Passive immunotherapy for N-truncated tau ameliorates the cognitive deficits in two mouse Alzheimer's disease models

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ABSTRACT

Clinical and neuropathological studies have shown that tau pathology better correlates with the severity of dementia than amyloid plaque burden, making tau an attractive target for the cure of Alzheimer's disease. We have explored whether passive immunization with the 12A12 monoclonal antibody (26-36aa of tau protein) could improve the Alzheimer's disease phenotype of two well-established mouse models, Tg2576 and 3xTg mice. 12A12 is a cleavage-specific monoclonal antibody which selectively binds the pathologically-relevant neurotoxic $NH_2$26-230 fragment (i.e. NH_2htau) of tau protein without cross-reacting with its full-length physiological form(s). We found out that intravenous administration of 12A12 monoclonal antibody into symptomatic (6-month-old) animals: (i) reaches the hippocampus in its biologically-active (antigen binding-competent) form and successfully neutralizes its target; (ii) reduces both pathological tau and Amyloid Precursor Protein/Amyloidβ metabolisms involved in early disease-associated synaptic deterioration; (iii) improves episodic-like type of learning/memory skills in hippocampal-based Novel Object Recognition and Object Place Recognition behavioural tasks; (iv) restores the specific upregulation of the Activity-regulated cytoskeleton-associated protein involved in consolidation of experience-dependent synaptic plasticity; (v) relieves the loss of dendritic spine connectivity in pyramidal hippocampal CA1 neurons; (vi) rescues the Alzheimer's disease-related electrophysiological deficits in hippocampal Long Term Potentiation at the CA3-CA1 synapses; (vii) mitigates the neuroinflammatory response (reactive gliosis). These findings indicate that the 20-22kDa NH_2-terminal tau fragment is crucial target for Alzheimer's disease therapy and prospect immunotherapy with 12A12 monoclonal antibody as safe (normal tau-preserving), beneficial approach in contrasting the early Amyloidβ-dependent and independent neuropathological and cognitive alterations in affected subjects.

Keywords: tau protein; tauopathies; Alzheimer's disease; tau cleavage; immunotherapy

Running title: N-truncated tau as target in AD therapy

Abbreviations: Alzheimer's Disease (AD); Amyloid Precursor Protein (APP); Amyloid-β peptides (Aβ); Long-Term Potentiation (LTP); Sodium Dodecyl Sulfate-PolyAcrylamide Gel Electrophoresis (SDS-PAGE); Staurosporin (STS); Amyloid precursor protein KM670/671NL Swedish mutation (APPSwe); induced Pluripotent Stem Cell (iPSC); CerebroSpinal Fluid (CSF); Transgenic (Tg); Wild-type (Wt).

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INTRODUCTION

Recent *in vitro* and *in vivo* data have highlighted a crucial role of proteolytic fragments of tau protein, in particular those derived from truncation at its N-terminal domain, in the initiation/progression of AD and other related tauopathies, thus paving the way for their potential use as therapeutic targets or as biomarkers for diagnosing dementia and/or monitoring disease progression (Avila et al., 2016a; Sebastián-Serrano et al., 2018; Quinn et al., 2018). On one hand, tau cleavage may generate amyloidogenic fragments that initiate its aggregation which, in turn, can cause toxicity (Wang et al., 2010). On the other hand, tau proteolysis may result in production of noxious, both intracellular and extracellular, truncated species which drive neurodegeneration independently of aggregative pathway(s) and in a fragment-dependent manner as a result of their deleterious action on pre- and/or post-synaptic functions and/or their secretion and transcellular propagation (Quinn et al., 2018).

Extracellular cleaved tau is toxic to neurons by increasing the Aβ production (Bright et al., 2015) and/or by impairing synaptic plasticity (Florenzano et al., 2017; Borreca et al., 2018; Fà et al., 2016; Hu et al., 2018). Hyperphosphorylation and caspase-3 cleavage of tau (Asp421), which promote aggregation, also favor the protein secretion *in vitro* (Plouffe et al., 2012). The amino-terminal projection domain of human tau -which interacts with the plasma membrane (Brandt et al., 1995) and undergoes early conformational changes in human tauopathies including AD (Combs et al., 2016, 2017)- is endowed with deleterious action(s), mainly at nerve endings (Ittner et al., 2010; King et al., 2006; Amadoro et al., 2012; Zhou et al., 2017). The N-terminus extremity of tau, despite the lack of the microtubule binding domains which abnormally aggregate to form Paired Helical Filaments (PHFs), is prone to come into higher order of oligomerization (Feinstein et al., 2016) and is specifically released into the extracellular space in an *in situ* tauopathy model (Kim et al., 2010), suggesting a potential role for molecular "templating" in the propagation of neurofibrillary lesions. Soluble C-terminally truncated tau species are also preferentially secreted from synaptosomes of AD brains (Sokolow et al., 2015) and in conditioned media from patient-derived induced pluripotent stem cell (iPSC) cortical neurons (Bright et al., 2015; Kanmert et al., 2015; Sato et al., 2018). Interestingly, although full- length tau is found in Cerebral Spinal Fluid (CSF) from healthy humans, a heterogeneous population of fragments -including the NH_2-terminal and/or prolin-rich domain- is mainly discernible in AD patients (Meredith et al., 2013; Johnson et al., 1997; Portelius et al., 2008; Amadoro et al., 2014; Cicognola et al., 2019; Chen et al., 2019). Exosomes-associated NH_2-derived tau fragments are also detected in CSF from AD patients (Saman et al., 2012) and a different CSF pattern of NH_2-derived tau fragments may reflect disease-specific

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neurodegenerative processes (Borroni et al., 2009). Consistently, passive immunotherapy with antibody targeting the N-terminal projection domain of full-length human tau has shown to be beneficial in AD transgenic mice by improving the cognitive deficits (Yanamandra et al., 2013; Dai et al., 2015; Subramanian et al., 2017) and blocking the seeding/spreading of tau pathology (Dai et al., 2018). Both intracerebroventricular (i.c.v.) infusion and peripheral administration of anti-tau antibodies specific for N-terminal 25-30 epitopes are curative in P301S mice model of tauopathy, by preventing the brain atrophy and ameliorating the motor/sensorimotor functions (Yanamandra et al., 2013, 2015). Immunization with antibody directed against the N-terminal end of full-length tau protein (Dai et al., 2017) significantly reduced the level of amyloid precursor protein (APP), Aβ40 and Aβ42 in CA1 region of AD animal models, indicating that tau-based immunotherapy is actually able to restore the Aβ-dependent and/or independent synaptic dysfunction(s) which occur at early stages in AD and other related dementias (Panza et al., 2016; Pedersen and Sigurdsson, 2015). However, albeit tau appears to be the main factor underlying the development and progression of AD (Kametani and Hasegawa et al., 2018; Castellani and Perry, 2019), its expression at physiological level is required for neuronal functions underlying learning and memory (Pooler et al., 2014; Regan et al., 2017) and its downregulation, even if moderate, has been proved to have deleterious effects, both *in vitro* and *in vivo* (Biundo et al., 2018; Velazquez et al., 2018). As a consequence, the development of selectively-targeting antibodies against pathogenic tau may have a unique therapeutic advantage by leading to valuable, beneficial effects -in the absence of unwanted consequences due to the "loss of function" of normal protein- in the cure of human, chronic neurodegenerative tauopathies which are sometimes expected to require long-term treatments with multiple and high dose administrations of drugs (Elmaleh et al., 2019; Kontsekova et al., 2014).

In this framework, we developed a neo-epitope antibody directed against the N-terminal sequence of human tau protein DRKD($_{25}$)-QGGYTMHQDQE (Amadoro et al., 2012) which encompasses a conserved cleavage-site sequence previously identified in cellular and animal AD models (Corsetti et al., 2008) and in human AD brains (Rohn et al., 2002). 12A12 (formerly Caspase-Cleaved protein-$NH_2$4268 tau antiserum, Amadoro et al., 2012) is a monoclonal antibody (mAb) which recognizes the newly-created Δ-$_{25}NH_2$tau(Q26-36aa)-terminus of degradation product(s) of tau without cross-reacting with the same aminoacidic stretch from full-length isoforms of intact, normal protein (Amadoro et al., 2019; Suppl.Fig.1). The pathologically-relevant NH_2tau 26-44aa stretch, which is the minimal active moiety of a neurotoxic 20-22kDa NH_2-derived tau peptide (aka NH_2htau), accumulates at AD presynaptic terminals (Amadoro et al., 2006, 2010, 2012; Corsetti et al., 2015) and is present in CSFs from living patients suffering from AD and other non-AD neurodegenerative diseases (Amadoro et al., 2014). Interestingly, this peptide is able to negatively

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impact on normal synaptic function(s) *in vitro* (Florenzano et al., 2017) and *in vivo* (Borreca et al., 2018), suggesting that its antibody-mediated selective clearance can have important clinical and translational implications in contrasting the earliest neuropathological and cognitive alterations associated with human tauopathies, including AD (Cicognola et al., 2019; Sokolow et al., 2015; Barthélemy et al., 2016a-b; Bright et al., 2015; Sato et al., 2018).

In this study, we explored the potentially-beneficial immunotherapeutic power of the 12A12mAb by means of its intravenous (i.v.) administration in two lines of AD transgenic animals with different genetic backgrounds, such as Tg2576 carrying the APP KM670/671NL Swedish mutation and 3xTg mice expressing the APPSWE, tauP301L, PS1M146V human transgenes. Relevantly, unlike other murine or humanized NH_2tau-directed immunotherapeutic antibodies (Dai et al., 2015, 2017, 2018; Yanamandra et al., 2013, 2015; Subramanian et al., 2017; Qureshi et al., 2018), 12A12mAb reacts with the 20-22kDa neurotoxic NH_2-truncated tau but not with the physiological full-length form of protein (Amadoro et al., 2012; Corsetti et al., 2008) advocating its *in vivo* use as safe, more harmless and personalized medicine treatment to slow progressing human tauopathies.

MATERIALS AND METHODS

Animals

All experiments involving animals were performed in accordance with the ARRIVE guidelines and were carried out in accordance with the ethical guidelines of the European Council Directive (2010/63/EU); experimental approval was obtained from the Italian Ministry of Health (protocol # 524/2017 PR; 554/2016-PR). Only male subjects were used to avoid changes in female hormone state that can affect cognitive data. All efforts were made to minimize the number of animals used and suffering.

One, 3- and 6-month-old, Tg2576 and 3xTg mice (Tg-AD) (n=8-10 per group/treatment) and age-matched wild-type (WT) controls (n=8-10 per group/treatment) were used in this study.

Heterozygous Tg2576 mice overexpressing the APP695 with the Swedish mutation (APP KM670/671NL, TgHuAPP695swe: Tg2576) in a hybrid genetic background (87% C57BL/6×12.5% SJL) (Hsiao et al., 1996) were subsequently backcrossed to C57BL/6xSJL F1 females and the offsprings were genotyped to confirm the presence of human mutant APP DNA sequence by PCR. Wild-type (WT) littermates were used as controls.

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The homozygous 3xTg mice harboring human APPSWE and tauP301L transgenes with knock-in PS1M146V under the control of the mouse Thy1.2 promoter were obtained from The Jackson Laboratory (https://www.jax.org/strain/004807). Mice were bred on the mixed C7BL/6;129X1/SvJ;129S1/Sv genetic background and genotypes were confirmed by PCR on tail biopsies (Oddo et al., 2003). B6129SF2/J strain mice, used as wild-type (WT) controls in the present study, were the offspring of a cross between C57BL/6J females (B6) and 129S1/SvImJ males (129S); they are commonly used as controls for genetically engineered strains generated with 129-derived embryonic stem cells and maintained on a mixed B6;129 background (https://www.jax.org/strain/101045). The housing conditions (four or five animals per cage) in pathogen-free facilities were controlled (temperature 22°C, 12 hrs light/12 hrs dark cycles, humidity 50%–60%) with *ad libitum* access to chow and water.

Immunization scheme

The N-terminal tau 12A12 antibody (26-36aa) was produced and characterized by Monoclonal Antibodies Core Facility (MACF) at EMBL- Monterotondo, Rome, Italy (Dott. Alan Sawyer), as previously described in Florenzano et al., 2017. 12A12mAb was purified from hybridoma supernatants according to standard procedures and its purity was determined using SDS-PAGE and Coomassie staining. In detail, the hybridoma supernatant was precipitated by ammonium sulfate (336g/l). After precipitation, the solution was centrifuged at 10 000g for 1 hr and the pellet was dissolved in PBS and dialyzed against the same buffer. The solution was centrifuged at 10 000g for 30 min and loaded on a HiTrap Protein G HP (GE Healthcare) equilibrated with PBS. The column was washed with PBS (5 column volumes). 121A12mAb was eluted with 0.1 M Glycine-HCl, pH 2.7. The fractions containing the antibody were neutralized by 1 M Tris-HCl, pH 9.0, collected and immediately dialyzed against PBS. 121A12mAb concentration was determined by measuring the absorbance at 280 nm. The average yield was 8 mg per liter of cell supernatant. 12A12mAb was ≥95% pure and contained ≤1 U/mg of endotoxin (LAL Chromogenic Endotoxin quantitation kit; Thermo Scientific).

To minimize experimental variability, all mice were initially grouped according to their body weight and age and mice from the same litter were finally assigned to different groups. For each animal strain (Tg2576, 3xTg), the grouped mice were randomized into: (1) wild-type mice treated with saline vehicle; (2) wild-type mice treated with 12A12mAb (30µg/dose); (3) age-matched Tg-AD mice treated with saline or nonspecific mouse IgG (normal mouse IgG, Santa Cruz sc-2025,

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30μg/dose); (4) age-matched Tg-AD mice treated with 12A12mAb (30μg/dose) or nonspecific mouse IgG (normal mouse IgG, Santa Cruz sc-2025, 30μg/dose). Animals were infused over 14 days with two weekly injections administered on two alternate days to the lateral vein of the tail. The dose and route of immunization were based on prior studies using AD transgenic mice (Castillo-Carranza et al., 2015). In details, mice were placed in a restrainer (Braintree Scientific), and an inch of the tail was shaved and placed in warm water to dilate veins. After injection via the lateral tail vein, mice were returned to home cages and kept under general observation. Abnormalities in overall health, home cage nesting, sleeping, feeding, grooming, body weight and condition of the fur of animals were noted.

Tissue collection, harvesting and preparation

For biochemical analysis, tissue sampling was carried out according to Mably et al., 2015 with some modifications. Briefly, two days following the last injection, animals were sacrificed by cervical dislocation to avoid anesthesia-mediated tau phosphorylation (Planel et al., 2007) and intracardially perfused with ice-cold phosphate-buffered-saline (PBS) using a 30ml syringe to remove blood contamination. Brains were collected, the meninges were carefully removed and dissected hippocampi were immediately frozen on dry-ice and, then, stored at -80°C until use.

Hippocampal total protein lysates were carried out according to Castillo-Carranza et al., 2015 with some modifications. In details, frozen mice hippocampi were diced and homogenized in phosphate buffered saline with a protease inhibitor mixture (Roche) and 0.02% NaN_3 using a 1:3 (w/v) dilution. Samples were then centrifuged at 10 000rpm for 10 min at 4°C and the supernatants were collected.

TBS extracts were carried out according to Mably et al., 2015 with some modifications. Frozen mice hippocampi were homogenized in 5 volumes (wt/vol) Tris-buffered saline (TBS), pH 7.4, plus proteases inhibitor cocktail (Sigma-Aldrich P8340) and phosphatase inhibitor cocktail (Sigma-Aldrich, Oakville, Ontario, Canada P5726/P2850) with 30 strokes of a glass Dounce tissue. The homogenate was centrifuged at 90 000g at 4°C for 1 hr. The supernatant (TBS extract) was removed and stored at -20°C.

Synaptosomes preparations were carried out as previously reported (Corsetti et al., 2015; Florenzano et al., 2017).

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Cloning, bacterial expression and purification of the 20-22kDa $NH_2$26-230 tau fragment (aka NH_2htau)

cDNA fragment coding for the aminoacids 26-230 of the isoform 4 of human tau protein (htau40) was cloned into the vector pET-11a (Novagen) suitable for the expression of recombinant proteins in BL21DE3 Gold E. coli cells. After induction with IPTG, recombinant protein in lysates from bacterial pellet was purified to homogeneity by a two-step procedure: step 1 was a HiCood Q Sepharose 16/10; step 2 was Hitrap Phenyl 5ml. Degree of protein purification was evaluated by Coomassie Brilliant Blue G-250 and checked by SDS-PAGE under reducing conditions by Western blotting with commercial human-specific NH_2-tau antibody (DC39N1 45-73aa) and with 12A12mAb (26-36aa). The molecular identity of purified peptide fraction was finally checked by electrospray ionization mass spectrometry (ESI-MS).

Detection of the NH_2htau fragment by 12A12mAb-based Enzyme-linked immunosorbent assay (ELISA)

High binding black 96 well plates (Costar 3925, Corning, NY) were coated overnight at 4°C following the addition of 5 µg/ml 12A12mAb capture antibody diluted in coating buffer (50mM $NaHCO_3$, pH9.6). Plates were washed with PBST (PBS containing 0.05% Tween-20) and incubated with 5% non-fat dry milk (w/v) in PBST at room temperature (RT) for 2-4 hrs while shaking to block nonspecific binding sites. Plates were washed with PBST and incubated (50 µl/well) overnight at 4°C while shaking with recombinant $NH_2$26-230 tau fragment standard curves prepared in assay buffer concentration of 5% milk (w/v) and 0.05% Tween-20 (v/v) in Phosphate Buffered Saline (PBS), pH 8. Plates were washed with PBST and incubated (50 µl/well) overnight at 4°C with rabbit H150 antibody (1-150aa; Santa- Cruz sc-5587) diluted to the final concentration of 2.5µg/ml in assay buffer concentration of 5% milk and 0.05% Tween-20 (v/v) in PBS. Plates were then washed with PBST and added with 50 µl/well of rabbit Horseradish Peroxidase (HRP)-conjugated secondary antibody for 1hr at RT. Plates were washed with PBST and developed at room temperature (RT) using TMB substrate (T0440; Sigma-Aldrich, Oakville, Ontario, Canada). Luminescence counts were measured using Packard TopCount (PerkinElmer, MA). Log-transformed luminescence counts from individual samples were interpolated to concentration using a second-order polynomial fit to the respective standards (GraphPad Prism 5.00, GraphPad Software, San Diego).

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Detection of i.v.-delivered 12A12mAb in TBS brain extracts

The concentration of i.v. delivered anti-tau 12A12mAb was measured in TBS brain extracts according to Mably et al., 2015 with some modifications. A solid-phase Enzyme-Linked Immunosorbent Assay (ELISA) was performed on the plate-immobilized synthetic $NH_2$26-44aa which was used as catching antigenic peptide, being the minimal AD-relevant (Borreca et al., 2018), active moiety of the parental longer $NH_2$26-230 (Amadoro et al., 2004, 2006). Clear 96 well high-binding plates (Costar 3925, Corning, NY) were coated (50 µl/well) of 5 µg/ml synthetic $NH_2$26-44aa in coating buffer (0.05M Carbonate-Bicarbonate, pH9.6) overnight at 4°C. Wells were washed twice with PBST and loaded (50 µl/well) with (i) the standard curve prepared by making serial dilutions of 12A12mAb (250-0.12 ng/ml), (ii) the TBS extracts diluted 1/50, 1/10, 1/2, 1/1.3 or (iii) blanks diluted in assay buffer concentration of 5% milk and 0.05% Tween-20 (v/v) in PBS overnight at 4°C. Plates were then washed with PBST and added with 50 µl/well of rabbit Horseradish Peroxidase (HRP)-conjugated secondary antibody for 1hr at RT. Plates were washed with PBST and developed at room temperature (RT) using TMB substrate (T0440; Sigma-Aldrich, Oakville, Ontario, Canada). Luminescence counts were measured using Packard TopCount (PerkinElmer, MA). Log-transformed luminescence counts from individual samples were interpolated to concentration using a second-order polynomial fit to the respective standards (GraphPad Prism 7.00, GraphPad Software, San Diego).

Cell culture, treatment and protein lysates preparation

SH-SY5Y human neuroblastoma cells were cultured and terminally-differentiated into post-mitotic neurons according to Corsetti et al., 2008. Culture treatment and protein lysates preparation were carried out by using standard procedures, according to Borreca et al., 2018.

Western blot analysis and densitometry

Western blot analysis and densitometry were carried out by using standard procedures, according to Borreca et al., 2018.

The following antibodies were used:

anti-Aβ/APP protein 6E10 (4-9aa) mouse MAB1560 Chemicon; anti-Alzheimer precursor protein 22C11 (66-81aa of N-terminus) mouse APP-MAB348 Chemicon Temecula-CA; anti-pan tau protein H150 (1-150aa of N-terminus) rabbit sc-5587 Santa Cruz Biotechnology ; anti-pan tau

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protein DC25(microtubule binding repeat) mouse T8201 Sigma-Aldrich; tau 21 (21-36aa of N-terminus) rabbit AHB0371 Biosource International (U.S.A.); anti-N-tau (45-73 aa) DC39N1 mouse T8451 Sigma-Aldrich; neuronal marker β III tubulin antibody mouse ab78078 (clone 2G10) Abcam; GAPDH antibody (6C5) mouse sc-32233 Santa Cruz Biotechnology; Arc (Activity-regulated cytoskeleton-associated protein) (C-7) mouse sc-17839 Santa Cruz Biotechnology; Glial Fibrillary Acidic Protein (GFAP) antibody rabbit Z0334 Dako; Iba1 antibody rabbit Wako 016-20001 (for WB).

Novel Object Recognition test (NOR)

Two days after the last i.v. injection, mice run the novel object recognition (NOR) task (Antunes and Biala, 2012) to check the hippocampal-dependent episodic memory (Bevins and Besheer, 2006; Akkerman et al., 2012a-b). The entire task was performed in three consecutive sessions during the same day (one-day version), according to previously-published protocol (Borreca et al., 2018).

Object Place Recognition test (OPR)

The object place recognition (OPR) paradigm involves the activity of the hippocampus and is used to test the short-term memory (Vogel-Ciernia and Wood, 2014). The animals, which underwent the NOR paradigm with a training and test session, were tested in the OPR paradigm 24 hrs later, with a separated training and test session. The objects used for the OPR were different from those used previously for the NOR test in order to avoid possible confounding effects. The entire behavioural task including 3 phases (a common habituation phase, a training phase and a test phase) was performed by using standard protocol (Lesburguères et al., 2017).

Spontaneous alternation (Y-maze) test

Evaluation of short-term working memory was carried out by using the spontaneous alternation version of the Y-maze, which involves different brain structures ranging from the hippocampus to the prefrontal cortex. Y-maze testing also indicates overall activity, or hyperactivity, based on the number of arm entries Spontaneous alternation, expressed as a percentage, was calculated by dividing the number of entries into all 3 arms on consecutive choices (correct choices) by number of arm entries subtracted by 2, then multiplying the quotient by 100 (Hiramatsu et al., 1997; Wall and

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Messier, 2002). A high spontaneous alternation rate is indicative of sustained working memory because the animals must remember which arm was entered last to know not to reenter it.

Energy metabolism

Energy expenditure (EE) and oxygen consumption (VO_2), were measured by an indirect calorimeter system (TSE PhenoMaster/LabMaster System®) in vehicle- or 12A12mAb-treated mice by a constant air flow of 0.35 l/min. Mice were adapted for 6 hrs to the metabolic chamber before the start of recording, and VO_2 was measured every 30 min in each mouse, starting at 7:00 PM and ending automatically after 4 days (96 hrs later). Room temperature was kept constant (22°±1°C). The EE for each sample point was evaluated across the 48 hrs of total recording. Locomotor activity was assessed during the indirect calorimetric assay by the number of infrared beams broken. Each cage of the calorimeter system is equipped with the InfraMot® device that uses "passive infrared sensors" to detect and record the motor activity of the mouse by the body-heat image and its spatial displacement across time. Any type of body movement was detected and recorded as activity counts. EE was also analyzed by considering animals' steady conditions or lack of motor activity (resting EE, REE; only values between 0 and 2 activity counts were included).

Golgi-Cox Staining and Dendritic Spine Analysis

Two days after the last i.v. injection, mice were sacrificed with a lethal dose of anesthetic (Zoletil/Rompun 800 mg/kg and 100 mg/Kg, respectively) and perfused transcardially with 0.9% saline solution. Brains were dissected and immediately immersed in a Golgi-Cox solution (1% $K_2Cr_2O_7$, 1% $HgCl_2$, and 0.8% K_2CrO_4) at room temperature for 6 days, according to a previously described protocol (Gibb and Kolb, 1998; Rosoklija et al., 2014). On the seventh day, brains were transferred in a 30% sucrose solution for cryoprotection and then sectioned with a vibratome. Staining and dendritic spine analysis were carried out according to standard criteria (Leuner et al., 2003; Horner and Arbuthnott, 1991) and by using previously-published method (Borreca et al., 2018). Statistical comparisons were made on single mouse values obtained by averaging the number of spines counted on neurons of the same mouse.

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Electrophysiological recordings

Two days after the last i.v. injection of 12A12mAb, mice were anesthetized by halothane or isofluorane inhalation and decapitated. The brain was rapidly removed and put in ice-cold cutting solution (in mM: 124 NaCl, 3.2 KCl, 1 NaH_2PO_4, 26 $NaHCO_3$, 2 $MgCl_2$, 1 $CaCl_2$, 10 glucose, 2 sodium pyruvate and 0.6 ascorbic acid, bubbled with 95% O_2-5% CO_2, pH 7.4). Electrophysiological recordings were performed on hippocampal coronal slices (400 μm thick) by using standard procedures (Podda et al., 2016; Nobili et al., 2017).

Statistical analysis

In box-and-whisker plots the centre lines denoted median values, edges were upper and lower quartiles, whiskers showed minimum and maximum values and points were individual experiments. Other data were expressed as means±standard error of the mean (S.E.M.). All data were representative of at least three separate experiments (n=independent experiments). Statistically significant differences were calculated by one-way or two-way analysis of variance (ANOVA) followed by Bonferroni's, Fisher's and Dunnett's post-hoc tests for multiple comparison among more than two groups. $p<0.05$ was accepted as statistically significant (*$p<0.05$; **$p<0.01$; ***$p<0.0005$; ****$p<0.0001$). All statistical analyses were performed using GraphPad Prism 7 software.

Data availability

The datasets used and/or analyzed during the current study and detailed protocols/experimental procedures are available from the corresponding author on reasonable request. Western blotting fill-size images can be found in Supplementary materials.

RESULTS

Intravenously (i.v.)-injected anti-NH_2htau 12A12mAb is biologically active in the animals' hippocampus.

Tg2576 and 3xTg mice -two well-established animal AD models (Hsiao et al., 1996; Oddo et al., 2003) which express the human APP695 with Swedish mutations (K670N-M671L), alone or in combination with MAPT P301L and PSEN1 M146V respectively- were analyzed because these transgenic animals are recognized to display progressive tau-dependent, hippocampus-based

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synaptic and cognitive impairments (Castillo-Carranza et al., 2015; Oddo et al., 2006; Amar et al., 2017). The hippocampal parenchyma was examined in the present study, since this vulnerable cerebral area: (i) selectively and disproportionally degenerates at early stages of Mild Cognitive Impairment (MCI) prior to the clinical diagnosis of full-blown dementia (West et al., 1994; Honer et al., 1992; Gomez-Isla et al., 1996; Kordower et al., 2001; Scheff et al., 2006 a-b); (ii) preferentially develops tau neuropathology into synaptic compartments, whose initial deterioration is considered the best correlate of cognitive decline in AD symptomatology by critically subserving the transition from normal aging to MCI (Braak and Braak, 1991, Arriagada et al., 1992; Markesbery et al., 2006, 2010; Guillozet et al., 2003; Spires-Jones and Hyman, 2014; Pooler et al., 2014).

Before addressing the possible benefits offered by systemic delivery of the cleavage-specific 12A12mAb (Δ-$_{25}NH_2$tau(Q26-36aa)-terminus), we determined an appropriate lifetime at which Tg-AD mice could be employed for antibody immunization experiments. In light of these considerations, the *in vivo* level of the pathogenic 20-22kDa NH_2htau was measured by Western blotting SDS-PAGE analyses carried out on synaptosomal preparations from hippocampi of wild-type and AD transgenic animals of both genetic backgrounds at three ages (1,3,6-month-old Tg2576 and 3xTg). As shown in Fig. 1A-B, the signal intensity of 12A12mAb-positive NH_2htau immunoreactivity band was virtually undetectable in 6-month-old cognitively-intact controls but appeared to be upregulated in diseased animals (one-way ANOVA followed by Dunnett's post-hoc test for multiple comparisons Tg2576 $F_{(3,12)}=13.34$ $p=0.0004$; 3-months-old Tg2576 *versus* 6-months-old wild-type ***$p<0.0005$, 6-months-old Tg2576 *versus* 6-months-old wild-type **$p<0.01$; 3xTg $F_{(3,12)}=76.79$ $p<0.0001$ 1-month-old 3xTg *versus* 6-months-old wild-type *$p<0.05$, 3-months-old 3xTg *versus* 6-months-old wild-type ****$p<0.0001$, 6-months-old 3xTg *versus* 6-months-old wild-type ****$p<0.0001$). Consistent with previous investigations from rodent preparations (Rohn et al., 2002; Corsetti et al., 2008) and human nerve terminals specimens (Amadoro et al., 2010, 2012; Corsetti et al., 2015; Sokolow et al., 2015), the steady-state expression level of the neurotoxic 20-22kDa NH_2htau truncated fragment significantly increased and time-dependently accumulated starting from 1 month of age into synaptic-enriched fractions of cognitively impaired older animals from both AD transgenic mouse models. The specific ability of 12A12mAb in binding the 20-22kDa NH_2htau fragment *in vitro*, both in recombinant and native forms, was checked by Western blotting and Enzyme-Linked Immunosorbent Assay (ELISA) (Suppl. Fig. 1).

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Having ascertained that the NH_2htau accumulated into hippocampal synapses under pathological conditions in association with progressive disruption of animals' memory/learning function(s), we investigated whether the 12A12mAb could be exploited to systemic tau-directed immunization regimen. In particular, we ascertained whether 12A12mAb was able to gain access to the cerebral parenchyma after its peripheral administration, an optimal prerequisite for local engagement of the pathogenic target and its successful neutralization/clearance *in vivo*. To this aim, 6-month-old mice from these two different strains (Tg-AD) were infused over 14 days with two weekly injections of 12A12mAb (30µg/dose) administered on two alternate days to the lateral vein of the tail. Both age-matched wild-types and *naive* (i.e. not-immunized) transgenic AD counterparts, which were sham-infused under the same experimental conditions with vehicle (saline) only, were also included as negative controls. By probing with anti-mouse IgG used as primary antibody, Western blotting analysis (Fig. 1C) carried out on hippocampal protein extracts from the three experimental groups (wild-type, *naive* Tg-AD, Tg-AD+mAb which, importantly, were sacrificed and thoroughly perfused with PBS in order to make sure that their brains were free of blood contaminations) showed that 12A12mAb-injected 3xTg animals exhibited high levels of cerebral mouse IgG when compared to not-vaccinated, saline-treated controls. This qualitative finding is in line with previous reports on the ability of other, intravenously-administered anti-tau antibodies to cross the blood brain barrier (BBB) of diseased transgenic mice (about 0.1% of delivered total amount), likely owing to its age-related impairment and increased permeability (Asuni et al., 2007; Mably et al., 2015; Blair et al., 2015; Bennett et al., 2018).

Next, to confirm that peripherally-delivered 12A12mAb was actually able to bind the NH_2htau *in vivo*, we carried out Enzyme-Linked Immunosorbent Assay (ELISA) quantitative test on TBS-soluble fractions isolated from hippocampi of wild type, *naive* 3xTg and 3xTg+mAb animals after 14 days i.v. injection. Healthy, wild-type mice infused with 12A12mAb under the same experimental conditions (wild-type+mAb) were also included to ascertain whether 12A12mAb could enter the brain from periphery despite the intact BBB and/or the lack of tau pathology into the CNS. It's worth underlying that: (i) the ELISA test aimed at assessing the cerebral amount of injected 12A12mAb is based on the plate-immobilized synthetic $NH_2$26-44aa which, being the minimal AD-relevant (Borreca et al., 2018) active moiety of the parental longer $NH_2$26-230 (Amadoro et al., 2004, 2006), was used as catching peptide; (ii) only the free (i.e. unoccupied) antibody can readily bind to its immobilized specific antigen and be measured, whereas the tau-bound antibody is not detectable. As shown in Fig.1D, a sizeable proportion of the injected 12A12mAb was unbound and biologically-active (antigen-binding competent) in 3xTg brains, being able to recognize the synthetic plate-immobilized recombinant tau peptide. Interestingly, the

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levels of i.v.-administered 12A12mAb were significantly lower in 3xTg+mAb experimental group than in wild-type+mAb counterpart (two-way ANOVA analysis followed by Bonferroni's post-hoc test for multiple comparisons, genotype x treatment interaction $F=_{(1,24)}=28.92$ $p<0.0001$; wild-type+saline *versus* Tg-AD+saline n.s. $p>0.99$; ****$p<0.0001$ for all other pair comparisons), indicating that a higher fraction of this antibody is actually bound *in vivo* to the endogenously-generated NH_2htau antigen -and thus less available for capture in *in vitro* ELISA assay- into the hippocampi from diseased animals than in healthy controls. Similar results were also found in 6-month-old Tg2576 animals from the other genetic background which were analyzed and treated under the same experimental conditions (data not shown).

Collectively, these findings demonstrated that: (i) the pathological tau truncated at its N-terminal domain early accumulates into hippocampal synapses from Tg-AD, suggesting that it might contribute to the age-dependent disruption of animals' memory and learning functions; (ii) after its i.v. injection, 12A12mAb can be actively up-taken into the brain because an appreciable percentage of free and antigen binding-competent form of antibody is present into the hippocampus both from healthy controls and 3xTg immunized mice, regardless of the integrity of their BBB and/or the presence of tau pathology; (iii) 12A12mAb does not aspecifically interact, neither in wild-type nor in 3xTg, with the large amount of intracellular full-length normal tau which is routinely released during procedure of samples homogenization, in line with our previous *in vivo* observations advocating its cleavage-specificity towards the NH_2htau truncated fragment (Amadoro et al., 2012, Suppl. Fig. 1); (iv) 12A12mAb is not in limiting amount and, thus, endowed with potential therapeutic effect (*in vivo* target-engagement) because after immunization it is locally detectable in its active/antigen-competent state into mouse brains, with higher level in the wild-type controls than in diseased 3xTg ones.

12A12mAb passive immunization reduces both pathological tau and APP/Aβ metabolisms into synaptic compartments from treated AD transgenic mice at the prodromal stage of neuropathology.

Co-occurrence between tau and Aβ pathology has been described to take place within neuronal processes and nerve ending compartments at early stages of AD (Takahashi et al., 2010; Amadoro et al., 2012; Spires-Jones and Hyman, 2014; Rajmohan and Reddy, 2017). In the preclinical models of Tg2576 and 3xTg, Aβ exerts its synaptotoxicity, at least in part, via tau, but both separate and synergistic neurodegenerative mechanisms have been also described in these two experimental

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paradigms (Nisbet et al., 2015; Polanco et al., 2018; Li and Gotz, 2017). Recent *in vitro* and *in vivo* evidence have demonstrated that Aβ and tau pathology -in addition to their direct and/or indirect interaction (Castillo-Carranza et al., 2015; Dai et al., 2017, 2018; Rajamohamedsait et al., 2017)- can damage the synaptic terminals in an Amyloid Precursor Protein (APP)-dependent manner suggesting that its increased expression level *per se* should be considered as an additional therapeutic target to preserve the integrity and function of crucial neuronal networks (Schreurs et al., 2018; Gulisano et al., 2018; Kametani and Hasegawa, 2018).

In view of these considerations, we investigated whether the antibody-mediated neutralization of pathogenic NH_2-truncated tau following i.v. 12A12mAb infusion could mitigate *in vivo* the occurrence of neurochemical derivatives from the abnormal APP and tau metabolisms which are largely recognized to compromise the AD nerve terminals at prodromal disease stages (Braak and Del Tredici, 2015). To this aim, Western blotting SDS-PAGE analyses (Fig.2, Tg2576; Fig.3, 3xTg) were carried out on hippocampal synaptosomal preparations from the three experimental groups (wild-type, *naive* Tg-AD, Tg-AD+mAb) of both genetic backgrounds (3-month-old Tg2576 and 3xTg) by probing with both 12A12mAb and specific commercial antibodies detecting the AD-like, site-specific tau hyperphosphorylation at Ser202/Thr205 epitope (AT8) (Goedert et al., 1995) and the accumulation of soluble 6E10-positive APP/Aβ derivatives (Teich et al., 2015). As shown in Fig.2A, 3A -and in line with Fig.1A,B- the intensity signal of the neurotoxic 20-22kDa NH_2htau truncated fragment was markedly increased in saline-treated, *naive* Tg-AD mice when compared to non-transgenic wild-type controls (one-way ANOVA followed by Bonferroni's post-hoc test for multiple comparisons $F_{(2,18)}$ =117.5 $p<0.0001$ Tg2576; $F_{(2,18)}$=34.54 $p<0.0001$ 3xTg; ****$p<0.0001$ Tg2576 *versus* wild-type; ****$p<0.0001$ 3xTg *versus* wild-type). Importantly, the 12A12mAb treatment significantly reduced the synaptic load of 20-22kDa NH_2htau form(s) in Tg-AD animals from both strains indicating that this antibody, after i.v. delivery, successfully engaged/intercepted its target into hippocampus with consequent neutralization/clearance *in vivo* (one-way ANOVA followed by Bonferroni's post-hoc test ****$p<0.0001$ Tg2576+mAb *versus* Tg2576; ****$p<0.0001$ 3xTg+mAb *versus* 3xTg). Following 12A12mAb immunization, the AT8 immunoreactivity was strongly inhibited in Tg-AD animals (one-way ANOVA followed by Bonferroni's post-hoc test $F_{(2,18)}$=23.72 $p<0.0001$ Tg2576; $F_{(2,18)}$=42.18 $p<0.0001$ 3xTg; Tg2576 *versus* wild-type ****$p<0.0001$; Tg2576+mAb *versus* wild-type n.s. $p=0.7913$; Tg2575+mAb *versus* Tg2576 ***$p<0.0005$; 3xTg *versus* wild-type ****$p<0.0001$; 3xTg+mAb *versus* wild-type n.s. $p=0.3747$; 3xTg+mAb *versus* 3xTg ****$p<0.0001$), proving that the anti-truncated tau antibody was able to downregulate the extent of tau neuropathology *in vivo* (Fig. 2C, 3C). A drastic decline and/or disappearance of the expression levels of 6E10-positive APP/Aβ specie(s) (i.e. 4kDa Aβ monomer,

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14kDa low-molecular weight Aβ oligomers or APP C-terminal fragment (βCTF)) was also clearly observed in Tg-AD hippocampal synapses from treated experimental groups of both genetic backgrounds (Fig. 2E, 3E/F) (one-way ANOVA followed by Bonferroni's post-hoc test $F_{(2,18)}$ =104.7 p<0.0001 Tg2576; $F_{(2,18)}$ =115.8 p<0.0001 3xTg; Tg2576 *versus* wild-type ****p<0.0001; Tg2576+mAb *versus* wild-type n.s. p=0.0536; Tg2576+mAb *versus* Tg2576 ****p<0.0001; ****p<0.0001 for all pair comparisons from 3xTg). Importantly, the steady-state expression level of total tau detected by probing with H150 and DC25 (Fig.2B, 3B), two commercial anti-pan tau antibodies binding both murine and human tau isoforms (Um et al., 2011; Lee et al., 2010; Zilka et al., 2006; Schroeder et al., 2016), was unchanged in synapses from AD transgenic animals after 12A12mAb immunization regimen, with significantly higher level of total tau detected in 3xTg in comparison with wild-type controls due to the presence of both endogenous and human transgenic proteins (one-way ANOVA followed by Bonferroni's post-hoc test $F_{(2,18)}$=0.3014 p=0.7434 Tg2576; $F_{(2,18)}$=22.8 p<0.0001 3xTg; n.s. p>0.999 for all pair comparisons from Tg2576; 3xTg *versus* wild-type***p<0.0005; 3xTg+mAb *versus* wild-type ****p<0.0001; 3xTg+mAb *versus* 3xTg n.s. p=0.1577). These findings are consistent with tau cleavage-specificity of 12A12mAb which selectively binds *in vivo* the neurotoxic NH_2htau truncated specie(s) (Amadoro et al., 2012; Corsetti et al., 2015) without showing any cross-reaction towards the full-length form of protein (Suppl.Fig.1). Finally, as visualized by 22C11 commercial antibody (Fig.2D, 3D), 12A12mAb immunization resulted to act upstream on Aβ production by normalizing the disease-associated upregulation in the expression level of APP full-length holoprotein in both transgenic AD mice models (one-way ANOVA followed by Bonferroni's post-hoc test $F_{(2,18)}$=46.07 p<0.0001 Tg2576; $F_{(2,18)}$=97.33 p<0.0001 3xTg; Tg2576 *versus* wild-type ****p<0.0001; Tg2576+mAb *versus* Tg2576 ****p<0.0001; Tg2576+mAb *versus* wild-type n.s. p=0.999; ****p<0.0001 3xTg for all comparisons). Interestingly, this evidence supports more recent studies which suggest a prominent, causal role of APP accumulation in triggering synaptotoxicity and tauopathy (Schreurs et al., 2018; Kametani and Hasegawa, 2018),

Collectively, these results demonstrate that: (i) when i.v. administrated to young (3-month-old) Tg2576 and 3xTg mice -two well-established AD animal models showing tau-dependent neuropathology (Castillo-Carranza et al., 2015; Oddo et al., 2006; Amar et al., 2017)- the cleavage-specific 12A12mAb is able to reach an appreciable concentration into the hippocampal parenchyma ensuing an effective binding/degradation of the pathologic 20-22kDa NH_2htau form(s); (ii) the *in vivo* antibody-mediated removal of the 20-22kDa NH_2htau form(s) alleviates the detrimental alterations of both APP/Aβ and tau metabolisms (i.e. AT8 tau hyperphosphorylation, APP/Aβ

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species accumulation and processing) commonly occurring at the earliest stage of AD progression into nerve endings; (ii) the 12A12mAb-mediated immunodepletion of the toxic 20-22kDa NH_2htau form(s) takes place in the absence of any significant change in the stability/turnover of normal full-length tau protein which is known to be endowed with important physiological functions into synaptic compartments (Pooler et al., 2014; Regan et al., 2017) and whose reduction, even if partial, is extremely harmful in terminally-differentiated post-mitotic neurons *in vivo* (Biundo et al., 2018; Velazquez et al., 2018).

Cognitive performance is significantly improved in symptomatic AD transgenic mice after i.v. 12A12mAb delivery.

Having established that classical molecular determinants underlying the phenotypic AD manifestations are strongly reduced at early/presymptomatic stages of neuropathology following peripheral administration of 12A12mAb, cognitive functioning of symptomatic Tg-AD animals (6-month-old Tg2576 and 3xTg) was analyzed under the same schedule of treatment by means of a comprehensive behavioural test battery (Fig.4, Tg2576; Fig.5, 3xTg).

The novel object recognition task (NOR) is a paradigm which is considered an appropriate readout for measures of learning/memory impairment in transgenic and non-transgenic animal models of tauopathies, including AD (Polydoro et al. 2009; Lanté et al., 2015). Relevantly, the NOR behavioural task: (i) involves brain areas such as transentorhinal/entorhinal/perirhinal cortices and hippocampus which are pathologically relevant in this field, being affected by neurofibrillary tau changes at early stages of disease (Braak and Braak, 1991; Bengoetxea et al., 2015; Sankaranarayanan et al., 2015; Lasagna-Reeves et al., 2011, 2012); (ii) is a non-aversive learning paradigm, avoiding the potential confounds of using differential rewards or punishments, able to evaluate the hippocampal-dependent episodic memory (Antunes and Biala, 2012; Leger et al., 2013) which is the first type of memory affected in AD patients (Grayson, et al., 2015; de Toledo-Morrell et al., 2007; Salmon and Bondi, 2009; Reed et al., 1997; Zola and Squire, 2001). Owing to innate and spontaneous preference of mice towards novelty, any increase in exploration of the novel object (NO) during the test session is to be ascribed to the animal's ability in discriminating it from the familiar one (FO). This parameter was quantified as preference index (PI), which is calculated as the percentage of time spent exploring the new object over the total time spent exploring the two objects. In the recognition session, a preference index for the NO above 50% indicated that the NO was preferred, below 50% that the FO was preferred and at 50% that no object was preferred (Hammond et al., 2004).

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Interestingly (Fig. 4A, 5A), Tg2576 and 3xTg mice receiving 12A12mAb showed a significant rescue in short-term memory deficits when tested in this hippocampal-dependent task, being able to distinguish NO from FO (Tg2576+mAb PI=58.6%; 3xTg+mAb PI=66.41%) just as wild-type, healthy non-transgenic mice (B6SJL PI=59.44%; C57 PI=68.0%, respectively). On the other hand, saline-treated/*naive* Tg-AD mice from both strains exhibited a poor performance when evaluated in NOR test because they spent the same time in exploring the NO *versus* the FO (Tg2576 DI=48.51%; 3xTg DI=50.48%, respectively). Accordingly, a two-way ANOVA of behavioural data (treatment x object discrimination) indicated significant difference between the three experimental groups of both strains ($F_{(1,32)}$=6.60 p=0.01 for Tg2576; $F_{(2,56)}$=3.4 p=0.04 for 3xTg) with the novel object being preferred from 12A12mAb-infused AD transgenic animals (Fisher's post-hoc test NO *versus* FO Tg2576+mAb: **p<0.01; 3xTg+mAb: ***p<0.0005) which behaved in the same manner of wild-type, non-transgenic ones (Fisher's post-hoc test NO *versus* FO B6SJL: ***p<0.0005; C57: ***p<0.0005). Conversely, not-immunized AD mice from both genetic backgrounds did not discriminate between NO and FO object and displayed defective mnestic abilities without any preference for NO (Fisher's post-hoc test Tg2576: p=0.61; 3xTg: p=0.32). Furthermore, these results were not due to an intrinsic inability of animals to interact with the objects because no significant difference (treatment x object discrimination) was measured during training phase among the animals' cohorts from both strains which explored both objects for the same length of time and without any particular preference toward a side of the cage (two-way ANOVA analysis $F_{(2,32)}$=0.087 p=0.916 for Tg2576 background; $F_{(2,52)}$=1.09 p=0.34 for 3xTg mice; Fisher's post-hoc test LO *versus* RO B6SJL: p=0.53, Tg2576: p=0.20, Tg2576+mAb: p=0.30; Fischer's post-hoc test LO *versus* RO C57: p=0.72, 3xTg: p=0.91, 3xTg+mAb: p=0.11).

In addition to the objects' recognition memory, the hippocampal formation is devoted to store information about places in the organism's environment, their spatial relations, and the existence of specific objects in specific places (spatial memory) (O'Keefe and Conway, 1978; Broadbent et al., 2004; Manns and Eichenbaum, 2009). Accordingly, immunized and not-immunized animals from the three experimental groups run the Object Place Recognition (OPR) task, another hippocampal-dependent paradigm which examines the memory/learning ability of mice in spatial relationships, rather than in objects recognition, by calculating the time spent in discriminating the spatially displaced "old familiar" object relative to the stationary "old familiar" object (Vogel-Ciernia and Wood, 2014; Antunes and Biala, 2012). Rodents normally display a clear preference for the object moved to a novel place (displaced object, DO) in comparison to the object that remains in the same (familiar) place (stationary object, SO), which confirms their proficiency for remembering which spatial locations have or have not been engaged earlier (Warburton et al., 2013).

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Again (Fig.4B, 5B), cognitive impairment of mice from the two genetic backgrounds (Tg2576 and 3xTg) was relieved following i.v. 12A12mAb injection because immunized animals were able to distinguish DO from SO (Tg2576 PI=73.26%; 3xTg PI=69.07%) by performing in spatial novelty memory task just as wild-type, healthy non-transgenic ones (B6SJL PI=79.71%; C57 PI=71.48%, respectively). On the other hand, saline-treated *naive* AD transgenic mice showed no preference for the moved object as they spent nearly equivalent amounts of time exploring the DO and SO which confirms that these not-immunized animals from both strains have object location memory dysfunction (Tg2576 home-cage PI=48.29%; 3xTg home-cage PI=52.53%, respectively). Consistently, a two-way ANOVA of behavioural data (treatment x object discrimination) indicated significant difference between the three animals' cohorts in both strains analyzed ($F_{(2,20)}$=9.68 p=0.001 for Tg2576; $F_{(2,50)}$=33.11 p<0.001 for 3xTg) with the DO being preferred from 12A12mAb-immunized AD mice (Fisher's post-hoc test DO *versus* SO Tg2576+mAb: *p<0.05, 3xTg+mAb: ***p<0.0005) which behaved in the same manner of wild-type, non-transgenic ones (Fisher's post-hoc test DO *versus* SO B6SJL: ***p<0.0005, C57: ***p<0.0005). In contrast, *naive* Tg2576 and 3xTg mice displayed no difference between DO and SO object with no obvious preference for DO (Fisher's post-hoc test Tg2576: p=0.76; 3xTg: p=0.35). Besides, regardless of the genetic background, no variation (treatment x object discrimination) was measured during the training phase among the three experimental groups which explored both objects for the same length of time and without any particular preference toward a side of the cage (two-way ANOVA analysis $F_{(2,20)}$=0.47 p=0.63 for Tg2576 background; $F_{(2,52)}$=0.79 p=0.46 for 3xTg mice; Fisher's post-hoc test LO *versus* RO B6SJL: p=0.58, Tg2576: p=0.76, Tg2576+mAb: p=0.47; Fischer's post-hoc test LO *versus* RO C57: p=0.24, 3xTg: p=0.86, 3xTg+mAb: p=0.68).

After assessing the object discrimination and spatial memory, we also tested mice in the spontaneous alternation by employing the Y-maze, an hippocampal-dependent episodic-like behavioural test for measuring their willingness to explore new environments (exploratory tendency). Animals are started from the base of the Y-shaped apparatus placed horizontally and allowed to freely explore all three arms. The number of arm entries and the number of triads are recorded in order to calculate the percentage of alternation (Deacon and Rawlins, 2006; Borchelt and Savonenko, 2008) which is based on the fact that the rodent tends to choose the arm not visited before, reflecting memory (spatial-based working memory) of the previous choice (Paul et al., 2009).

Interestingly (Fig.4C), in line with previous literature findings (Yassine et al., 2013; Deacon et al., 2008; King and Arendash, 2002), the spontaneous alternation task did not reliably detect cognitive deficits in Tg2576 mice at 6 months of age because no difference was found in their working-

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memory performance in comparison to cognitively-intact, wild-types, both in spontaneous alternation and total entries into the arms (spontaneous alternation one way ANOVA $F_{(2,12)}=0.15$ $p=0.86$; Fisher's post-hoc test wild-type *versus* Tg2576 $p=0.99$; Tg2576 *versus* Tg2576+mAb $p=0.68$; Total Entries $F_{(2,12)}=0.28$ $p=0.76$; Fisher's post-hoc test wild-type *versus* Tg2576 $p=0.81$; Tg2576 *versus* Tg2576+mAb $p=0.72$). All three groups of mice alternated between arms above chance level (22.2%), indicating that neither cohort showed impairment in this test. On the other hand (Fig.5C) and in line with previous reports (Ameen-Ali et al., 2017; Spires-Jones and Knafo, 2012), although disability was clearly discernible in *naïve,* cognitively-impaired 3xTg at 6-month of age when tested in comparison to age-matched wild-types (Spontaneous alternation one way ANOVA $F_{(2,28)}=7.44$ $p=0.025$; Total entries $F_{(2,28)}=18.01$ $p=0.00001$), no significant improvement in their reference and working-memory/learning abilities was detected following systemic injection with 12A12mAb (Fisher's post-hoc test analysis, Total entries: wild-type *versus* 3xTg ***$p<0.0005$; wild-type *versus* 3xTg+mAb ***$p<0.0005$; 3xTg *versus* 3xTg+mAb $p=0.19$; Spontaneous alternation wild-type *versus* 3xTg ***$p<0.0005$; wild-type *versus* 3xTg+mAb *$p<0.05$; 3xTg *versus* 3xTg+mAb $p=0.17$). In this framework, it's worth stressing that, in contrast to 3xTg characterized by genetically-driven tau pathology, Tg2576 mice express human APP (K670N/M671L)PS1(M146V) transgene in endogenous background of murine not-mutated tau. Therefore, the discrepancy in results between two different transgenic AD rodent models, each having its own characteristics, may be due to the aggressive phenotype of the human tau-overexpressing 3xTg strain, which is likely to require a more optimized immunization regimen (antibody dosage, time of treatment, timing of administration) in order to fully prevent and/or delay its robust cognition symptomatology. Alternatively, the reversible nature of *in vivo* tau neuropathology could be restricted within strain-specific temporal window(s) because of the complex and multifactorial feature of AD pathology involving a wide range of inflammatory, oxidative, neurodegenerative causative mechanisms (Webster et al., 2014; Velazquez et al., 2018).

Finally, to rule out the possibility that the *in vivo* enhancement of cognitive skills in immunized animals involved an effect of 12A12mAb treatment on body energy homeostasis known to influence their sensorial-motor abilities, metabolic rate (energy expenditure, EE) from vehicle- or antibody-infused non-transgenic wild-type mice was assessed by means of indirect calorimetry during 2 days of continuous analysis/recording. As shown in Suppl.Fig.2A, an unpaired t-test of EE data revealed no significant difference between the two experimental groups (vehicle-treated animals: ($M=25.29$; $SEM=\pm0.61$) or mAb-treated animals: ($M=24.92$; $SEM=\pm0.64$ $t_{(142)}=0.42$). Furthermore, the EE analysis in resting conditions (REE) -i.e. by considering only the EE values

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generated in the absence of motor activity (i.e. 0–2 counts)- did not show any variation in heat production from 12A12mAb-treated healthy wild-type mice, thus corroborating the important finding that intra-caudal injection either with vehicle alone (M=18.51; SEM=±0.43), or with antibody (M=18.08; SEM=±0.52 $t_{(142)}$=0.42), was ineffective in altering the whole body REE, whatever the physical motor activity involved ($t_{(118)}$=0.63) (Suppl.Fig.2B).

In keeping with this finding, no change in recognition memory performance was detected when vehicle- or 12A12mAb-treated WT mice were evaluated in the NOR paradigm (Suppl.Fig.2C-D), further indicating that the immunization regimen *per se* did not affect cognitive functions under non-pathological settings. Two-way ANOVA of time (sec.) of exploration of FO *versus* NO showed no significant difference for treatment factor ($F_{(1,12)}$=0.28 p=ns), significant object novelty factor ($F_{(1,12)}$=18.74 p<0.001) and no significant effect of the interaction between treatment and object novelty ($F_{(1,12)}$=0.08 p=ns). Post-hoc Tukey's test for object novelty (FO *versus* NO) further confirmed that both vehicle-treated (**p<0.01) and mAb-treated (**p<0.01) wild-type animals exhibited intact recognition memory (Suppl.Fig.2C). No difference was found between the DI of vehicle-treated and mAb-treated wild-type groups (unpaired sample t-test: vehicle-treated and mAb-treated ones ($t_{(6)}$=1.672, p=ns, Suppl.Fig.2D), thus demonstrating that 12A12mAb-induced injection did not impair recognition memory in non-transgenic mice. In agreement with the cleavage-selectivity of antibody (Suppl.Fig.1), the 12A12mAb treatment appeared to be avoid of potential adverse side-effects in discriminatory skills when injected in healthy animals, notwithstanding its ability of penetrating the animals' BBB and/or successfully accessing to hippocampus in biologically-active state (Fig.1D).

Likewise, no difference in cognitive performance was detected when sham-immunized 6-month-old Tg2576 mice (i.e., animals administered with IgG isotype control, at the same dosage and period of time) were tested for performance in NOR paradigm in comparison with their vehicle-treated counterparts. Two-way ANOVA analysis on time (sec.) of exploration of FO *versus* NO displayed no significant difference for object factor ($F_{(1,8)}$ =0.66 p=ns) and treatment factor ($F_{(1,8)}$=0.67 p=ns) in vehicle- and IgG-treated transgenic mice (Suppl.Fig.3A). Moreover, the unpaired sample t-test of DI data showed that neither vehicle nor IgG administration improved the deficit of recognition memory (compare Suppl. Fig.3B with Suppl. Fig.2C), thus confirming the lack of ability of transgenic animals in discriminating between FO and NO (($t_{(4)}$=0.05, p=ns). Similar results were detected following IgG infusion in 6-month-old 3xTg mice when compared to not-immunized transgenic counterparts (data not shown).

Active behaviour, such as exploring a novel environment, induces the expression of the immediate-early gene Arc (Activity-regulated cytoskeletal associated protein, or Arc/Arg3.1) in several brain

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regions, including the hippocampus. Arc messenger ribonucleic acid (mRNA) is quickly induced and dynamically up-regulated by behavioural experience and the protein is selectively translated into activated dendrites, being required for the memory consolidation of an early initial potentiation of synaptic transmission into a lasting form of long-term potentiation (LTP) (Plath et al., 2006; Korb and Finkbeiner, 2011; Ramirez-Amaya et al., 2005, 2013). Interestingly and consistent with their cognitive enhancement in behavioural assessments (Fig. 4-5), Western blotting analyses performed on hippocampal synaptosomal-enriched preparations isolated from post-trained animals (Suppl.Fig.4A-B) showed that the stimulus-driven, steady-state expression level of Arc was significantly upregulated in 12A12mAb-immunized Tg2576 and 3xTg mice when compared to their saline-treated cognitively-impaired counterparts (one-way ANOVA followed by Bonferroni's post-hoc test $F_{(2,18)}=34.81$ $p<0.0001$ Tg2576; $F_{(2,18)}=33.32$ $p<0.0001$ 3xTg; Tg2576+mAb *versus* Tg2576 ****$p<0.0001$; Tg2576+mAb *versus* wild-type n.s. $p=0.1441$; 3xTg+mAb *versus* 3xTg ***$p<0.0005$; 3xTg+mAb *versus* wild-type **$p<0.01$). Conversely and in line with their scarce performance in novelty-based cognitive tasks (Fig. 4-5), *naïve* AD transgenic animals -which were not systemically infused with 12A12mAb- displayed marked defects in the experience-dependent induction of Arc expression, and then in their processes of memory/learning trace consolidation following its initial acquisition, as shown by the finding that immunoreactivity signal of protein in their synaptic fractions was significantly lower than that from healthy wild-type controls (one-way ANOVA followed by Bonferroni's post-hoc test ****$p<0.0001$ for Tg2576 *versus* wild-type and for 3xTg *versus* wild-type).

Collectively these experiments indicate that passive immunization with 12A12mAb, which selectively targets the neurotoxic NH_2htau fragment(s) *in vivo*, significantly improves cognition in symptomatic (6-month-old) Tg-AD animals by rescuing their instinctual and innate preference for novelty (object recognition and object location skills) when tested in two pathologically-relevant, hippocampal-dependent behavioural tasks.

Loss in dendritic spine density is prevented in hippocampal CA1 region from 12A12mAb-infused 6-month-old Tg-AD animals.

Dendritic spines, the sites of excitatory synapses protruding from dendritic shafts, are cellular morphological specializations devoted to memory-forming processes in neurons (Segal, 2005). Being extremely dynamic structures, modification in their number, size and/or shape is an important index of synaptic plasticity occurring in response to external environmental inputs (Pignataro et al., 2015). As a consequence, loss of dendritic arborization (length/complexity) in vulnerable neuronal

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networks, although occurring along different spatio-temporal patterns among commonly used transgenic animal models, undoubtedly represents one of the earliest changes of structural plasticity which critically contributes over time to the disruption of neuronal network with consequent appearance of cognitive dysfunction in AD and other related dementias (Spires-Jones and Knafo, 2012; Dorostkar et al., 2015; Knobloch and Mansuy, 2008; Cochran et al., 2014). Therefore, in order to complement our behavioural findings, we assessed the neuroanatomical effect of passive immunization with 12A12mAb on dendritic connectivity from 6-month-old AD animals. To this aim, hippocampal sections from mice of the three experimental groups were stained by Golgi-Cox impregnation procedure followed by quantitative assessment of dendritic spine density (number of spines per unit length) along both apical and basal compartments of individual CA1 pyramidal neurons (Fig. 6).

As shown in Fig. 6A and in line with previous works reporting in Tg2576 an early decline of dendritic boutons which undergo dystrophy and shrinkage (Lanz et al., 2003; Jacobsen et al., 2006; D'Amelio et al., 2011), the spine loss was detectable at the age of 6-months in apical compartments of CA1 hippocampal neurons from this genetic background when animals were compared to non-transgenic controls. Importantly, in 12A12mAb-immunized AD group the apical spine density was significantly ameliorated up to the level of saline-injected cognitively-intact wild-types (one-way ANOVA followed by Fisher's post-hoc test ($F_{(2,\ 8)}$=10,828, p=,00530; **p<0.01 wild-type *versus* Tg2576; **p<0.01 Tg2576+mAb *versus* Tg2576), indicating that treatment was strongly effective in blocking/preventing the dendritic degeneration. Interestingly, no difference was detected when spines were counted in the basal compartment of CA1 neurons from the three experimental cohorts (one-way ANOVA $F_{(2,\ 8)}$=0,71926, p=0,51611), suggesting that age-related spine changes in Tg2576 mice initially involve the apical dendritic arbors with no apparent effect on basal dendrites of CA1 pyramidal neurons which are more likely to be affected only later, when their structural plasticity and stability (formation and elimination) is completely impaired (Spires-Jones et al., 2007).

On the other hand and in stark contrast with previous literature findings (Bittner et al., 2010), we found out (Fig. 6B) that the reduction in the spines density, both in apical and basal compartments of individual CA1 pyramidal neurons, already started from the age of 6 months in cognitively-impaired 3xTg mice (one-way ANOVA followed by Fisher's post-hoc test apical: $F_{(2,\ 7)}$=18,697, p=0,00156; basal: $F_{(2,\ 7)}$=13,404, p=0,00404) which exhibited lower values in dendritic protrusions counts when compared with their age-matched, non-transgenic wild-types. Remarkably, degeneration of dendritic spine structures was robustly decreased in immunized 3xTg mice (apical:

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**p<0.01 3xTg +mAb *versus* 3xTg; **p<0.01 wild-type *versus* 3xTg; basal: **p<0.01 3xTg+mAb *versus* 3xTg; *p<0.05 wild-type *versus* 3xTg) pointing out that -possibly as result of increased afferent inputs to the CA1 from other neighboring hippocampal areas and/or as a local positive effect in the CA1 region- 12A12mAb treatment was able to mitigate the age-related pathology of post-synaptic connections from symptomatic 6-month-old 3xTg mice, both in their apical and basal compartments.

Systemic administration of 12A12mAb also normalizes the AD-related electrophysiological alterations of Tg-AD animal models.

In order to investigate whether 12A12mAb immunization, in addition to its protective actions on AD-related behavioural and neurochemical and neuroanatomical abnormalities, was also able to exert an effect on electrophysiological correlate(s) of the memory/learning processes, hippocampal synaptic transmission and plasticity in the Schaffer collateral pathway were compared between transgenic and wild-type animals from both genetic backgrounds (Fig. 7 A,B,C,D for Tg2576; E,F,G,H for 3xTg).

We first recorded basal synaptic transmission and the strength of pre-synaptic Schaffer collaterals activation (i.e. axonal depolarization) from CA3-to-CA1 synapses in acute brain slices from 6-month-old wild-type and age-matched Tg2576 animals treated with saline-vehicle or 12A12mAb, respectively. To this aim, we first generated input/output (I/O) curves by measuring the field excitatory postsynaptic potentials (fEPSPs) elicited in the stratum radiatum of the CA1 area after stimulation of the Schaffer collaterals at increasing intensities. As shown in Fig.7A-B and in line with previous investigations reporting no change in basal synaptic transmission in this transgenic AD model at 6 months of age (Chapman et al., 1999; Nobili et al., 2017), the I/O curves displayed a similar trend among the three experimental groups (two-way repeated-measures ANOVA for stimulus intensity x experimental group followed by Bonferroni's post-hoc test $F_{(12,282)}$ =0.8409 p=0.6082; n.s. p>0.05 for all comparisons).

Next, we investigated the presynaptic function by assessing paired-pulse facilitation (PPF), a short-term plasticity paradigm which inversely depends on Ca^{2+}-dependent presynaptic changes in neurotransmitter release probability at nerve endings (Manabe et al., 1993; Debanne et al., 1996; Dobrunz and Stevens, 1997; Dobrunz et al., 1997; Thomson, 2000; Zucker and Regehr, 2002). Again (Fig.7C), short-term potentiation was almost identical among the three animals' cohorts (two-way repeated-measures ANOVA for paired-pulse interval x experimental group, followed by

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Bonferroni's post-hoc test $F_{(10,170)}$=0.51 p=0.8839; n.s. p>0.05 for all comparisons), consistent with previous results referring no significant dissimilarity in PPF between 6-month-old Tg2576 and wild-type littermates (Nobili et al., 2017; Jung et al., 2011).

In contrast to the basic synaptic transmission (input-output relationship and PPF), the "classical" NMDA receptor-dependent long-term potentiation (LTP) paradigm at Schaffer collaterals/CA1 synapses -a long-lasting enhancement of the strength/efficacy of excitatory synaptic transmission which is widely used in investigations on numerous APP/Aβ models of AD (Rowan et al., 2003; Shankar et al., 2008)- turned out to be significantly compromised in 6-month-old Tg2576 mice in comparison to age-matched wild-types (Fig.7D). Following the induction of LTP by delivery of trains of high-frequency stimulation (HFS) at half-maximal intensity, fEPSP slopes appeared to decay down to baseline in 6-month-old Tg2576 animals so that no persistent, activity-driven potentiation was measurable starting from 30 min after induction which was indicative of an impaired function of hippocampal Schaffer collaterals/CA1 synapses. Importantly and in keeping with improvement of cognitive performance in hippocampal-dependent behavioural assessments, peripheral administration of 12A12mAb was able to mitigate *in vivo* the disease-related LTP deficiency of symptomatic Tg2576 animals, as shown by the fact that the LTP amplitude calculated after application of HFS was significantly increased in immunized experimental group when compared to *naive* cognitively-impaired counterpart (one-way ANOVA followed by Bonferroni's post-hoc test $F_{(2,21)}$=19.38 p<0.0001; ***p<0.0005 Tg2576 *versus* wild-type; *p<0.05 Tg2576+mAb *versus* Tg2576; Tg2576+mAb *versus* wild-type **p<0.01). Moreover, these electrophysiological investigations further corroborated the finding that the disruption of synaptic plasticity in hippocampal Schaffer collateral commisural pathway from this AD model was more likely due to altered post-synaptic signaling pathways, given that no alteration in PPF was contextually detected in transgenic animals at 6 months of age (Nobili et al., 2017; Jung et al., 2011; Jacobsen et al., 2006; Chapman et al., 1999).

In contrast with results from symptomatic Tg2576 mice, in 3xTg paradigm the I/O relationship revealed a significant reduction of fEPSP slopes evoked by increasing stimulation intensities (Fig.7E-F) when 6-month-old transgenic animals were compared to wild-type counterparts (two-way repeated-measures ANOVA for stimulus intensity x experimental group followed by Bonferroni's post-hoc test $F_{(12,204)}$=5.812 p<0.0001; *p<0.05 and **p<0.01 wild-type *versus* 3xTg for paired comparisons). Most importantly, cumulative distributions of fEPSP slopes within the range of 100 μA and 300 μA of stimulus amplitude were shifted to higher values in 12A12mAb-immunized AD group in contrast to *naive*, cognitively-impaired counterpart, indicating that

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antibody treatment positively influenced the fast glutamatergic transmission in this genetic background (#$p<0.05$, ##$p<0.01$, ###$p<0.001$ for 3xTg *versus* 3xTg+mAb for paired comparisons with Bonferroni's post-hoc test). No change in PPF short-term plasticity (Fig.7G) was found among the three experimental cohorts (two-way ANOVA paired-pulse interval x genotype followed by Bonferroni's post-hoc test $F_{(12,198)}=0.3464$ $p=0.9792$ n.s. $p>0.05$ for all comparisons), consistent with previous data showing that the abnormalities in pre-synaptic release machinery were not discernible between 6-month-old 3xTg and age-matched wild-types when measured in a facilitation electrophysiological paradigm (Oddo et al., 2003). In a way similar to Tg2576, hippocampal slices from 6-month-old 12A12mAb-injected 3xTg animals displayed a strong potentiation after HFS bout, pointing to a strong protective action evoked *in vivo* by the antibody treatment on the cellular/molecular correlate(s) of their memory/learning processes (one-way ANOVA followed by Bonferroni's post-hoc test $F_{(2,33)}=7.018$ $p=0.0029$; **$p<0.01$ 3xTg *versus* wild-type; *$p<0.05$ 3xTg+mAb *versus* 3xTg; 3xTg+mAb *versus* wild-type n.s. $p>0.05$). Notably, when LTP was calculated in 6-month-old 3xTg mice (Fig.7H), a lower post-tetanic potentiation was found against wild-types suggesting that, in this AD strain, the LTP reduction in magnitude and persistence was more likely due to deficits of induction (either pre- and/or post-synaptic), in line with structural and functional modifications observed both in their basal synaptic transmission and dendritic spine density (Fig.6B).

Taken together, these electrophysiological recordings indicate that disruption of excitatory synaptic transmission and plasticity detected at 6 months of age in hippocampal CA3-CA1 circuit of these two genetically distinct Tg-AD animal models, although manifests at different rate and involves non-overlapping causative mechanism(s), was significantly rescued following *in vivo* peripheral administration of 12A12mAb.

Expression levels of inflammatory astroglial and microglial markers are also downregulated in 6-month-old 12A12mAb-immunized Tg-AD animals.

The inflammatory response -which is one of the earliest manifestations of neurodegenerative tauopathies, including AD (Yoshiyama et al., 2007; Wes et al., 2014; Leyns et al., 2017 ; Bellucci et al., 2004; Ishikawa et al., 2018)- may act as a double-edged sword being either detrimental or protective depending on the context (Schlachetzki and Hull, 2009). On one hand, activated glial cells contribute to the AD pathogenesis by means of diverse mechanisms including complement-mediated synapse removal, non-cell autonomous spreading of pathological seeds/conformers,

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extracellular release of inflammatory mediators such as pro-inflammatory cytokines, complement components, chemokines, free radicals and gliotransmitters which, in turn, trigger neurodegeneration. On the other hand, astro- and microglial reactions are endowed with beneficial role in AD environment by stimulating the digestion/clearance of pathogenic Aβ and tau species and, then, by preventing their accumulation into insoluble cerebral lesions, the senile plaques and neurofibrillary tangles.

To get further insights into protective effect evoked by i.v. 12A12mAb-based immunization in Tg-AD mice, the extent of inflammatory response was assessed on hippocampi from 6-month-old Tg2576 and 3xTg mice of the three experimental groups (wild-type, *naive* Tg-AD, Tg-AD+mAb). Western blotting analysis (Fig.8) were carried out on animals' total extracts by probing with antibodies which detect the glial fibrillary acidic protein (GFAP) and Iba1, whose cell type-specific steady-state expression levels are recognized as indicative of active astrogliosis and microgliosis respectively (Sydow et al., 2016). As shown in Fig.8A-B and regardless of the different genetic background, the immunoreactivity signals of these two classical inflammatory markers were strongly increased in saline-treated, *naive* Tg-AD mice in comparison to wild-type controls, in line with previous findings reporting a prominent astrocytic and microglial activation in hippocampal parenchyma from these animal models (Olabarria et al., 2010, 2011; Vogels et al., 2019). Remarkably, the gliosis detected in 12A12mAb-injected Tg-AD mice turned out to be significantly downregulated compared to their *naïve* counterparts (one-way ANOVA followed by Bonferroni's post-hoc test GFAP: $F_{(2,21)}=169.4$ $p<0.0001$ Tg2576 ****$p<0.0001$ for all comparisons; $F_{(2,18)}=53.88$ $p<0.0001$ 3xTg; ****$p<0.0001$ 3xTg *versus* wild-type; ****$p<0.0001$ 3xTg+mAb *versus* 3xTg; n.s. $p>0.05$ 3xTg+mAb *versus* wild-type; Iba1: $F_{(2,21)}=38.43$ $p<0.0001$ Tg2576 ****$p<0.0001$ Tg2576 *versus* wild-type; ****$p<0.0001$ Tg2576+mAb *versus* Tg2576; n.s. $p>0.05$ Tg2576+mAb *versus* wild-type; $F_{(2,18)}=273$ $p<0.0001$ 3xTg ****$p<0.0001$ for all comparisons), indicating that: (i) the neuron-derived, extracellularly-released 20-22kDa NH_2htau form(s) is more likely to be endowed with non-cell autonomous action by contributing to the glial cells activation; (ii) the neuroprotective effect of 12A12mAb appears to be, at least in part, due to its modulatory role at the glia-neurons interplay.

Taken together these findings indicate that sub-chronic i.v. delivery of 12A12mAb into the hippocampus is devoid of potentially adverse inflammatory effects associated to classical immunization regimen by limiting the local activation of neuroglia which is *per se* both a consequence to the disease process and a contributor to the synaptic pathology and neuronal

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damage (Perry et al., 2010; Zotova et al., 2010; Schwab et al., 2010; Block et al., 2007; Edison et al., 2008; Yoshiyama et al., 2007).

DISCUSSION

Accumulating evidence have suggested that the detrimental effects of Aβ are dependent on tau pathology (Rapoport et al., 2002; Roberson et al., 2007; King et al., 2006; Vossel et al., 2010; Shipton et al., 2011; Ittner et al., 2010; Bloom, 2014; Desikan et al., 2011; Nussbaum et al., 2012) and that tau, rather than Aβ (Murray et al., 2015), serves a prominent role in early synaptic decline and cognitive impairment (Pontecorvo et al., 2017; Busche et al., 2019; Arriagada et al., 1992; Nelson et al., 2012; Ashe and Zahs, 2010). Independently of its ability of seeding aggregation, abnormal extracellular/intracellular tau is *per se* neurotoxic (Diaz-Hernandez et al., 2010; Medina and Avila, 2014a-b; Hu et al., 2018) and propagates trans-synaptically along interconnected neuronal networks in a stereotypical manner which strongly correlates with the development of clinical symptoms during AD progression (Pooler et al., 2013; Mohamed et al., 2013; Yamada et al., 2017). These pathologically relevant findings represent the rationale which advocates the employment of tau-based strategies (Li and Gotz, 2017; Congdon and Sigurdsson, 2018) as promising disease-modifying intervention of slow progressing AD and other human dementias (Jadhav et al.,2019; Novak et al., 2018a), especially in view of the disappointing outcomes from Aβ-targeting pharmacological and immunological approaches (Agadjanyan et al., 2017; Sigurdsson, 2008; Schroeder et al., 2016; Doody et al., 2014; Salloway et al., 2014; Giacobini and Gold, 2013). In this connection, tau-directed passive immunotherapy -which relies on the specific, epitope-directed antibody-mediated depletion/clearance of its toxic species (Congdon and Sigurdsson, 2018; Sigurdsson, 2008; Pedersen and Sigurdsson, 2015; Li and Gotz, 2017)- has been recently recognized as a feasible, valuable approach to reduce the neuropathology and to improve the memory/learning abilities of experimental animal models of tauopaties (Boutajangout et al., 2011; Chai et al., 2011; Yanamandra et al., 2013, 2015; Castillo-Carranza et al., 2014; d'Abramo et al., 2013; Subramanian et al., 2017; Dai et al., 2015, 2018). However, several reasons have hindered the clinical success of tau-targeting approaches which are currently under investigation (Novak et al., 2018a; Elmaleh et al., 2019; Giacobini and Gold, 2013). To this regard, recent reports of ongoing trials indicate that the potential flaws include: (i) study design with the medical care given too late when neuronal damage is already present in a considerable extent so that drugs/modulators are unable to compensate adequately for the detrimental effects; (ii) systemic toxicity owing to long-term and multiple administrations of drugs/modulators used at high doses which can interfere with the neuronal physiology (i.e. affect the normal cellular metabolism and/or impact on the immune

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surveillance); (iii) inadequate brain bioavailability of medicaments against the target substrate and/or the biochemical pathway, even after crossing the BBB; (iv) adverse risks of inflammatory response, such as cerebral microhemorrhages (Elmaleh et al., 2019).

Here, we show that systemic administration with 12A12mAb -which selectively recognizes the human tau at D_{25}(DRKD_{26}QGGYTMHQDQEGDTDAGLK_{44}), a known N-terminal truncation protein site (Quinn et al., 2018) previously identified both in cellular and animal AD paradigms (Corsetti et al., 2008) and in human AD brains (Rohn et al., 2002; Amadoro et al., 2012; Corsetti et al., 2015)- rescues the neurochemical, anatomical, behavioural and electrophysiological alterations underlying the AD phenotype in two well-established transgenic mouse strains, such as preclinical Tg2576 and 3xTg models. Of particular relevance is the fact that the transgenic Tg2576 mice expressing human mutant APP (K670N/M671L), in contrast to 3xTg harboring PS1(M146V), APP(Swe), and tau(P301L) transgenes, display an endogenous genetic background of murine not-mutated tau. Furthermore, since treatments started when synaptic deterioration is evident but extensive neurodegeneration has not yet developed turn out to be the most effective in preventing the disease-associated brain atrophy and related cognitive impairment (Bokde et al., 2009; Elmaleh et al., 2019), our experimental evaluations are carried out on symptomatic animals which are employed at early-middle stages (6-month-old) of pathology progression, when their hippocampi are not largely compromised with massive neuronal loss. It's also worth underlining that the 12A12mAb we employed in the present study is specific for the pathological truncated tau because it selectively binds *in vivo* the neurotoxic AD-linked $NH_2$26-230 fragment (i.e. NH_2htau) without showing any significant cross-reaction towards the intact, physiological form of protein, in line with our previous investigations (Amadoro et al., 2012). To this point, biochemical and functional outcomes *in vivo* measures further confirm that 12A12mAb: (i) does not specifically interact with the abundant intracellular pool of endogenous normal full-length tau protein whose steady state level is unchanged in hippocampus after its i.v. delivery in Tg-AD mice regardless of the genetic backgrounds; (ii) is harmless when injected in healthy, cognitive-intact wild-type mice, despite the ability of successfully penetrating/reaching the brain in its biologically-active (antigen-competent) state under physiological settings. Remarkably, the cleavage-specific 12A12mAb -which selectively binds 20-22kDa NH_2htau without unproductive and deleterious cross-reaction towards the physiological intact tau- appears to be potent tool by providing measurable changes on AD brain physiopathology which result in significant improvement of the synaptic and cognitive deficits in affected animals, even after its short-term (14 days) i.v. delivery. Conversely, there is proof that the use of other therapeutic anti-tau antibodies binding all forms of tau is more likely to result in considerable reduction of its effective dose available *in vivo* against the target toxic tau species with

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consequent requirement of more aggressive and prolonged applications (Novak et al., 2018a). Furthermore, our results may have important clinical implications by prospecting the non-invasive i.v. delivery route of 12A12mAb as effective and safe disease-modifying approach in contrasting the earliest neuropathological and cognitive alterations of subjects which suffer the chronically-developing human AD and non-AD tauopathies characterized by an increased burden of tau truncation. In post-mitotic neurons, tau is endowed with important functions beyond the control of microtubule integrity and dynamics (Sotiropoulos et al., 2017) and the treatment with tau-targeting antibodies may have undesirable adverse side-effects due to "loss-of-function" of full-length protein (Rosenmann et al., 2006; Rozenstein−Tsalkovich et al., 2013; Bakota et al., 2017). Although we cannot rule out the later development of gliosis following prolonged immunization regimen, from a translational perspective another interesting finding of the present study is that the sub-chronic i.v. treatment with 12A12mAb is sufficient *per se* to drive a robust therapeutic effect in the absence of increased microglia and astrocyte activation which, on the contrary, appears to be critical for the mechanism of action of at least a few Aβ-directed antibodies (Bard et al., 2000; Wilcock et al., 2004) leading as byproduct to excessive deleterious stimulation of local inflammatory response (Lemere, 2013; Wisniewski and Goni, 2015). We find no obvious evidence of neuroinflammatory response which is known to cause mortality in wild-type mice when actively immunized with various fragments of tau (Rosenmann et al., 2006; Rozenstein-Tsalkovich et al., 2013). Furthermore, the evidence that passive immunization with 12A12mAb can normalize *in vivo* the APP/Aβ dysmetabolism in two independent genetic backgrounds overexpressing human mutated APP (K670N/M671L) not only unveils a novel and potential connection between tau and APP/Aβ, whereby toxic tau can upstream affect APP/Aβ pathology in damaging synapses, but also -and more importantly- highlights the 20-22kDa NH_2-terminal tau fragment as crucial target for AD therapy starting from its earliest stages which are characterized by initial disruption of synaptic functions in the absence of frank neuronal loss. Therefore, this study prospects the peripheral administration of the humanized counterpart of murine 12A12mAb as a novel, promising multi-targeted intervention in preventing disease-associated cognitive deterioration in human beings suffering AD-related dementias, being endowed with higher clinical potentialities than those altering either neuropathology alone (Lambracht-Washington and Rosenberg, 2013; Rosenmann et al., 2006; Bakota et al., 2017; Oddo et al., 2006).

Concerning the mechanism(s) of action involved in the beneficial power of 12A12mAb immunization, in the present study we are unable to anticipate whether tau-directed therapeutic

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effects offered by i.v. delivery of 12A12mAb involve only the extracellular or both intracellular and extracellular pool of toxic truncated tau because we did not collect and analyze the level of NH_2htau fragment in CSF or interstitial fluid (ISF) and plasma. It's worth noting that the N-terminal, but not C-terminal, fragments of tau including the 20-22kDa NH_2htau form(s), are mainly secreted from synaptosomes of AD brains (Sokolow et al., 2015) and detected both in CSF from AD patients (Meredith et al., 2013; Johnson et al., 1997; Portelius et al., 2008; Amadoro et al., 2014; Cicognola et al., 2019; Chen et al., 2019) and in conditioned media from patient-derived induced pluripotent stem cells (iPSC) cortical neurons (Bright et al., 2015; Kanmert et al., 2015; Sato et al., 2018). The $NH_2$26-44 aminoacidic stretch, which is the minimal biological active moiety of parental 20-22kDa NH_2-truncated tau form(s) (Amadoro et al., 2010, 2012; Corsetti et al., 2015) has been recently recognized as one among the potentially-targetable tau epitopes for promising AD immunotherapeutic interventions, being largely represented into CSF samples (Barthélemy et al., 2016a-b; Sato et al., 2018) and in autoptic specimens from affected subjects (Borreca et al., 2018). Interestingly, previous *in vitro*, *ex-vivo* and *in vivo* experiments from our research group have demonstrated that this short peptide when extracellularly-administered to hippocampal neurons dynamically perturbs the plasma membranes –mainly of distal axonal compartments (Perini et al., 2019)- by exerting a deleterious action on synaptic connectivity and plasticity being more likely internalized only after prolonged incubation times (Florenzano et al., 2017; Borreca et al., 2018). Moreover, studies have shown that tau antibodies can be readily taken up by neurons, promote the intracellular sequestration/clearance of pathological species by means of different mechanisms and prevent their release into the extracellular space followed by consequent spreading throughout the brain (Pedersen and Sigurdsson, 2015; Shamir et al., 2016; Krishnamurthy et al., 2011; Congdon et al., 2013; Collin et al., 2014; Gu et al., 2013; Asuni et al., 2007). After its i.v. administration in both healthy and disease mice, the 12A12mAb in circulation seems to be able to successfully penetrate the hippocampus and engage *in vivo* its target at a sufficient level to exert biologically-relevant neuroprotective effects. The N-terminal region of tau, despite the lack of the microtubule binding domains which abnormally aggregate to form paired helical filaments (PHFs), is able to undergo higher order of oligomerization (Feinstein et al., 2016) and, in this framework, the binding of 12A12mAb to the NH_2htau may also prevent the trans-synaptic propagation of detrimental insoluble tau. Therefore, both extracellular and intracellular interaction between 12A12mAb and the NH_2htau might be plausible routes by which immunization directed against this harmful, AD-relevant N-truncated tau specie(s) operates *in vivo*. Furthermore, although the immune system has been increasingly recognized as an important player in the immunotherapeutic approaches (Congdon and Sigurdsson, 2018; Katsinelos et al., 2019), the finding that the cognitive skills

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improvement of 12A12mAb-injected AD transgenic mice are paralleled by a strong and concomitant reduction of the disease-associated cerebral level of reactive gliosis further supports recent results showing that (i) antibody-mediated targeting of pathological tau *in vivo* does not necessarily required engagement of microglia that may *per se* induce deleterious neuroinflammation (Lee et al., 2016); (ii) the neuroprotective mechanism action evoked by tau-based immunotherapy is more likely to rely on the direct neutralization of toxic extracellular species and/or on preventing their uptake by neurons (Congdon et al., 2013; Gu et al., 2013). In this regard, glial activation and neuroinflammation have been reported to severely impact on tau pathology directly, by participating to tau aggregation and degradation and spreading (Hopp et al., 2018; Bolos et al., 2017; Yuan et al., 2016; Asai et al., 2015), or indirectly, through a non-cell autonomous effect on neuronal signaling via cytokine and complement factor and gliotransmitter secretion (Liddelow et al., 2017; Litvinchuk et al., 2018; Piacentini et al., 2017) and upregulation of senescence-associated genes (Bussian et al., 2018) and synapses pruning (Vogels et al., 2019; Marttinen et al., 2018).

Another challenging question is whether the neuroprotection offered by 12A12mAb can be further ameliorated *in vivo* following its prolonged administration, especially in more severe 3xTg animal model, or sustained even after its discontinuing immunization. Further investigations will be needed to better clarify the dose-dependent effect of 12A12mAb treatment on pathology and cognitive performance of AD transgenic mice and how long the beneficial effect can last beyond the period of the immunization.

It's also worth stressing that -although mouse and human tau aminoacidic sequences are similar- there are 14 amino acid differences in the N-terminal region (Hernandez et al., 2019; Andorfer et al., 2003; Bright et al., 2015). Nevertheless, the extreme N-terminal sequence of tau protein starting at D25 encompasses a not-canonical caspase(s) cleavage-site sequence (Kumar et al., 2014; McStay et al., 2008) which has been identified both in cellular (human SY5Y and rat PC12) and animal (AD11 mice) AD models (Corsetti et al., 2008; Rohn et al., 2002) and in human AD brains (Amadoro et al., 2012; Rohn et al., 2002; Quinn et al., 2018). Moreover results from *in vitro* experiments and transgenic animal models have shown that truncation plays a causative role in remodeling the highly-flexible conformational ensemble of intrinsically disordered protein tau into AD-like pathological conformations (Novak et al., 2018b). Conformational changes involving the amino-terminus of tau early occur in AD and other related tauopathies (Garcia-Sierra et al., 2003). Consistently, Mukrasch et al., have demonstrated that -although the largest part of tau441 aminoacid sequence is devoid of any ordered structure- the N-terminal 50 residues of protein favour

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a compact conformation, as indicated by strong contacts within the residue stretch 1–20 and from this region to residues 30–50 (Mukrasch et al., 2009). Therefore it's reasonable to hypothesize that, although the amino acid sequence of human and murine tau surrounding this epitope is divergent, 12A12mAb is more likely to recognize the newly-generated, sequence- and structural-based immunoreactive determinants whose formation requires pathological truncation occurring under AD conditions at D25 residue both in human and rodent tau (Corsetti et al., 2008; Rohn et al., 2002; Quinn et al., 2018; Amadoro et al., 2019). In support of this finding and in line with the experimental evidence that temporary secondary structures occur in causal relation with tau neuropathology progression, both in isolated domains of the full-length protein and of some of its fragments (Mukrasch et al., 2009; Avila et al., 2016b; Fichou et al., 2019), by means of MD simulations and SAXS experiments we have recently demonstrated that the short sequence including the 26-44 of N-terminal region of human tau- but not its reverse counterpart (tau44-26 peptide)- undergoes isolated β-bridges, α-helices and 3-helix which involve the Y29,T30,Q33,D34,Q35,E36 aminoacid residues (Perini et al., 2019). Importantly, these aminoacid residues are present both in murine and primate tau sequence. Besides, the fact that 12A12mAb does not change the expression level of full-length tau but selectively reduces the endogenously-produced 20-22 kDa tau fragment in both AD strains, as we showed in Western blotting Fig. 2-3, further strengthens the notion that a local conformational element (i.e. sequence- and structure-based immunoreactive epitope) is more likely to underlie its *in vivo* specificity in targeting the neo-epitope of the N-derived truncated pathological tau specie(s), both in human and mouse. Finally, since the epitopes cannot be predicted reliably from antigen primary amino acid sequences because some novel epitopes can arise exclusively due to the alteration of the molecule's conformation (Opuni et al., 2018), further experiments of immunoprecipitation followed by mass spectrometry and alanine epitope scanning mapping are needed to identify the crucial binding residues and the precise structure of N-terminal of tau protein that are directly involved in the interaction with 12A12mAb.

Concerning the interplay occurring *in vivo* between APP/Aβ and tau pathologies, according to the classical Aβ cascade hypothesis aberrant changes of tau metabolism are considered downstream of Aβ pathology which acts as initial trigger (Hardy and Selkoe, 2002; De Strooper and Karran, 2016). Consistently, compelling studies have demonstrated that Aβ can potentiate tau abnormalities (Bolmont et al., 2007; Gotz et al., 2001; Lewis et al., 2001; Oddo et al., 2004) and that an enhanced neuropathology occurs following *in vivo* interaction between Aβ and tau (Pontecorvo et al., 2017; Lewis et al., 2001; Gotz et al., 2001; Hurtado et al., 2010; Bennett et al., 2017; Jacobs et al., 2018;

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Quiroz et al., 2018; He et al., 2018). In this regard, our findings highlighting a novel mechanistic interplay between APP/Aβ and tau at synapses fit more well with other studies showing that changes in tau metabolism precede Aβ pathology in aged and AD brains (Braak and Del Tredici, 2004; Braak et al., 2013; Schonheit et al., 2004; Jack et al., 2013) and that the removal of pathogenetic species of tau can prevent *in vivo* the deleterious effect of both Aβ and tau (Oddo et al., 2006; Castillo-Carranza et al., 2015; Dai et al., 2017, 2018; Rajamohamedsait et al., 2017). Remarkably, the spreading/propagation of tau neuropathology into the Aβ plaque-bearing cerebral cortex is associated with the transition from the preclinical (asymptomatic) to the clinical (symptomatic) stage of AD (Delacourte et al., 1999; Wang et al., 2016; Pontecorvo et al., 2017). Furthermore, although the tau pathology to evolve to full-blown AD requires the concomitant presence of Aβ pathology (Braak and Del Tredici, 2011; Crary et al., 2014; Duyckaerts, 2011; Jack et al., 2013), the failure of anti-Aβ therapies in preventing the disease progression suggests that AD pathogenesis might be driven by tau independently of Aβ (Giacobini and Gold, 2013). However, whether Aβ is necessary for tau neurotoxicity or whether the reverse is true is still an open question (Ashe and Zahs, 2010). On the other hand, recent data also suggests that tau and Aβ may be independent processes and reciprocally interact over the evolution of AD (Mondragón-Rodríguez et al., 2010; Small and Duff, 2008). In this context, co-occurrence between tau and Aβ within neuronal processes and synaptic compartments have been described in AD (Hoover et al., 2010; Ittner et al., 2010; Zempel et al., 2010; Miller et al., 2014; Amadoro et al., 2012) and synaptic abnormalities occur in aging Tg2576 and 3xTg mice (Spires-Jones et al., 2007; Ameen-Ali et al., 2017; Nisticò et al., 2012). Aβ and tau pathologies exert synergistic effects on neuronal morphology/function (Rhein et al., 2009) particularly at synapses (Takahashi et al., 2010; Ittner et al., 2010; Hoover et al., 2010; Amadoro et al., 2012) believed to initiate AD progression (Selkoe, 2002), indicating that passive immunization with 12A12mAb can contribute to improve disease-associated mnestic disabilities at its early phases by preventing both pathognomonic toxic proteins from damaging synaptic connectivity in pathologically-relevant vulnerable neuronal circuits. Furthermore, a recent hypothesis also suggests that synaptic dysfunction in AD is triggered by impairment of APP metabolism which further progresses via tau pathology (Schreurs et al., 2018; Gulisano et al., 2018; Kametani and Hasegawa, 2018). Consistently, an increased level of APP and/or its C-terminal fragments are able to induce axonal and synaptic defects (Rusu et al., 2007; Rodrigues et al., 2012; Deyts et al., 2016; Xu et al., 2016) associated with mis-localization of tau (Blurton-Jones and Laferla, 2006; Hochgrafe et al., 2013). Overexpression of APP promotes *per se* the seeded aggregation of intracellular tau in cultured cell, suggesting that APP, rather than Aβ, can work as a receptor of abnormal tau fibrils (Takahashi et al., 2015) by accelerating *in vivo*

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internalization in neurons (Holmes et al., 2013; Mirbaha et al., 2015) followed by pathological accumulation and propagation. Besides, both soluble/prefibrillar extracellular toxic Aβ and tau can damage the synaptic terminals in APP-dependent manner (Puzzo et al., 2017; Wang et al., 2017), suggesting a translation potential of 12A12mAb for APP-targeted therapy in patients.

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Concerning the routes by which the 12A12mAb-mediated removal of the NH_2htau can affect the cross-talk between Aβ and tau neuropathology or interfere upstream with APP metabolism and/or its processing *in vivo*, both cell- and non-cell autonomous action mechanisms (Alasmari et al., 2018) should be taken into account by operating in alternative but not mutually-exclusive manners and by acting at different transcriptional (Zhang et al., 2018; Bright et al., 2015), translation (Borreca et al., 2016; Asuni et al., 2014; Meier et al., 2016; Koren et al., 2019) and post-translational (Amadoro et al., 2012) levels. Furthermore, variations in the type of mechanism(s) engaged *in vivo* by 12A12mAb in two APP mouse models analyzed and/or dissimilarity in their temporal progression of plaque deposition (Ameen-Ali et al., 2017) are more likely to account for the difference in the magnitude of antibody effect(s) on APP/Aβ mis-processing. At the present, *in vitro*, *ex-vivo* and in *in vivo* experiments are being performed by our research group to better clarify this important issue.

In conclusion, the present investigation not only highlights a novel dynamic positive feed-forward regulation between APP/Aβ and N-truncated tau *in vivo* by reinforcing the concept of pathological tau as main therapeutic target of AD but also hopefully helps to design more efficacious and safety tau-directed interventions by prospecting the 12A12mAb as beneficial and disease-modifying approach for the cure of AD and other tauopathies.

Figure legend

Figure 1. The i.v.-injected 12A12mAb anti-tau antibody is biologically active into the animals' hippocampus.

A-B) Western blot analysis carried out on hippocampi from Tg2576 and 3xTg AD mice at different ages (1-, 3-, 6-months-old) and from 6-month-old wild-type by probing with 12A12mAb (left). β-III tubulin was used as loading control. Arrows on the right side indicate the molecular weight (kDa) of bands calculated from migration of standard proteins. Full uncropped blots are available in Suppl. Fig. 5. Pooled data and relative densitometric quantifications are reported on the right. In this

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and all other Figures, in box-and-whisker plots the centre lines denote median values, edges are upper and lower quartiles, whiskers show minimum and maximum values and points are individual experiments. Statistically significant differences (see details in the main text) were calculated by ANOVA followed by post-hoc test for multiple comparison among more than two groups. $p<0.05$ was accepted as statistically significant (*p <0.05; **p<0.01; ***p<0.0005; ****p<0.0001).

C) Western blotting analysis was carried out by probing with anti-mouse IgG as primary antibody (Thermo-Fisher 10400C) on hippocampal protein extracts (40µg) from animals of the three experimental groups (wild-type, *naive* 3xTg, 3xTg+mAb) which underwent i.v. injection with saline or 12A12mAb (see details in methods). β-III tubulin was used as loading control. Arrows on the right side indicate the molecular weight (kDa) of bands calculated from migration of standard proteins. Full uncropped blots are available in Suppl. Fig. 5. Notice that 3xTg animals, which are systemically i.v. injected for 14 days with 12A12mAb (see details in methods), exhibit high levels of cerebral mouse IgG when compared to not-vaccinated controls confirming that a fraction of mAb injected into the tail vein is present in the hippocampal parenchyma. Asterisks point to the light and heavy antibody chains (25kDa and 50kDa, respectively).

D) Brain levels of anti-tau antibody 12A12mAb were evaluated by enzyme-linked immunosorbent assay (ELISA) in the TBS-soluble fraction of hippocampal homogenates from wild-type and 3xTg mice that i.v. received saline or 12A12mAb for 14 days (see details in methods). The ELISA used to measure the anti-tau antibody relies on the plate-immobilized recombinant $NH_2$26-44aa tau which, being the minimal AD-relevant (Borreca et al., 2018) active moiety of the parental longer $NH_2$26-230 (Amadoro et al., 2004, 2006), was used as catching peptide. Notice that a significant portion of the 12A12mAb in 3xTg brains is bound to endogenous NH_2htau and does non-specifically interact with the large amount of intracellular tau released during homogenization. Statistically significant differences (see details in the main text) were calculated by ANOVA followed by post-hoc test for multiple comparison among more than two groups. $p<0.05$ was accepted as statistically significant.

Figure 2. Reduction of the hippocampal NH_2htau in Tg-AD (Tg2576) mice immunized with 12A12mAb ameliorates the disease-associated synaptic neuropathology.

A-B-C-D-E-F) Representative blots (n=5) of SDS-PAGE Western blotting analysis (left) on isolated synaptosomal preparations from hippocampal region of animals from three experimental groups (wild-type, Tg-AD and Tg-AD+mAb) of Tg2576 strain to assess the content of the NH_2htau

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fragment (A), total tau full-length (B), AT8-phosphorylated tau (C), APP holoprotein (D), Aβ monomeric and oligomeric species (E). Data were quantified for molecular weight size ranges for each antibody and normalized to β-III tubulin which was used as loading control (F) and relative densitometric quantifications are reported (right). Arrows on the right side indicate the molecular weight (kDa) of bands calculated from migration of standard proteins. Full uncropped blots are available in Suppl. Fig. 6. Notice that changes in levels of total tau are not statistically significant. Statistically significant differences (see details in the main text) were calculated by ANOVA followed by post-hoc test for multiple comparison among more than two groups. $p<0.05$ was accepted as statistically significant (*p <0.05; **p<0.01; ***p<0.0005; **** p<0.0001).

Figure 3. Reduction of the hippocampal NH_2htau in Tg-AD mice (3xTg) immunized with 12A12mAb ameliorates the disease-associated synaptic neuropathology.

A-B-C-D-E/F-G) Representative blots (n=5) of SDS-PAGE Western blotting analysis (left) on isolated synaptosomal preparations from hippocampal region of animals from three experimental groups (wild-type, Tg-AD and Tg-AD+mAb) of 3xTg strain to assess the content of the NH_2htau fragment (A), total tau full-length (B), AT8-phosphorylated tau (C), APP holoprotein (D), Aβ monomeric and oligomeric species (E/F). Data were quantified for molecular weight size ranges for each antibody and normalized to β-III tubulin which was used as loading control (G) and relative densitometric quantifications are reported (right). Arrows on the right side indicate the molecular weight (kDa) of bands calculated from migration of standard proteins. Full uncropped blots are available in Suppl. Fig. 7. Notice that changes in levels of total tau are not statistically significant. Statistically significant differences (see details in the main text) were calculated by ANOVA followed by post-hoc test for multiple comparison among more than two groups. $p<0.05$ was accepted as statistically significant (*p <0.05; **p<0.01; ***p<0.0005; **** p<0.0001).

Figure 4. Improved cognition in Tg-AD (Tg2576) mice immunized with 12A12mAb.

A-B-C) 14 days after i.v. 12A12mAb immunization, the *in vivo* effect of NH_2htau removal on cognitive performance was investigated in animals from the three experimental groups (wild-type, Tg-AD and Tg-AD+mAb) of Tg2576 genetic background in the novel object recognition (NOR)(A), Object Place Recognition (OPR)(B) and Y-maze(C) tasks(top to bottom).

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For NOR (A) and OPR (B): right and left histograms respectively represent the preference index (%) of corresponding values measured during the test trial among animals from the different experimental groups (wild-type, Tg-AD and Tg-AD+mAb) of Tg2576 genetic background. The columns refer to objects presented during training (LO= left object; RO=right object) and test trial (FO familiar object; NO=novel object; DO=displaced object; SO=stationary object). Analysis of preference index (%) measured as time spending in the exploration of the novel/displaced object/(time spending in the exploration of novel/displaced object+time spending in the exploration of familiar/stationary object)X100. Data were expressed as means±S.E.M. (n=6-10). Values are means of at least three independent experiments and statistically significant differences (see details in the main text) were calculated by ANOVA followed by post-hoc test for multiple comparison among more than two groups. $p<0.05$ was accepted as statistically significant (*<0.05; **p<0.01; ***p<0.0005; ****p<0.0001).

For Y-maze (C): right and left histograms, respectively, represent the total entries (the total arm entries correspond to the total number of arms entered) and the spontaneous alternation (the number of alternations corresponds to the successive entries into 3 different arms in overlapping triplet sets) of animals from the different experimental groups (wild-type, Tg-AD and Tg-AD+mAb) of Tg2576 genetic background. The percentage alternation was calculated as the ratio between number of correct triplets (e.g. ABC) and total entrances minus 2, multiplied by 100. Values are means of at least three independent experiments and statistically significant differences (see details in the main text) were calculated by ANOVA followed by post-hoc test for multiple comparison among more than two groups. $p<0.05$ was accepted as statistically significant (*p <0.05; **p<0.01; ***p<0.0005; ****p<0.0001).

Figure 5. Improved cognition in Tg-AD (3xTg) mice immunized with 12A12mAb.

A-B-C) 14 days after i.v. 12A12mAb immunization, the *in vivo* effect of NH_2htau removal on cognitive performance was investigated in animals from the three experimental groups (wild-type, Tg-AD and Tg-AD+mAb) of 3xTg genetic background in the novel object recognition (NOR)(A), Object Place Recognition (OPR)(B) and Y-maze(C) tasks(top to bottom).

For NOR (A) and OPR (B): right and left histograms respectively represent the preference index (%) of corresponding values measured during the test trial among animals from the different experimental groups (wild-type, Tg-AD and Tg-AD+mAb) of 3xTg genetic background. The columns refer to objects presented during training (LO= left object; RO=right object) and test trial

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(FO familiar object; NO=novel object; DO=displaced object; SO=stationary object). Analysis of preference index (%) measured as time spending in the exploration of the novel/displaced object/(time spending in the exploration of novel/displaced object+time spending in the exploration of familiar/stationary object)X100. Data were expressed as means±S.E.M. (n=6-10). Values are means of at least three independent experiments and statistically significant differences (see details in the main text) were calculated by ANOVA followed by post-hoc test for multiple comparison among more than two groups. $p<0.05$ was accepted as statistically significant (*<0.05; **p<0.01; ***p<0.0005; ****p<0.0001).

For Y-maze (C): right and left histograms, respectively, represent the total entries (the total arm entries correspond to the total number of arms entered) and the spontaneous alternation (the number of alternations corresponds to the successive entries into 3 different arms in overlapping triplet sets) of animals from the different experimental groups (wild-type, Tg-AD and Tg-AD+mAb) of 3xTg genetic background. The percentage alternation was calculated as the ratio between number of correct triplets (e.g. ABC) and total entrances minus 2, multiplied by 100. Values are means of at least three independent experiments and statistically significant differences (see details in the main text) were calculated by ANOVA followed by post-hoc test for multiple comparison among more than two groups. $p<0.05$ was accepted as statistically significant (*p <0.05; **p<0.01; ***p<0.0005; ****p<0.0001).

Figure 6. Immunization with 12A12mAb in Tg-AD mice is protective against the dendritic spines density loss which affects the memory and learning processes.

A-B) Comparative photomicrographs of Golgi-stained hippocampal CA1 neurons showing dendritic segments from animals from three experimental groups (wild-type, Tg-AD and Tg-AD+mAb) of both strains (Tg2576, 3xTg) (left, refers to CA1 pyramidal neurons dendrites scale bar: 5 µm). Box and whisker plots (right) depict the morphometric analysis of the dendritic spine density from the three experimental groups. Values are expressed as number of spines per 1 µm segment. Statistically significant differences (comparisons were made on single mouse values obtained by averaging the number of spines counted on neurons of the same mouse) were calculated by ANOVA followed by post-hoc test for multiple comparison among more than two groups. $p<0.05$ was accepted as statistically significant (*p<0.05; **p<0.01; ***p<0.0005; ****p<0.0001).

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Figure 7. Reduction of cognitive deficits in 12A12mAb-immunized Tg-AD mice correlates with an increased LTP.

A,D for Tg2576; E,H for 3xTg) Time plot of average fEPSP responses (A, E) and changes in magnitude of LTP at CA3-Ca1 synapses (D,H) were calculated among animals (n=6-10) from three experimental groups (wild-type, Tg-AD and Tg-AD+mAb) of both strains. At least 7 slices from 6 different mice were recorded for each experimental condition. Data are presented as the mean (±SEM). The traces above the plot show fEPSPs at baseline (1) and at 60 min after LTP induction (2). The box-whisker plots show pooled data. Statistically significant differences (see details in the main text) were calculated by ANOVA followed by post-hoc test for multiple comparison among more than two groups. $p<0.05$ was accepted as statistically significant (*p <0.05; **p<0.01; ***p<0.0005; **** p<0.0001).

B,C for Tg2576; F,G for 3xTg) Input/output curves show the fEPSP slopes plotted against the corresponding stimulus intensities recorded from hippocampal slices of animals (n=6-10) from three experimental groups (wild-type, Tg-AD and Tg-AD+mAb) of both strains (B,F). Comparison of paired-pulse facilitation (PPF) in animals (n=6-10) from three experimental groups (wild-type, Tg-AD and Tg-AD+mAb) of both strains (Tg2576, 3xTg) was also determined (C,G). PPF was induced by pairs of stimuli delivered at increasing interpulse intervals (20, 50, 100, 200,300, 500 msec). Data are presented as the mean (±SEM) facilitation ratio of the second response relative to the first response. Statistically significant differences (see details in the main text) were calculated by ANOVA followed by post-hoc test for multiple comparison among more than two groups. $p<0.05$ was accepted as statistically significant (*p<0.05; **p<0.01; ***p<0.0005; ****p<0.0001).

Figure 8. Inflammatory response (activation of astrocytes and microglia) is strongly downregulated in 12A12mAb-immunized Tg-AD mice.

A-B) Neuroinflammation processes (activation of astrocytes and microglia) were assessed on hippocampal extracts from animals from three experimental groups (wild-type, Tg-AD and Tg-AD+mAb) of both strains (Tg2576, 3xTg) by Western blotting analysis (left) for inflammatory proteins (GFAP, Iba1). Relative densitometric quantification of intensity signals (right) indicates lower levels of GFAP and Iba1 in Tg-AD mice+mAb compared to not-immunized Tg-AD. Glyceraldehyde-3-phosphate Dehydrogenase (GAPDH) housekeeping expression serves as loading control. Arrows on the right side indicate the molecular weight (kDa) of bands calculated from migration of standard proteins. Full uncropped blots are available in Suppl. Fig. 8. Values are from

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at least three independent experiments and statistically significant differences (see details in the main text) were calculated by ANOVA followed by post-hoc test for multiple comparison among more than two groups. $p<0.05$ was accepted as statistically significant (* $p<0.05$; ** $p<0.01$; *** $p<0.0005$; **** $p<0.0001$).

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Competing interests:

V.C., P.C., G.A: have intellectual property through CNR-EBRI regarding 12A12mAb.

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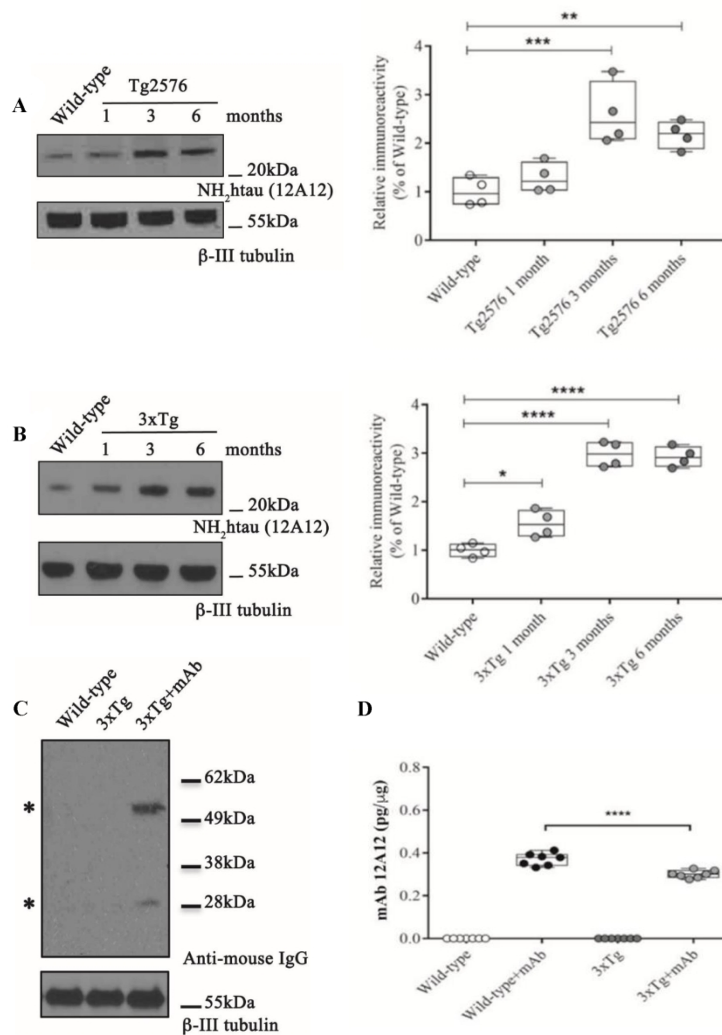

Figure 1. The i.v.-injected 12A12mAb anti-tau antibody is biologically-active into the animals' hippocampus.

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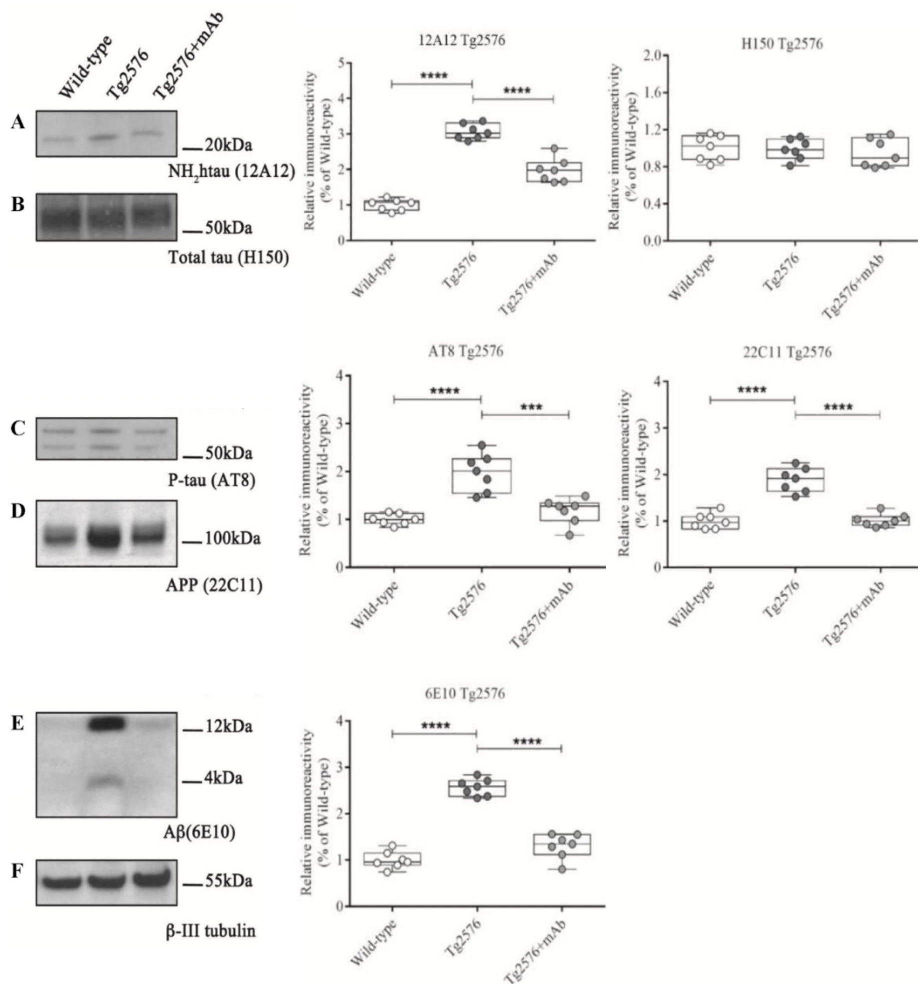

Figure 2. Reduction of the hippocampal NH2htau in Tg-AD (Tg2576) mice immunized with 12A12mAb ameliorates the disease-associated neuropathology.

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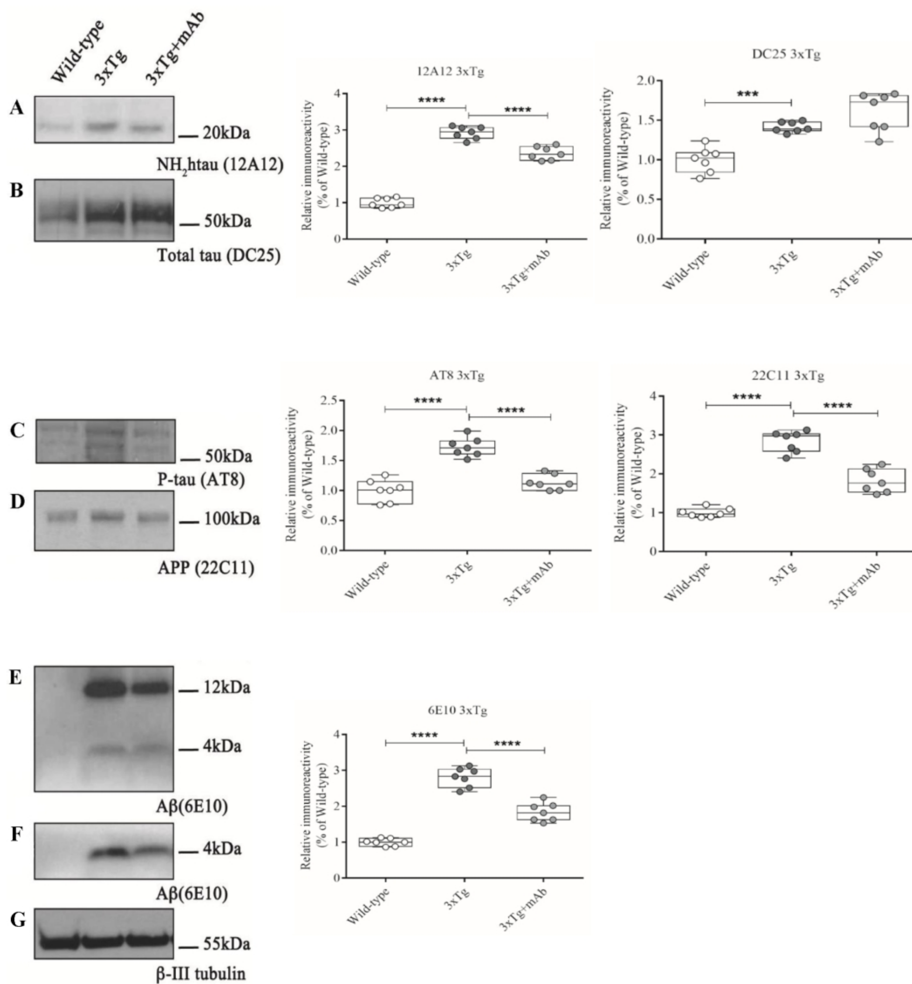

Figure 3. Reduction of the hippocampal NH2htau in Tg-AD (3xTg) mice immunized with 12A12mAb ameliorates the disease-associated neuropathology.

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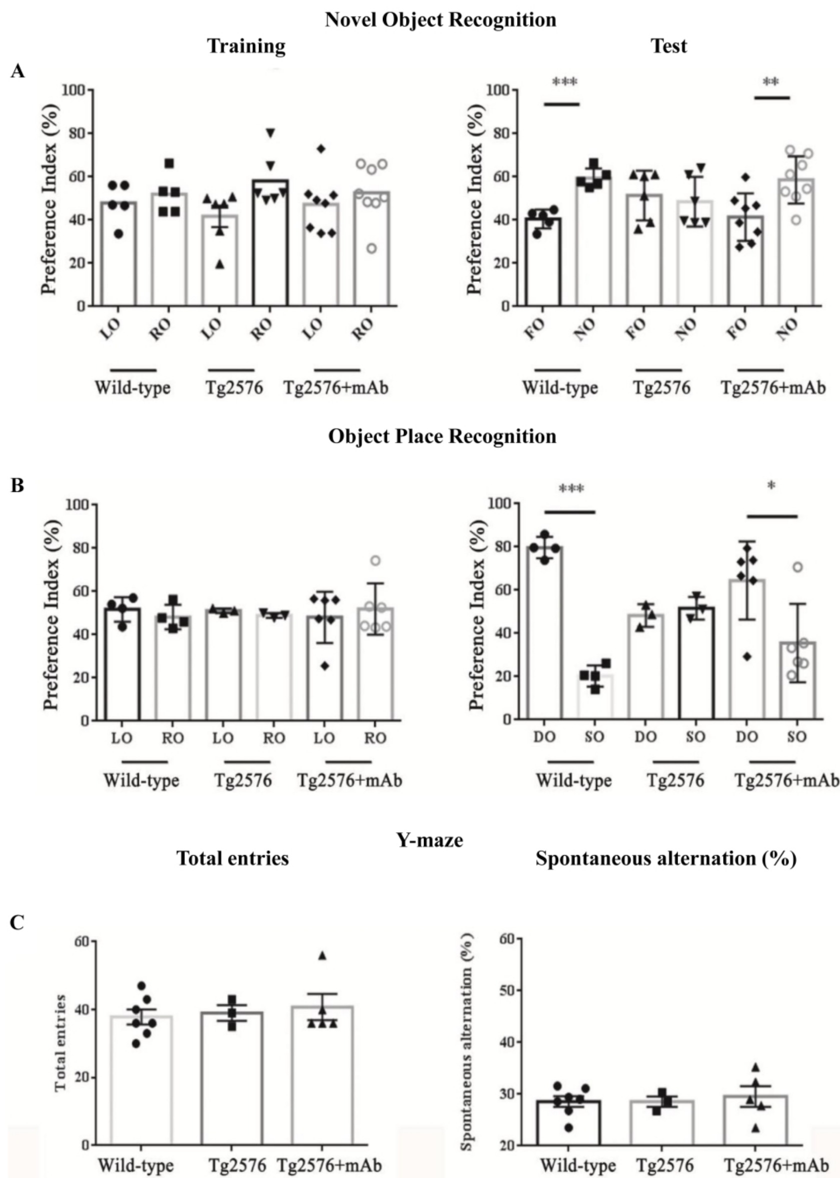

Figure 4. Improved cognition in Tg-AD (Tg2576) mice immunized with 12A12mAb.

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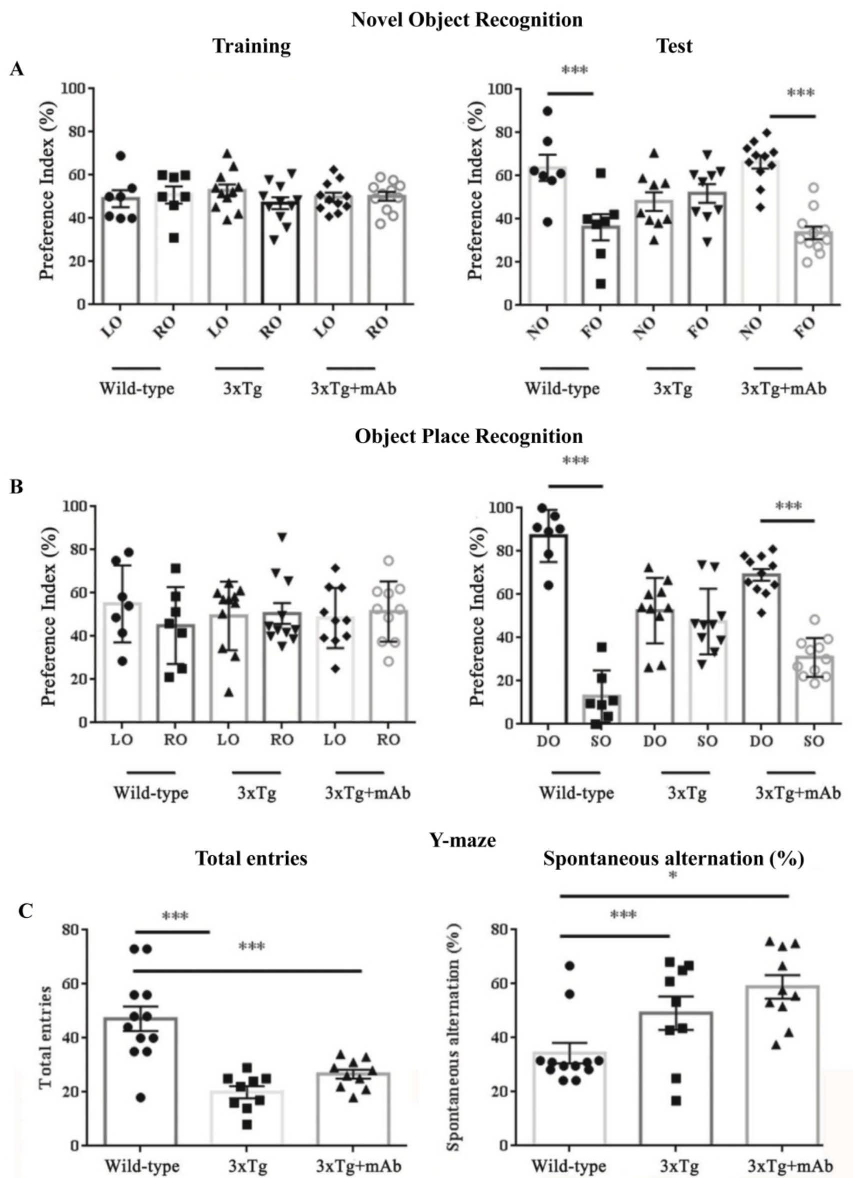

Figure 5. Improved cognition in Tg-AD (3xTg) mice immunized with 12A12mAb.

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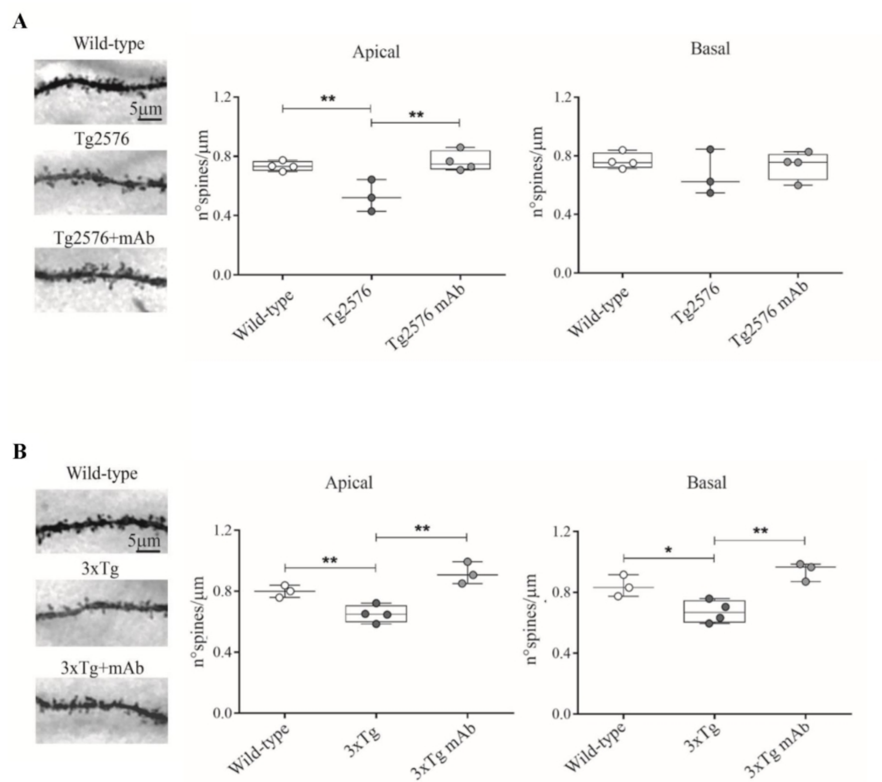

Figure 6. Immunization with 12A12mAb in Tg-AD mice is protective against the dendritic spines density loss which affects the memory and learning processes.

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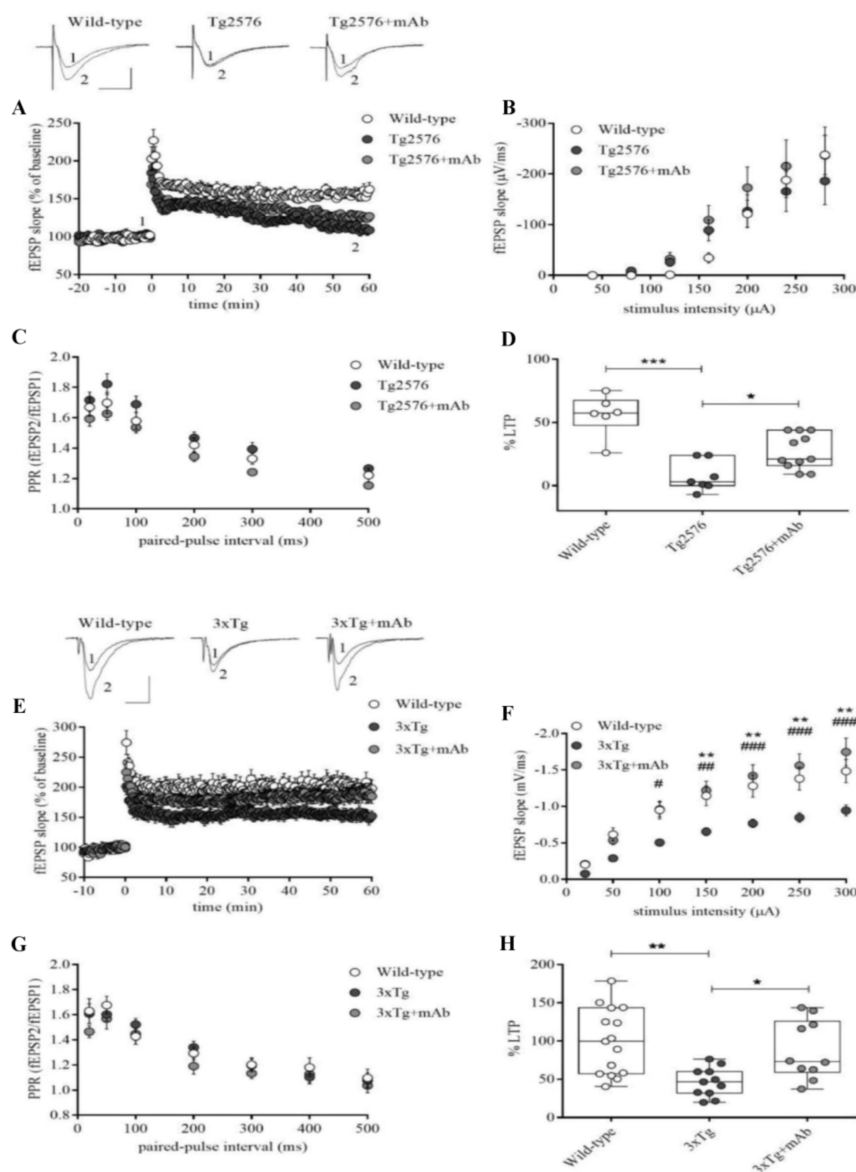

Figure 7. Reduction of cognitive deficits in 12A12mAb-immunized Tg-AD mice correlates with an increased LTP.

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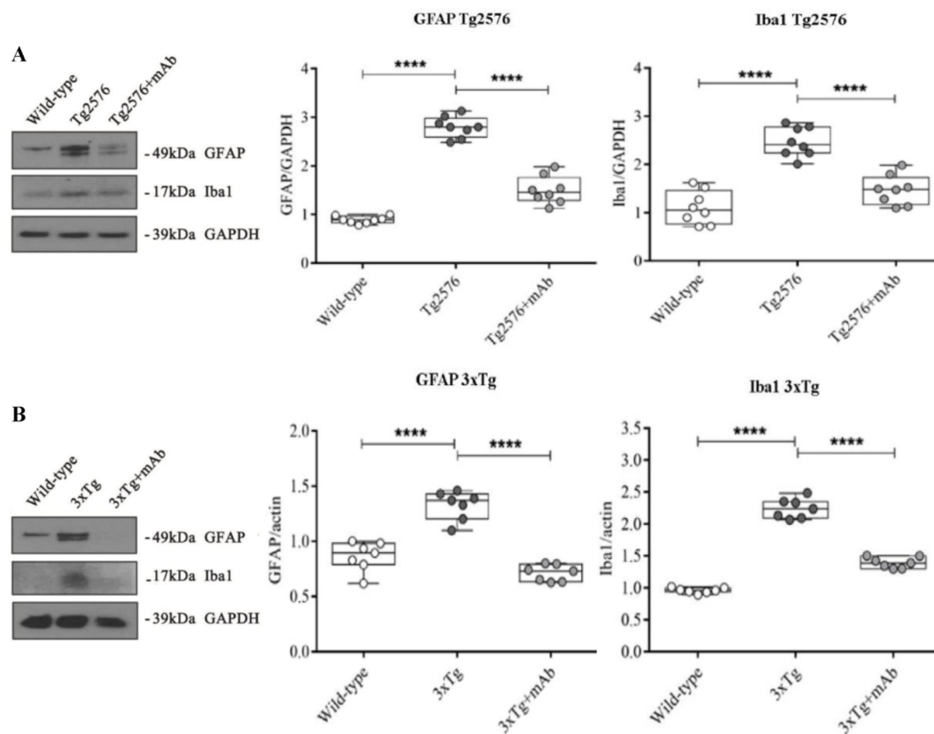

Figure 8. Inflammatory response (activation of astrocytes and microglia) is strongly downregulated in 12A12mAb-immunized Tg-AD mice.

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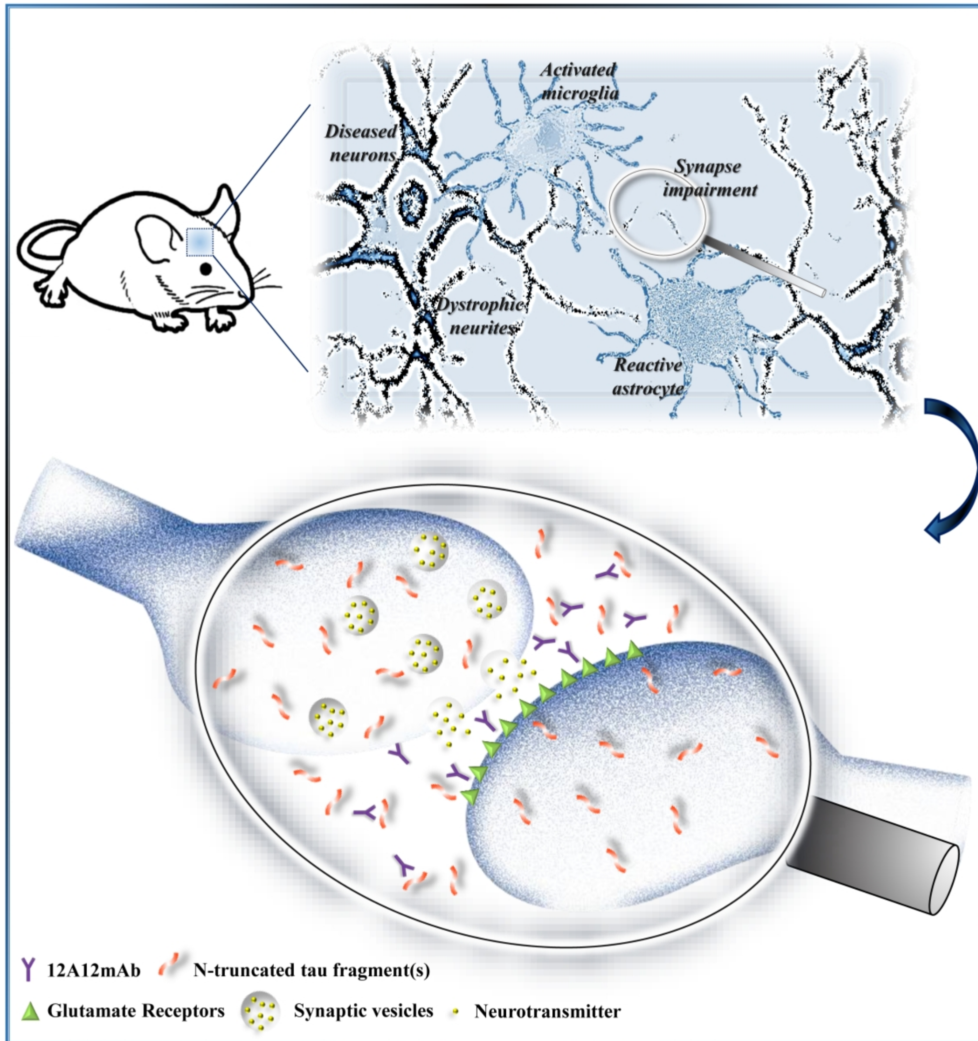

Graphical abstract

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Abbreviated summary:

Intercepting the pathologically-relevant species without interfering with the physiological form(s) of protein is one of the challenges of tau-directed immunotherapy. The present study shows that antibody-mediated selective neutralization of the toxic N-truncated tau fragment in hippocampi from two transgenic Alzheimer's Disease mice significantly improves the synaptic functions.

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