binding of recombinant Tau-His to ultrapure rat synaptic vesicles, 1 μ M Tau-His and 50 μ g/mL vesicles were incubated together in synaptic vesicle binding buffer containing 1 % BSA for 2 h at 4 °C in a 100 μ l binding reaction. After binding, 2 μ l of the vesicle suspension was dotted onto a glowdischarged, carbon-coated grid and let air dry. After drying, grids were blocked in 1 % BSA for 5 minutes followed by incubation with 20 nM of 5 nm Ni-NTA-Nanogold (Nanoprobes) for 30 minutes in SV buffer containing 1 % BSA. Grids were subsequently washed in SV buffer containing 8 mM imidazole before fixation in 2 % glutaraldehyde for 5 min. Grids were then washed in ultrapure water before negative staining with 1 % uranyl acetate. Samples were imaged on a Jeol JEM1400 transmission electron microscope.

15. Rat neuronal cultures.

For electrophysiology, isolated hippocampal neurons were plated on astrocyte micro-islands (Bekkers and Stevens, 1991). In short, hippocampi were dissected from embryonic day 18 (E18) Wistar rat embryos (Janvier Labs) of either sex and collected in Hanks Buffered Salts Solution (HBSS; Sigma), buffered with 7 mM HEPES. After removal of the meninges, hippocampi were minced and incubated for 15-20 minutes in 0.25 % trypsin in HBSS at 37 °C. After washing the neurons were triturated, counted and plated in Neurobasal (E18 neurons) medium (Invitrogen, Carlsbad, USA) supplemented with 2 % B-27 (Invitrogen), 1.8 % HEPES, 1 % glutamax (Invitrogen), 1 % Pen/Strep (Invitrogen) and 0.2 % β -mercaptoethanol. Neurons were plated at 2500 cells/cm² on micro islands of mouse glia. Glial islands were obtained by first coating glass coverslips with 0.15 % agarose. After drying and UV sterilization custom-made rubber stamps were used to print dots (islands, diameter 200-250 μ m) using a substrate mixture containing 0.25 mg/ml rat tail collagen and 0.4 mg/ml poly-D-lysine dissolved in 17 mM acetic acid; glial cells were plated at 4800 cells/cm². Neurons were transduced (DIV 5) with bicistronic AAV virus co-expressing Tau and GFP (to indicate transduced neurons), or transduced with control AAV virus expressing GFP alone. For competition experiments, transduced neurons expressing Tau_P301L were pretreated with 5 μ M Tat-NT^{Tau} or Tat-mCherry peptide for four hours before recording.

16. Electrophysiological Recordings of rat neurons.

Isolated neurons from Wistar rat embryos were recorded on DIV 11-13. The patch pipette solution contained (in mM): 136 KCl, 18 HEPES, 4 Na-ATP, 4.6 MgCl₂, 4 K₂-ATP, 15 Creatine Phosphate, 1 EGTA and 50 U/mL Phosphocreatine Kinase (300 mOsm, pH 7.30). The external medium used contained the following components (in mM): 140 NaCl, 2.4 KCl, 4 CaCl₂, 4 MgCl₂, 10 HEPES, 10 Glucose (300 mOsm, pH 7.30). Cells were whole-cell voltage clamped at -70 mV with a double EPC-10 amplifier (HEKA Elektronik, Lambrecht/Pfalz, Germany) under control of Patchmaster v2x32 software (HEKA Elektronik). Currents were low-pass filtered at 3 kHz and stored at 20 kHz. Pipette resistance ranged from 3 to 5 MΩ. The series resistance was compensated to 85 %. Only cells with series resistances below 15 MΩ were included for analysis. All recordings were made at room temperature. Spontaneous glutamatergic release (mEPSCs) was recorded at -70 mV while brief depolarization steps of the cell soma (from -70 to 0 mV for 1 ms) were used to initiate action potential dependent glutamatergic release (eEPSCs).

17. Adeno-associated Viral Vectors.

The cDNA of HA tagged wild type or P301L mutant human Tau (0N4R) was subcloned into a bicistronic adeno-associated viral serotype 6 (AAV6) vector containing eGFP cDNA. The coexpression of eGFP and HA-Tau was driven by a human synapsin-1 promoter (Genscript, Piscataway, NJ, USA). Recombinant AAV vectors were produced by S. Kügler as described previously (Taschenberger et al., 2013). Briefly, serotype 6 AAV vectors were propagated in HEK293 cells, purified by iodixanol step gradient ultracentrifugation and heparin affinity FPLC, followed by extensive dialysis against PBS. Genome copies were determined by quantitative real time PCR and purity > 99% by SDS gel electrophoresis and silver staining.

18. Statistics.

The results are shown as average $\pm$ s.e.m. Statistical testing was performed using GraphPad and SigmaPlot 12.3 (Systat Software Inc), Student t-test, One-Way ANOVA and Two-Way ANOVA. In figures, the significance levels are indicated by asterisks; * p<0.05; ** p<0.01; *** p<0.001.

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35 P301S tauopathy mouse model. *Neuron* 53, 337-351.

CLAIMS

1. A method for producing a compound that prevents the Tau-mediated early synaptic dysfunction comprising the steps of:
 - a) providing an *in vitro* system comprising N-terminal Tau protein domain as depicted in SEQ ID NO: 1 or a homologue with at least 65 % amino acid identity thereof, and synaptic vesicles,
 - b) administering a test compound to said *in vitro* system,
 - c) monitoring said N-terminal Tau protein domain binding to synaptic vesicles in said *in vitro* system, wherein, as compared to the binding under the same test conditions in the same system without the test compound, a reduction in the N-terminal Tau protein binding to synaptic vesicles identifies said test compound as a compound that prevents the Tau-mediated early synaptic dysfunction.
2. A method according to claim 1 wherein said *in vitro* system comprises immobilized synaptic vesicles using molecules with specificity for protein present on said synaptic vesicles and a buffer condition suitable to perform an immune-based assay.
3. A method according to claim 1 wherein said *in vitro* system comprises a buffer condition suitable to perform a vesicle sedimentation assay.
4. A method according to claim 1 wherein the *in vitro* system is a neuronal cell.
5. A method according to any one of claims 1 to 4 wherein said produced compound is a candidate for lead optimization preventing or rescuing early synaptic dysfunction in neuronal cells.
6. A method according to any one of claims 1 and 3 wherein said monitoring of the N-terminal Tau protein domain binding to the synaptic vesicles comprises a vesicle sedimentation assay followed by immune-based detection.
7. A method according to any one of claims 1 to 6 wherein said monitoring of the N-terminal Tau protein domain binding to synaptic vesicles comprises an immune-based assay.
8. A polypeptide inhibiting the binding of N-terminal Tau protein domain to synaptic vesicles
9. The polypeptide of claim 8, wherein said polypeptide comprises SEQ ID NO: 1 or a homologue with at least 65 % amino acid identity thereof and a cell penetrant carrier.
10. The polypeptide of claim 9, for use as a medicament.
11. The polypeptide of claim 9, for treatment of a Tauopathy, wherein said treatment prevents or reduces early synaptic pathology.
12. The polypeptide of claim 11, wherein said Tauopathy is Alzheimer's disease.
13. The polypeptide of claim 8, wherein said polypeptide comprises an antibody
14. The polypeptide of claim 13, wherein said antibody specifically binds an epitope within SEQ ID NO:1 or a homologue with at least 65 % amino acid identity thereof.

Figure 1

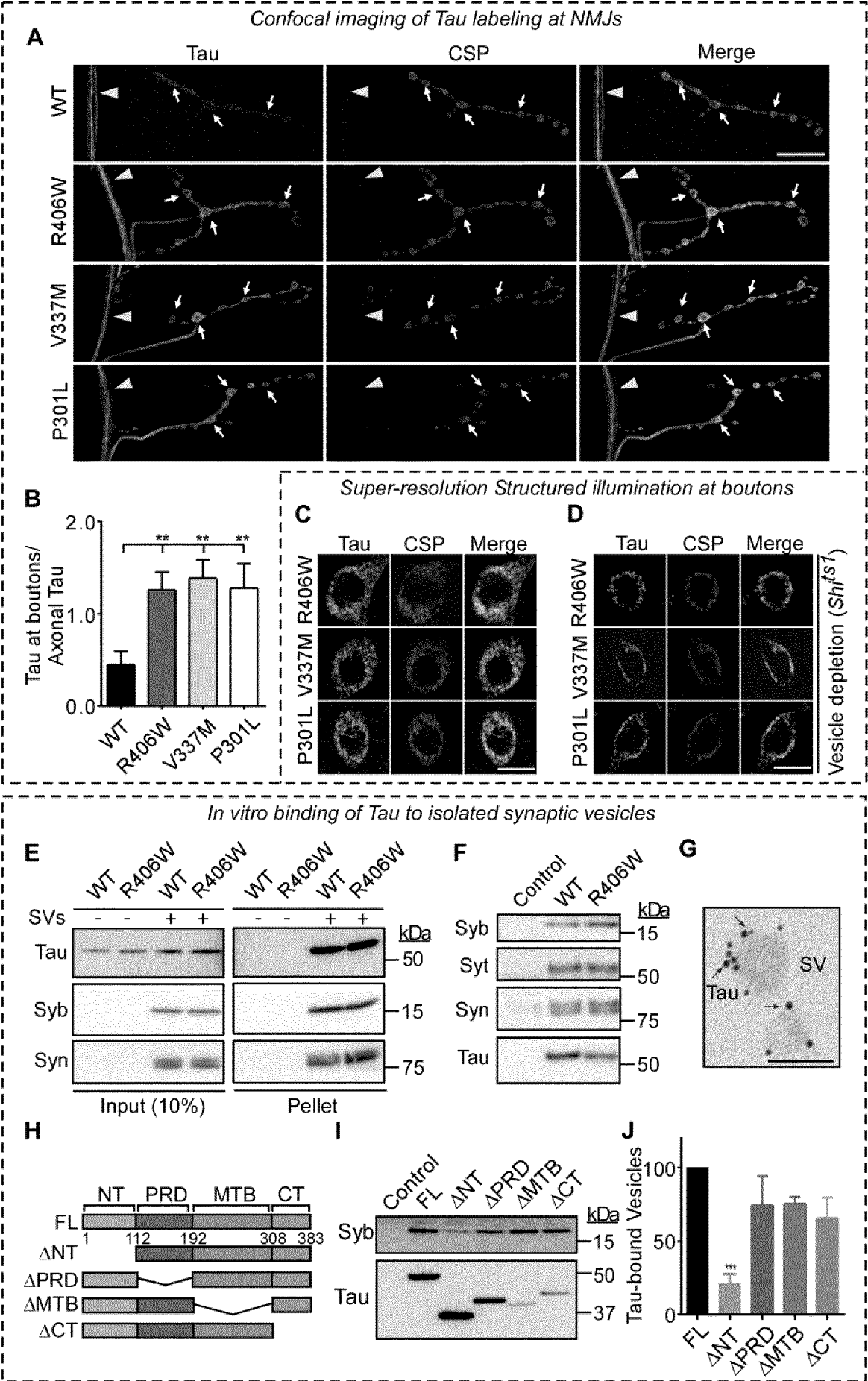


Figure 2

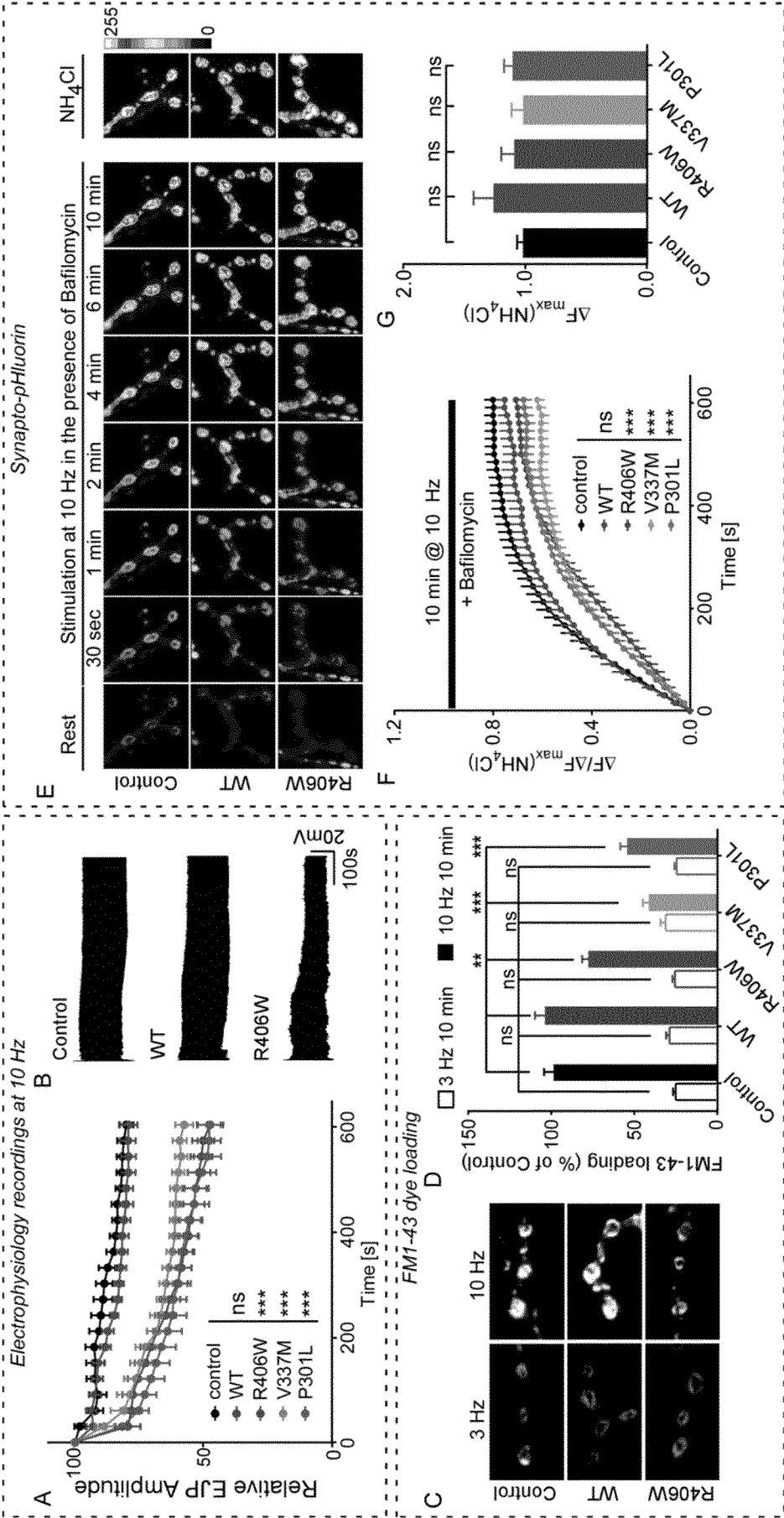


Figure 3

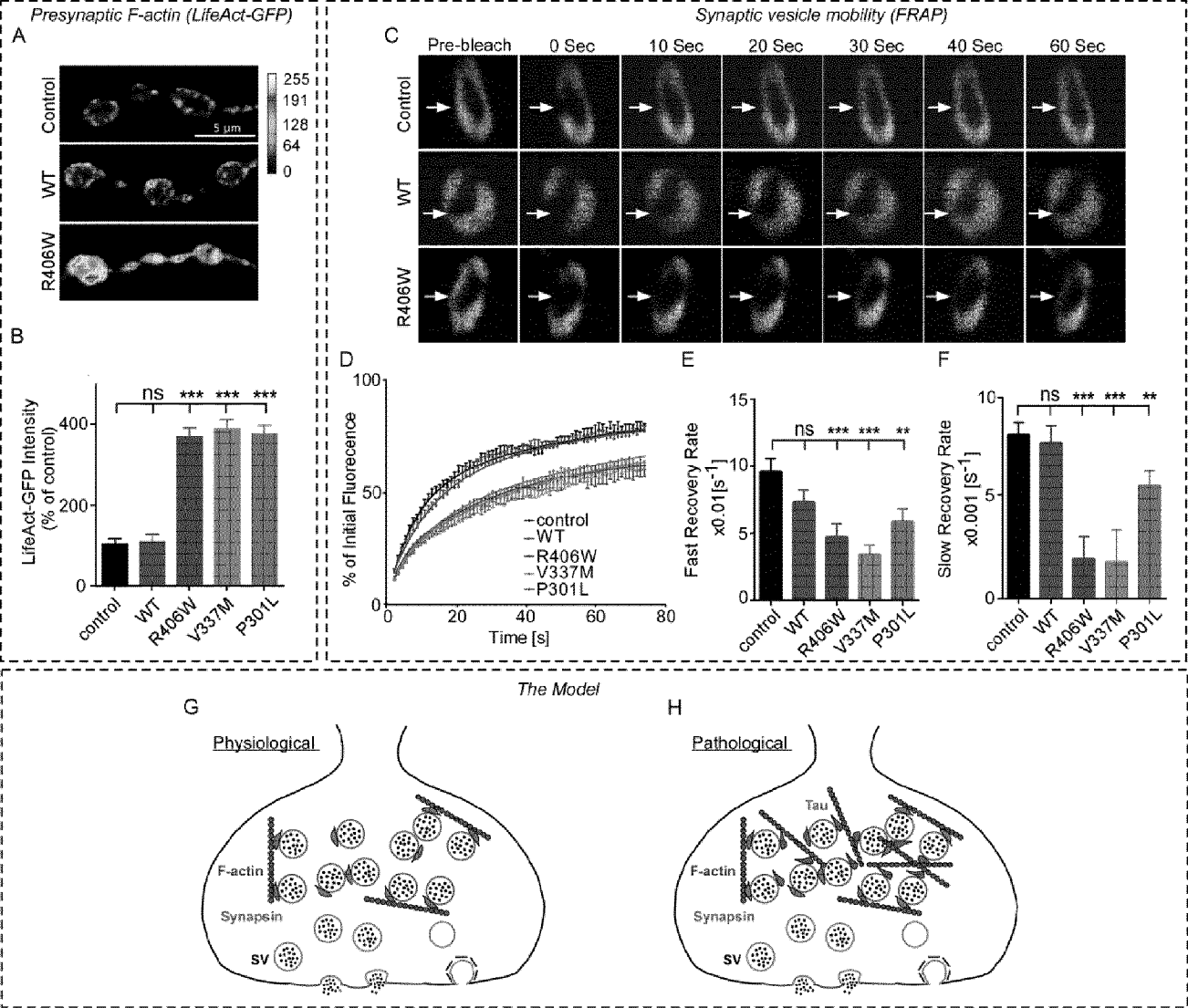


Figure 4

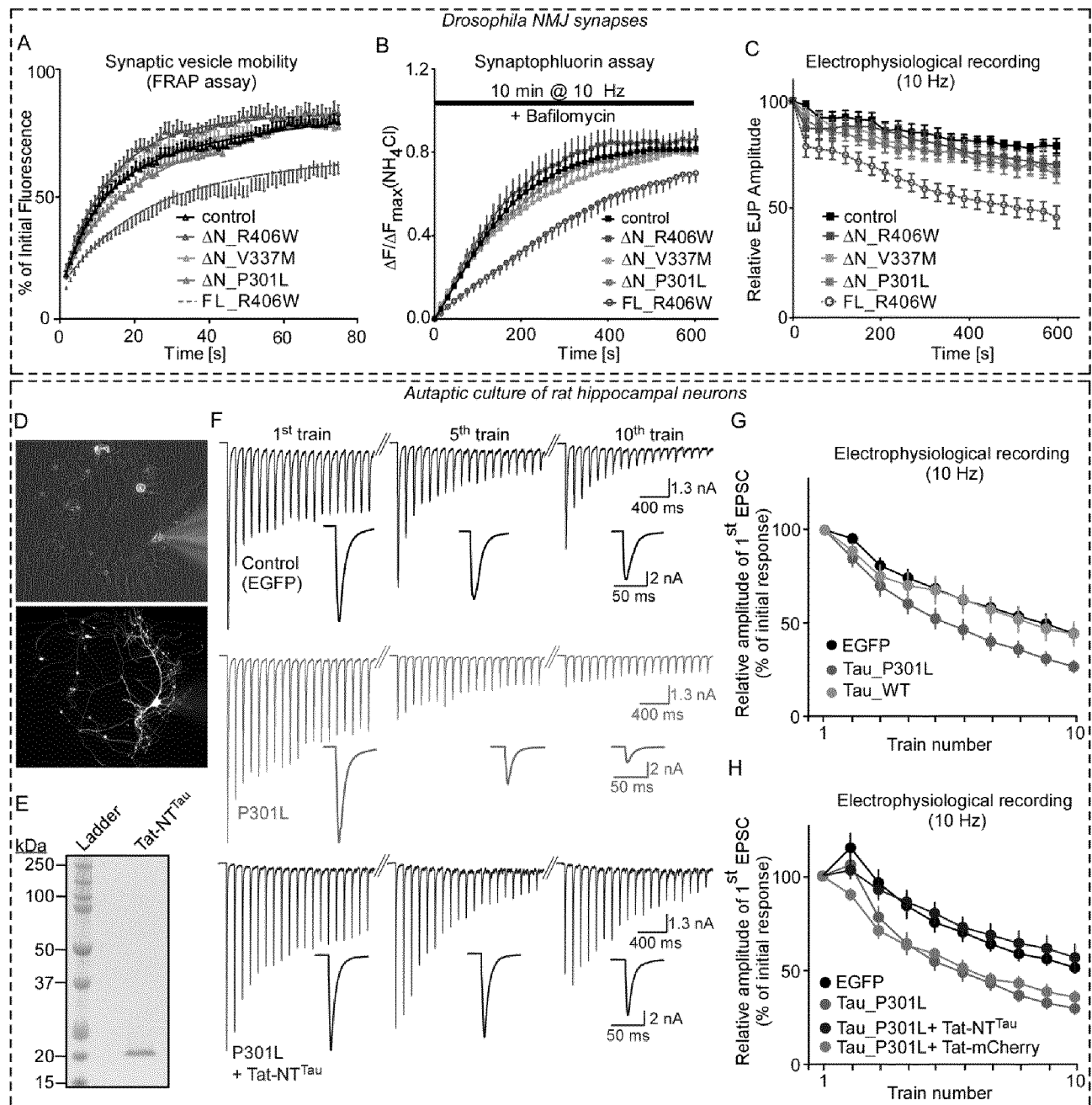


Figure 5

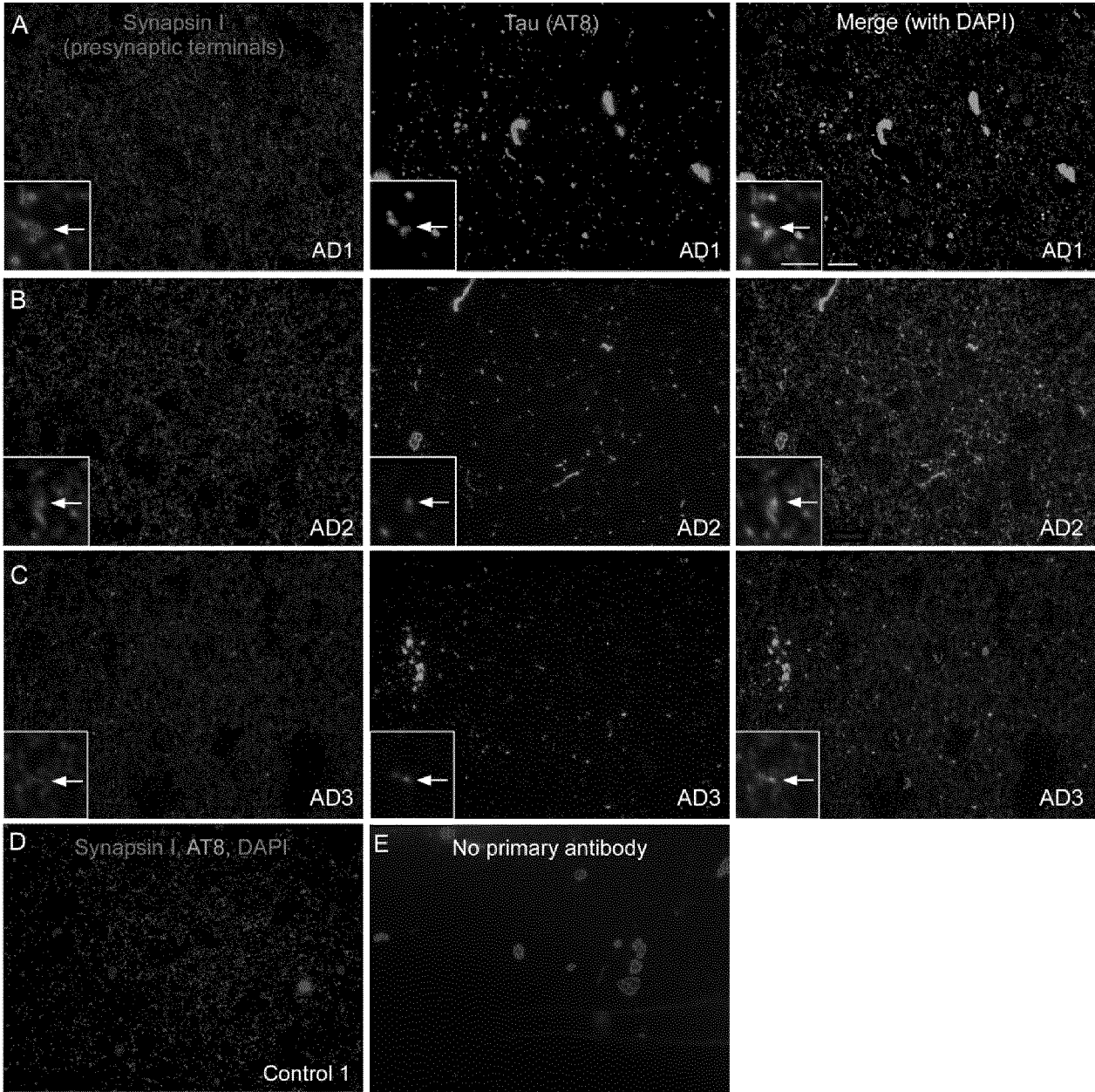


Figure 6

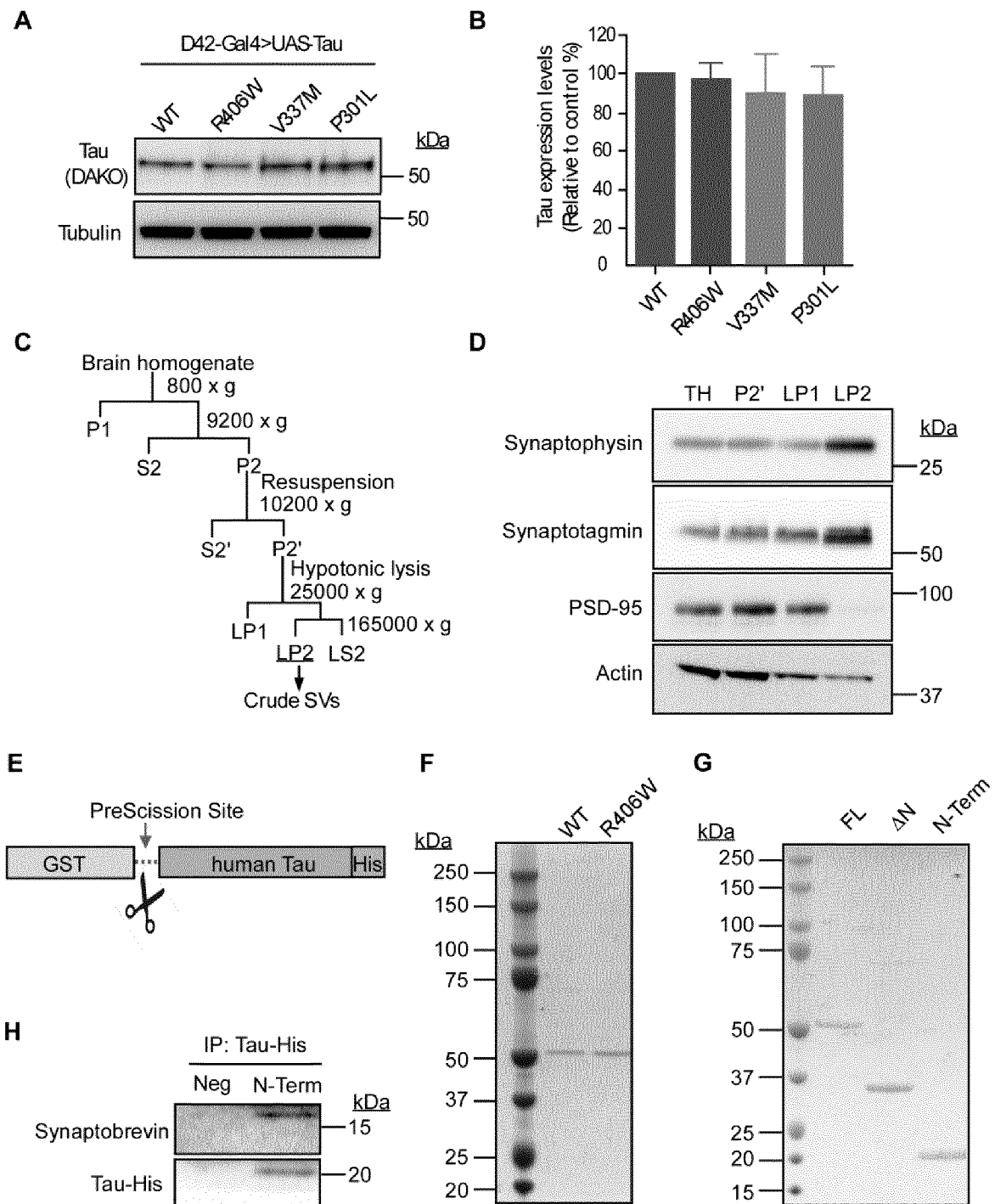


Figure 7

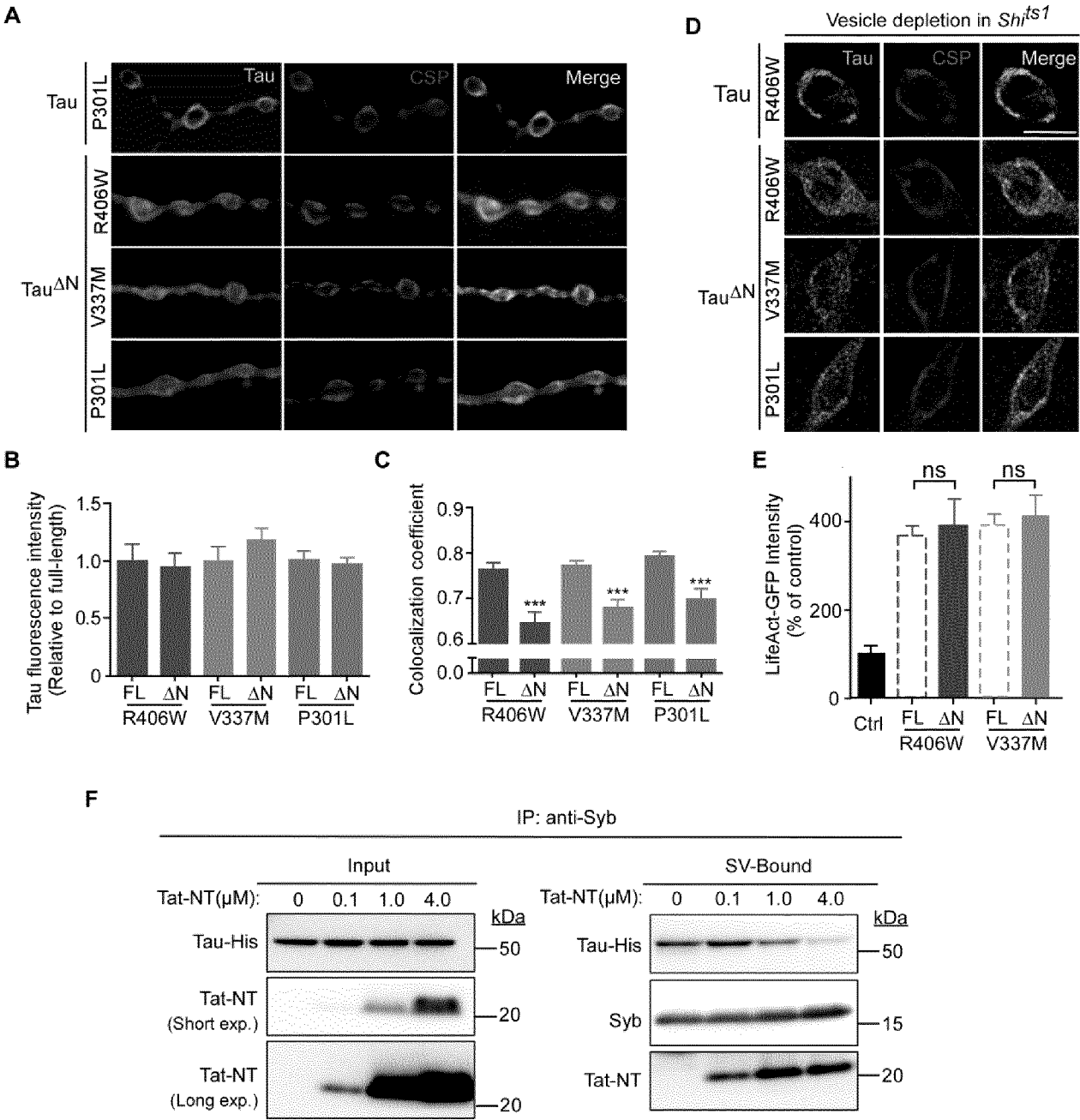
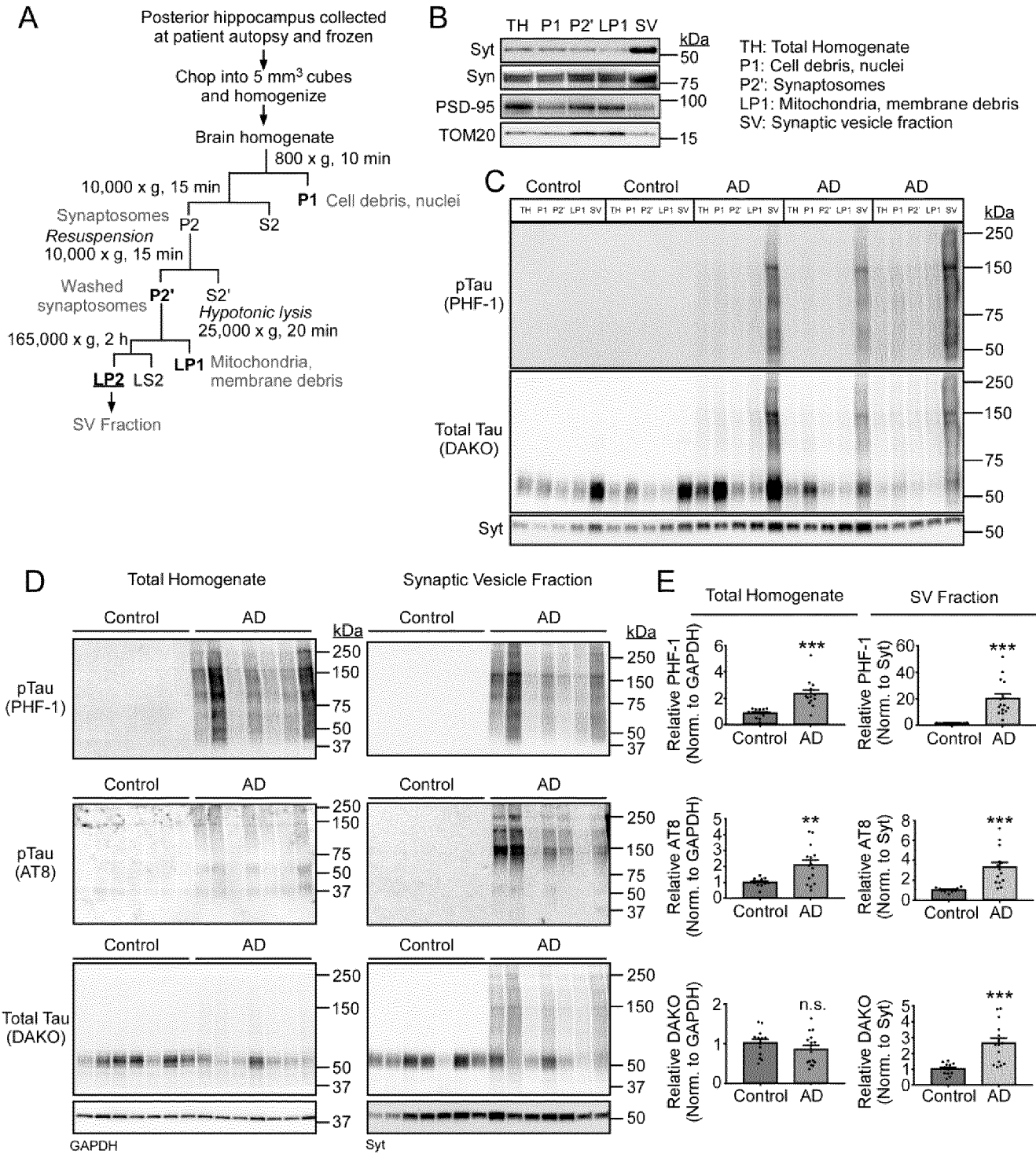


Figure 8



INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2017/061155

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K38/00 A61K39/00 G01N33/68
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, COMPENDEX, EMBASE, FSTA, INSPEC, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X A	WO 2015/122922 A1 (IPIERIAN INC [US]) 20 August 2015 (2015-08-20) SEQ ID NO : 73 in example 8 and figure 14A; example 9, table 2, figure 1A; clones 7299-M2, -M5 and -M9 of table 3 on page 149.	8,13,14 1-7,9-12
X A	US 2014/234214 A1 (GRISWOLD-PRENNER IRENE [US] ET AL) 21 August 2014 (2014-08-21) paragraph [0085]	8,13,14 1-7,9-12
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Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

13 July 2017

Date of mailing of the international search report

22/08/2017

Name and mailing address of the ISA/

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Jacques, Patrice

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2017/061155

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MANUELA POLYDORO ET AL: "Soluble pathological tau in the entorhinal cortex leads to presynaptic deficits in an early Alzheimer's disease model", ACTA NEUROPATHOLOGICA., vol. 127, no. 2, 24 November 2013 (2013-11-24), pages 257-270, XP055390328, BERLIN, DE ISSN: 0001-6322, DOI: 10.1007/s00401-013-1215-5 the whole document -----	1-14
A	BRIAN R. HOOVER ET AL: "Tau Mislocalization to Dendritic Spines Mediates Synaptic Dysfunction Independently of Neurodegeneration", NEURON, vol. 68, no. 6, 1 December 2010 (2010-12-01), pages 1067-1081, XP055390329, US ISSN: 0896-6273, DOI: 10.1016/j.neuron.2010.11.030 the whole document -----	1-14
A	CRISTIAN A LASAGNA-REEVES ET AL: "Tau oligomers impair memory and induce synaptic and mitochondrial dysfunction in wild-type mice", MOLECULAR NEURODEGENERATION, BIOMED CENTRAL LTD, LO, vol. 6, no. 1, 6 June 2011 (2011-06-06), page 39, XP021104126, ISSN: 1750-1326, DOI: 10.1186/1750-1326-6-39 the whole document -----	1-14
T	LUJIA ZHOU ET AL: "Tau association with synaptic vesicles causes presynaptic dysfunction", NATURE COMMUNICATIONS, vol. 8, 11 May 2017 (2017-05-11), page 15295, XP055390309, DOI: 10.1038/ncomms15295 the whole document -----	1-14

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2017/061155

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2015122922 A1	20-08-2015	CN 105939722 A	14-09-2016
		EP 3104870 A1	21-12-2016
		JP 2017512751 A	25-05-2017
		US 2016347804 A1	01-12-2016
		WO 2015122922 A1	20-08-2015

US 2014234214 A1	21-08-2014	US 2014234214 A1	21-08-2014
		US 2014294831 A1	02-10-2014
		US 2015050281 A1	19-02-2015
		US 2015232544 A1	20-08-2015
		US 2016355578 A1	08-12-2016
