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Abstract

Alzheimer's disease is a neurodegenerative disorder characterized by amyloid deposits and neurofibrillary tangles. Cholinergic dysfunction is also a main pathological feature of the disease. Nevertheless, the links between cholinergic dysfunction and neuropathological hallmarks of Alzheimer's are still unknown. In the present study, we aimed to further investigate Tau aggregation in cholinergic systems, in a Tau transgenic mouse model. THY-Tau22 mice have recently been described as a novel model of Alzheimer–like Tau pathology without motor deficits. This strain presents an age-dependent development of Tau pathology leading to synaptic dysfunctions as well as learning and memory impairments. In the present work, we observed that Tau pathology differentially affects cerebral structures. Interestingly, early Tau pathology was observed in both hippocampus and basal forebrain. Moreover, some morphological as well as functional alterations of the septohippocampal pathway suggest a disconnection between these two key brain regions in Alzheimer's disease. Finally, these data suggest that Tau pathology may participate to cholinergic degeneration.

Introduction

The definite diagnosis of Alzheimer's disease (AD) is based on the observation of characteristic brain lesions: senile plaques and neurofibrillary tangles. Each of these lesions is located in specific areas of the brain. The neurofibrillary pathology is due to the neuronal abnormal accumulation of tau protein isoforms, which normally play an important role in the polymerization of the microtubules (for reviews, [1-4]). Regarding the amyloid pathology, this latter is characterized by the extracellular accumulation of amyloid-beta peptide (Aβ), which is normally present in low concentrations, and formed following the sequential cleavage of its precursor, the amyloid precursor protein (APP). The normal function of this peptide, and of its precursor, still remains unknown (for reviews, [5,6]).

Despite intense research effort in this field, from the therapeutic point of view, AD is currently treated symptomatically and, at present there is no means to slow down or halt the degenerative processes. Former observations showing that Alzheimer's disease is accompanied by a decrease in the level of acetylcholine in the brain have led to the concept that preventing its degradation by inhibiting acetylcholinesterase, the enzyme responsible for the degradation of this neurotransmitter may be of clinical interest [7,8]. The current molecules used in AD treatment are indeed acetylcholinesterase inhibitors including donepezil, rivastigmine and galantamine (for review, [9]). Nevertheless, the links between Alzheimer neuropathological lesions and acetylcholine defects are still ill-defined. Some data have suggested that amyloid pathology may affect cholinergic systems but these data are still controversial [10-12]. For instance, on one hand, it has been shown that Aβ deposition in amyloid transgenic mice produces age-dependent effects on cortical and hippocampal choline acetyltransferase (ChAT) fiber networks and enzyme activity without any impact on the survival of cholinergic forebrain neurons [11]. On the other hand, the amyloid peptide (1-42) is able to induce neuronal death through the p75 neurotrophin receptor. This property may

explain the early and characteristic loss of cholinergic neurons in the septohippocampal pathway occurring in Alzheimer's disease [12].

These discrepancies may be related to the fact that defects in cholinergic systems could be rather related to Tau [13]. In AD, the pathway of neurofibrillary degeneration is well described. It starts from the hippocampal formation, reaches the polymodal association areas and then successively spreads to the unimodal association areas and the entire cerebral cortex [14-16]. However, the relationship between cholinergic systems and Tau pathology has been poorly explored. Interestingly, recent reports have shown that Tau aggregation in cholinergic neurons could also be found in individuals where the amyloid pathology is missing [17,18]. These data thus supported that Tau pathology found in cholinergic neurons is an early marker of the mild cognitive impairment-Alzheimer's disease continuum [18].

In the present study, we aimed to further investigate Tau aggregation in cholinergic systems, in our recently developed THY-Tau 22 transgenic mouse model, which nicely recapitulates the Alzheimer-type neurofibrillary degeneration in the absence of amyloid deposits [19,20].

Materials and methods

Animals

A stable colony of THY-Tau22 mice is maintained in our laboratory [19]. Male THY-Tau22 mice were bred with C57Bl6 females and the progeny was genotyped using PCR on DNA isolated from tail biopsy. In the present study, we have used heterozygous males THY-Tau22 and littermate wild type mice as controls. All experiments on animals were performed in compliance with, and following the approval of the local Animal Resources Committee, standards for the care and use of laboratory animals and with French and European Community rules.

Western Blot Analysis

For biochemical analysis and at each time point, we have used 5 THY-Tau22 mice and 5 littermate controls. At the time indicated, animals were sacrificed and brain removed. Several structures were dissected out (Cortex M1/S1, Striatum, Hippocampus and Cerebellum) using a coronal acrylic slicer (Delta Microscopies, France) at 4°C and stored at -80°C until use. Tissue was homogenized in 300 µl of RIPA buffer (Pierce, France) containing 0.5% w/v CHAPS, protease inhibitors (Complete, Roche, France) as well as phosphatase inhibitors (4 µg/mL okadaic acid and 5 µg/mL orthovanadate) using a Potter teflon glass homogenizer (70 strokes), sonicated and let under agitation for 1h at 4°C. Lysates were centrifuged at 12000g for 20 minutes at 4°C. The supernatant was removed and kept. The remaining pellet was homogenized in 100 µl of the same buffer, sonicated, let under agitation and centrifuged again. The resulting supernatant was pooled with the former one and proteins were quantified using the BCA system (Pierce). For Western Blot analysis, samples were diluted in NuPage sample buffer (Invitrogen) and denaturated at 100°C for 5 minutes. Then, 15 µg of proteins were loaded on 4-12% NuPAGE Novex gels (Invitrogen), and transferred to nitrocellulose or PVDF membranes and incubated with appropriate antibodies. Signals were visualized by chemiluminescence (ECL, Amersham Biosciences). Antibodies used for western blot were AT8, AT100 and HT7 (developed by Innogenetics and obtained from Perbio). AD2 and 988 are home-made antibodies and recognize pS396-404 and pS422 respectively.

Immunohistochemistry

To evaluate Tau pathology at an early time point in the THY-Tau22 model, 5 THY-Tau22 and 5 littermate controls were sacrificed at 3 months of age. Brains were fixed for 7 days in 4% paraformaldehyde, then incubated in 20% sucrose for 24 hours and finally kept frozen until use. Free-floating coronal sections (40µm) were obtained using a cryostat (Leica).

Sections of interest were used for free floating immunohistochemistry as previously described [19] and finally mounted on Superfrost slides respectively.

Retrograde transport

The quantification of septo-hippocampic retrograde transport was done by the stereotaxic injection of Fluorogold (FG) (2%) into the dentate gyrus/hilus in 7 THY-Tau22 mice and 7 littermate controles aged of 13 months. Three injections were made with 0.1µl FG in each hemisphere at the following coordinates AP -2.0 ML ± 1.3 DV -1.6, AP -2.3 ML ± 1.6 DV -1.65 and AP -2.6 ML ± 1.75 DV -1.65. After one week, mice were sacrificed and transcardially perfused sequentially with 0.9% NaCl and 4% paraformaldehyde in 0.1 mol/L phosphate-buffered saline (pH 7.4). Brains were postfixed for 2 days in 4% paraformaldehyde and cryoconserved as described above. Coronal sections were cut (40µm thick). Sections were mounted and coverslipped with Permafluor medium. Fluorescent cells were counted manually under a Zeiss Axiophot 2 microscope through the medial septum region. The mean number of immunopositive cells in each zone of the medial septum region was compared between wild-type and transgenic animals. Comparison between groups was performed using an unpaired Student t-test.

Results

THY-Tau22 mice express human 4-repeat tau mutated at sites G272V and P301S under a Thy1.2-promotor [19]. These mice display deficits in hippocampal synaptic transmission and impaired behaviour characterized by an increased anxiety, delayed learning from 3 months and reduced spatial memory at 10 months [19]. Conversely to other models, there are no sign of motor deficits or changes in motor activity over all investigated ages [19,23,24]. Importantly, starting from 3-6 months of age, Thy-Tau22 mice show, in the hippocampus, neurofibrillary tangle-like inclusions (Gallyas and MC1-positive) and hyperphosphorylation of Tau on several AD-relevant tau epitopes (AT8, AT100, AT180, AT270, pSer396 and pSer422) (Figure 1)[19]. Interestingly, western blot analysis demonstrated that brain areas are differentially affected by the Tau pathology (Figure 2). At 3 months of age hippocampus, M1/S1 cerebral cortex and striatum strongly expressed the transgene (HT7) and displays hyperphosphorylation (AD2, AT270). However, although already present in the hippocampus at early stage (3 months), abnormal Tau species (AT100, AP422) were faintly observed in the M1/S1 cerebral cortex and not in the striatum (Figure 2). Over the time, the pathology increases in the hippocampus and the cerebral cortex [19]. Thus, our transgenic model displays spatio-temporal features of Tau pathology as observed in AD, as well as several of the neurophysiological disturbances seen in this disorder [19,20].

In the present study, we have analyzed more carefully both AT8 and AT100 immunoreactivities in the basal forebrain of transgenic mice. As shown in figure 3, we have identified the presence of Tau pathology (both hyperphosphorylation with AT8 epitope and abnormal phosphorylation with AT100 epitope) in nuclei corresponding to the basal forebrain cholinergic neurons (BFCN) including nucleus basalis of Meynert, horizontal limb of the diagonal band and medial septum. In young animals (3 months old), a few AT100- and AT8-immunoreactive BFCN were identified (Figure 3E-G).

In order to evaluate whether Tau pathology could not only affect cholinergic neurons but also their projection to hippocampus, we have evaluated AT8 immunoreactivity on saggital sections. As shown in figure 4, septohippocampal fibers were immunoreactive, further indicating that Tau pathology affects cholinergic BFCN neurons and supporting that axonal transport within cholinergic neurons may be impaired. To functionally assess this latter hypothesis, FluoroGold, a retrograde tracer, was injected within the hippocampus of both THY-Tau22 and wild-type littermate controls. FluoroGold-labelled BFCN were then identified under fluorescent microscope in medial septum. There was a fifty percent decrease in FluoroGold-positive neurons in medial septum in THY-Tau22 compared to wild-type littermate controls (Figure 5).

Discussion

THY-Tau22 strain presents an age-dependent development of Tau pathology leading to synaptic dysfunctions as well as learning and memory impairments without motor deficits [19]. In the present work, we observed that early Tau pathology starts in both hippocampus and basal forebrain. Moreover, alterations in axonal transport likely induced by Tau pathology were also observed in the septohippocampal pathway, suggesting a disconnection between these two key brain regions.

In AD, Tau pathology is known to start within the hippocampal formation, namely the entorhinal cortex and the hippocampus, and then to spread to isocortical areas. More recently, an early contribution of Tau pathology in the nucleus basalis of Meynert has been suggested [17]. Indeed, in the brains of elderly individuals at Braak stages I or II and showing no amyloid deposit, AT8-immunoreactivity was found in cholinergic neurons of the nucleus basalis of Meynert [17] indicating a possible early dysfunction of the cholinergic pathway in

the course of the disease [18]. Our THY-Tau22 model appears to replicate, at least in part, this phenotype and further support the interest in this transgenic strain.

In humans, cholinergic neurons of the basal forebrain innervate different cortical regions and may be involved in memory formation, particularly within the hippocampus [25]. For instance, BFCN cholinergic inputs from medial septum (MS) to the hippocampus appear to be of critical importance in mediating mnemonic function [26]. This function of cholinergic neurons on the maintenance of memory function is, in part, supported by a cross-talk with the hippocampus since hippocampal neurons produce neurotrophins as Nerve Growth Factor which are retrogradely transported to the basal forebrain to sustain their survival (for review, [27]).

THY-Tau22 mice have been shown to display learning and memory dysfunctions using Morris Water Maze test [19]. The early learning alterations together with the later memory dysfunctions seen in this model fit well with the early Tau pathology found in the basal forebrain and the later pathology observed in the septo-hippocampal pathway. These data suggest that septo-hippocampic dysfunction may be involved in memory loss in this model. This is further supported by Fluoro-Gold experiments which demonstrate an impairment of the retrograde axonal transport. This observation is in line with the recent work showing that normal Tau proteins favour kinesin- and dynein-dependent axonal transport [28] and thus, the trophic support of hippocampus-secreted factors to cholinergic neurons is likely to be affected. Finally, it appears that THY-Tau22 mice well reflect the basal forebrain alterations seen in AD supporting that Tau pathology may participate to cholinergic defects observed in the AD patient. This mouse to human comparison is allowed by the observation that comparable impairments in memory function are found in basal forebrain-damaged rats and patients with dementia of the Alzheimer's type, suggesting that there might be important

homologies between rodents and humans in terms of basal forebrain-mediated memory function [29-31].

Therapies aiming to recover cholinergic functions, such as anticholinesterase molecule, are the major therapeutical strategy used in AD patients [9]. Their use only delays clinical symptoms. Given the early dysfunctions of cholinergic neurones in AD and their importance in learning and memory processes, it remains crucial to improve current treatments. Besides the fact that our model emphasizes the importance of Tau pathology in such defects, the comparable sequence of septo-hippocampic defect seen in THY-Tau22 mice and AD patients indicates that this model is an invaluable tool for studying molecular actors of cholinergic dysfunctions and uncovering new therapeutical targets.

Acknowledgements

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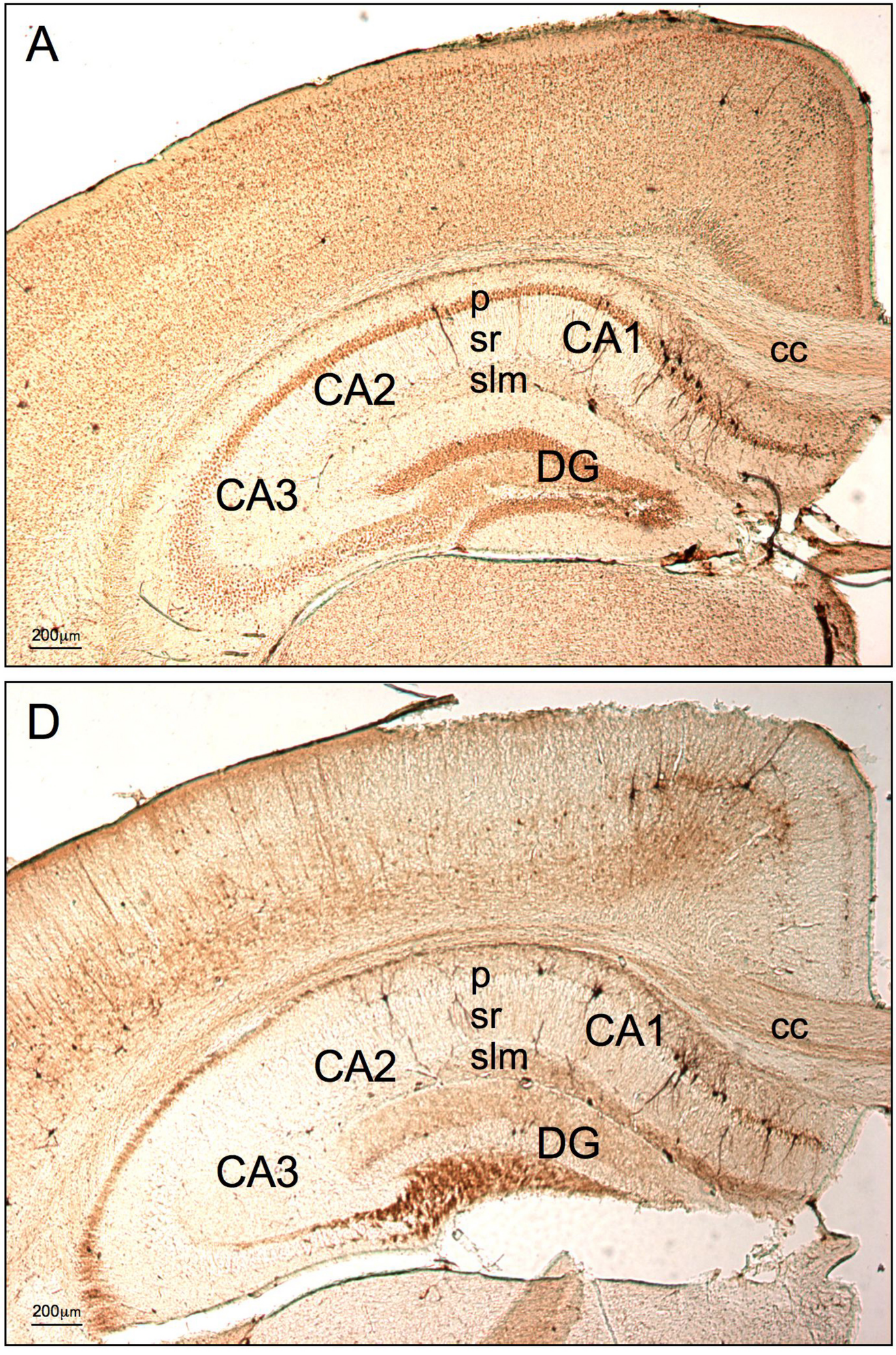
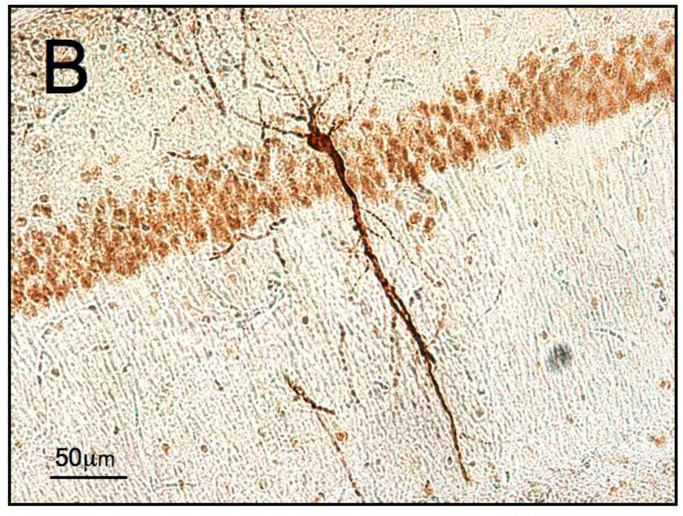
Figure 1

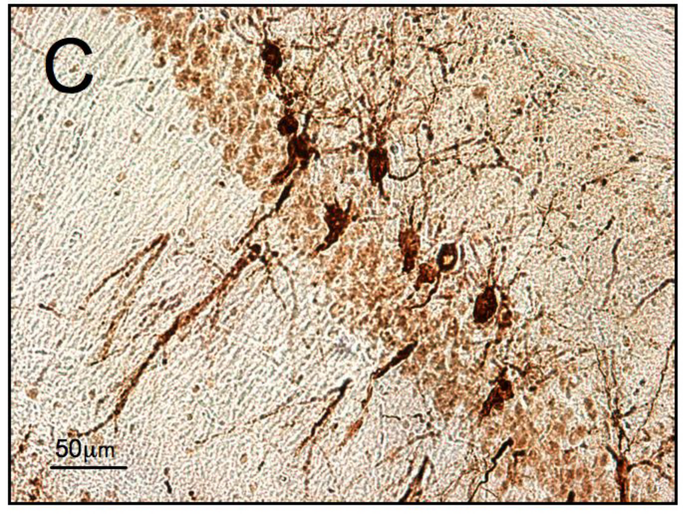
Tau phosphorylation mapping of the brain in young THY-Tau22 (3 months old) mice by immunohistochemistry.

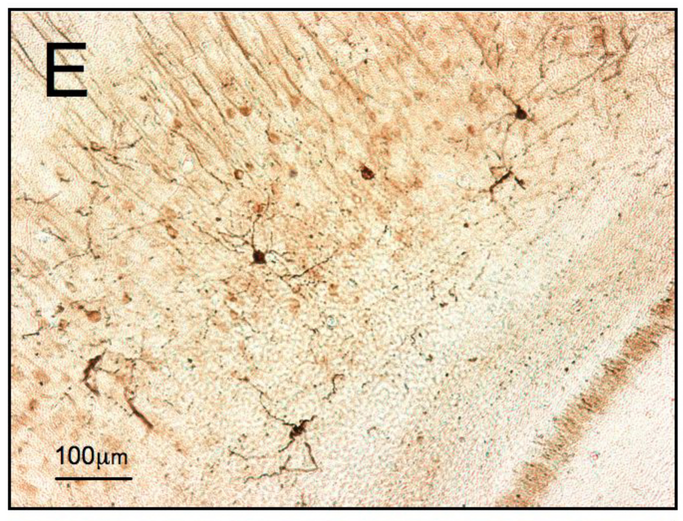
At three months old, AT100-immunoreactivity is rare and mostly located in the CA1 pyramidale layer (A-C). AT8-immunoreactivity is also found in the same hippocampal region with a greater extend and intensity (D-F). It is also found in other cortical areas (DE).

CA1–3, hippocampal fields CA1–3; cc, corpus callosum; DG, dentate gyrus; p, pyramidal cell layer; slm, stratum lacunosum moleculare; sr, stratum radiatum. Note the non-specific nuclear staining of AT100, which allows for a better cytoarchitecture of the hippocampus.









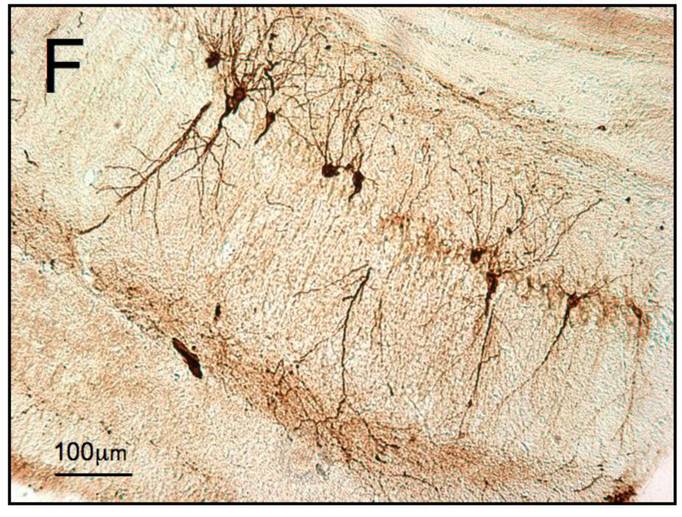


Fig. 1

Figure 2

Tau phosphorylation mapping of the brain in young THY-Tau22 (3.5 months old) mice by immunoblotting.

Immunoblot analysis of the major AD-relevant tau phosphorylation epitopes in different brain areas in three different animals. There is no major difference in Tau profiles among the three animals indicating that the Tau expression and phosphorylation does not strongly differ in same series of animals. Human Tau, as visualized by HT7 antibody as two main bands, is similarly expressed in all brain regions with the exception of the cerebellum. Tau hyperphosphorylation (AD2) gives a similar pattern of immunoreactivity than HT7. In cerebellum, AD2-immunoreactivity is also found whereas no other labelling is observed. AD2 is the only antibody, which recognizes both murine and human Tau. Only the upper band is visualized using antibodies against abnormal Tau phosphorylation (AT100 and 988) and it is mainly found in the hippocampus and weakly observed in cortex.

Cbl, cerebellum; Cx M1/S1, Motor and sensory cortex; Hipp, hippocampus; Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is used as a loading control.

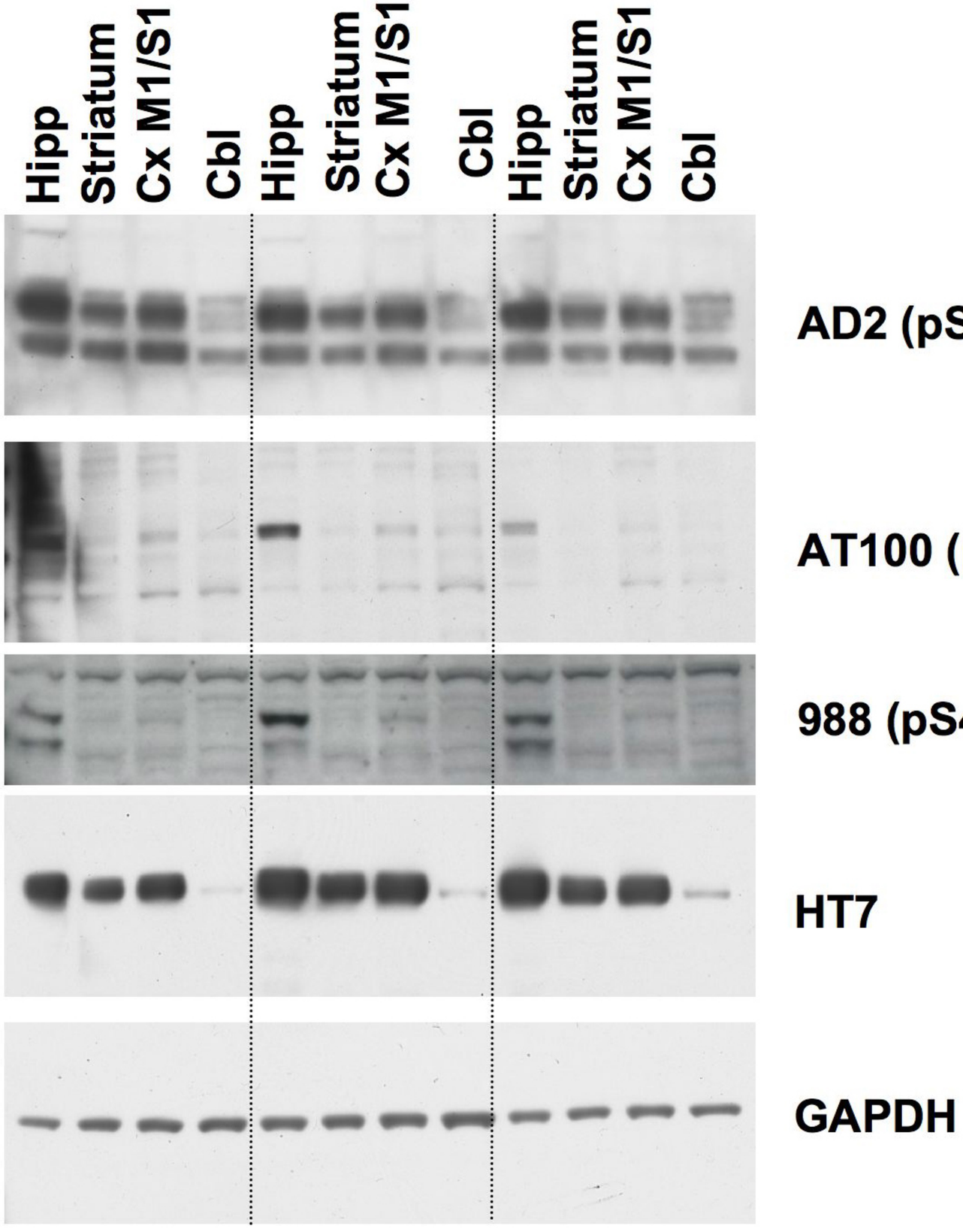

Hipp
Striatum
Cx M1/S1
Cbl
Hipp
Striatum
Cx M1/S1
Cbl
Hipp
Striatum
Cx M1/S1
Cbl
AD2 (pSer396-404)
AT100 (pThr212-214)
988 (pS422)
HT7
GAPDH

Fig. 2

Figure 3

Tau phosphorylation mapping of the basal forebrain in THY-Tau22 mice by immunohistochemistry.

Tau pathology in different basal forebrain areas (A and D, nucleus basalis of Meynert; B, horizontal limb of the diagonal band; C, medial septum) in THY-Tau22 (12 months old) mice. AT8 was used to visualize Tau hyperphosphorylation (A-C) and AT100 to detect AD-specific abnormal phosphorylation (D). In THY-Tau22 (3 months old), Tau pathology in found in rare basal forebrain cholinergic neurons (BFCN). AT100 was used to visualize Tau abnormal phosphorylation in degenerating BFCN (E,F) and AT8 to detect Tau hyperphosphorylation (G).

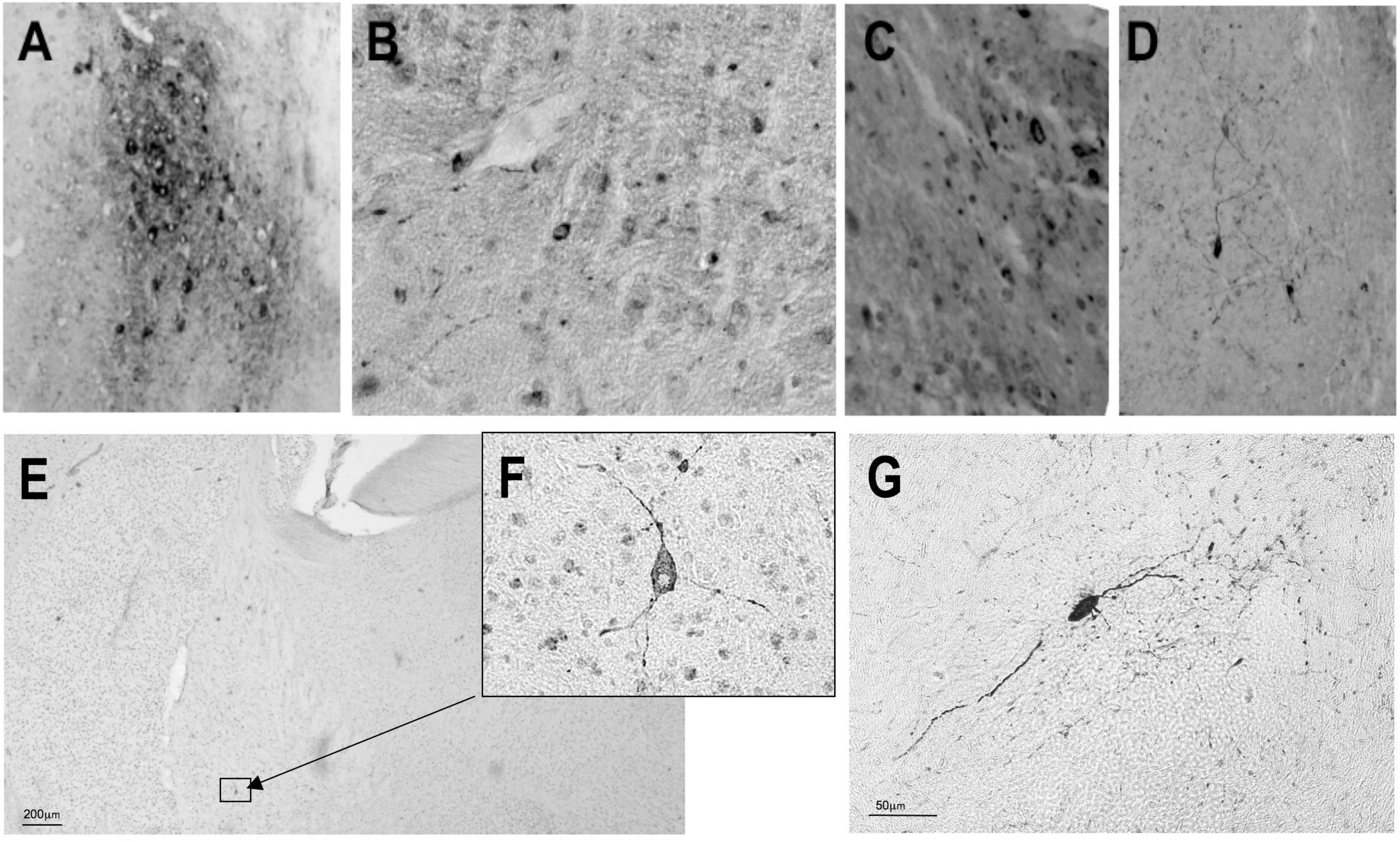


Fig. 3

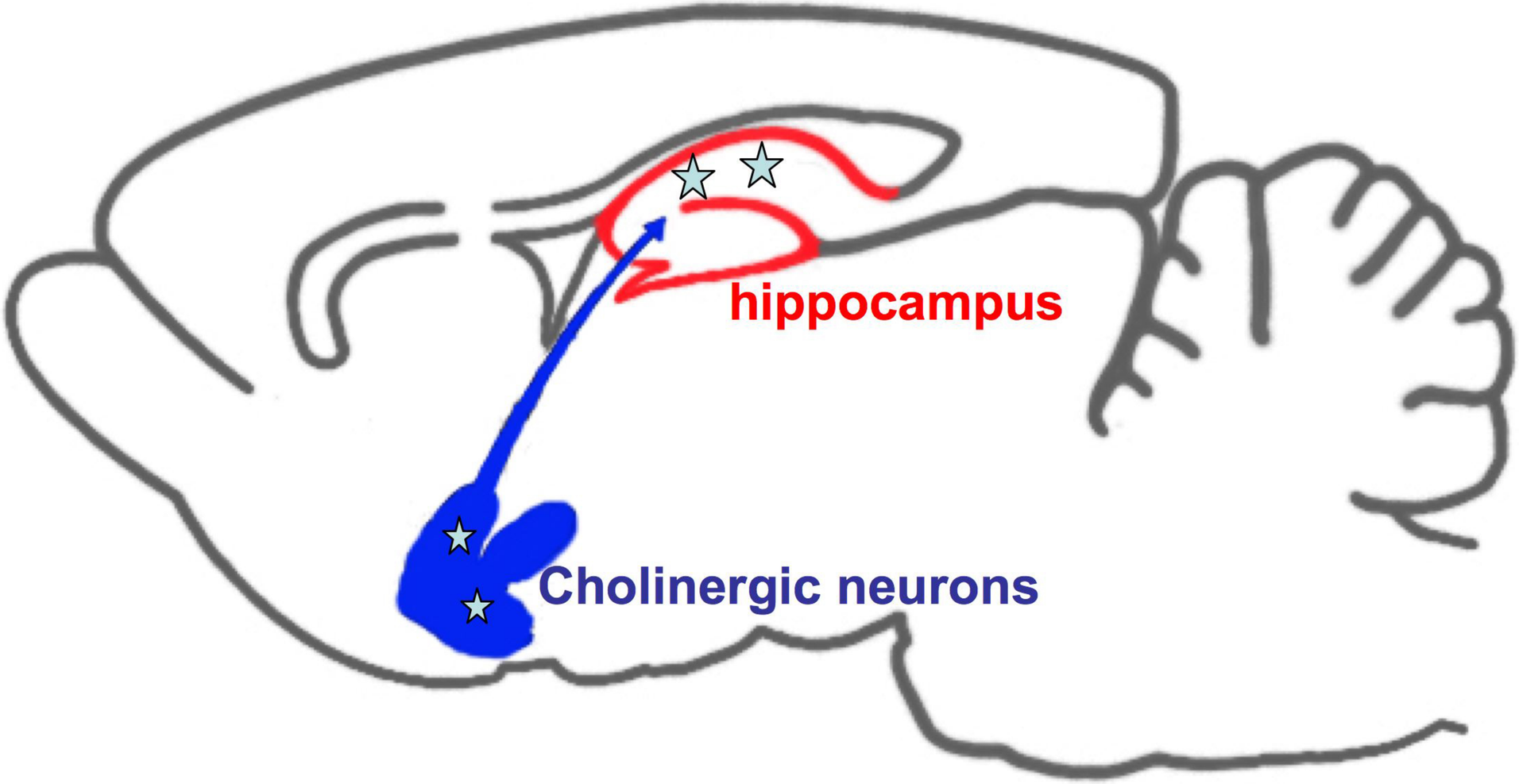
Figure 4

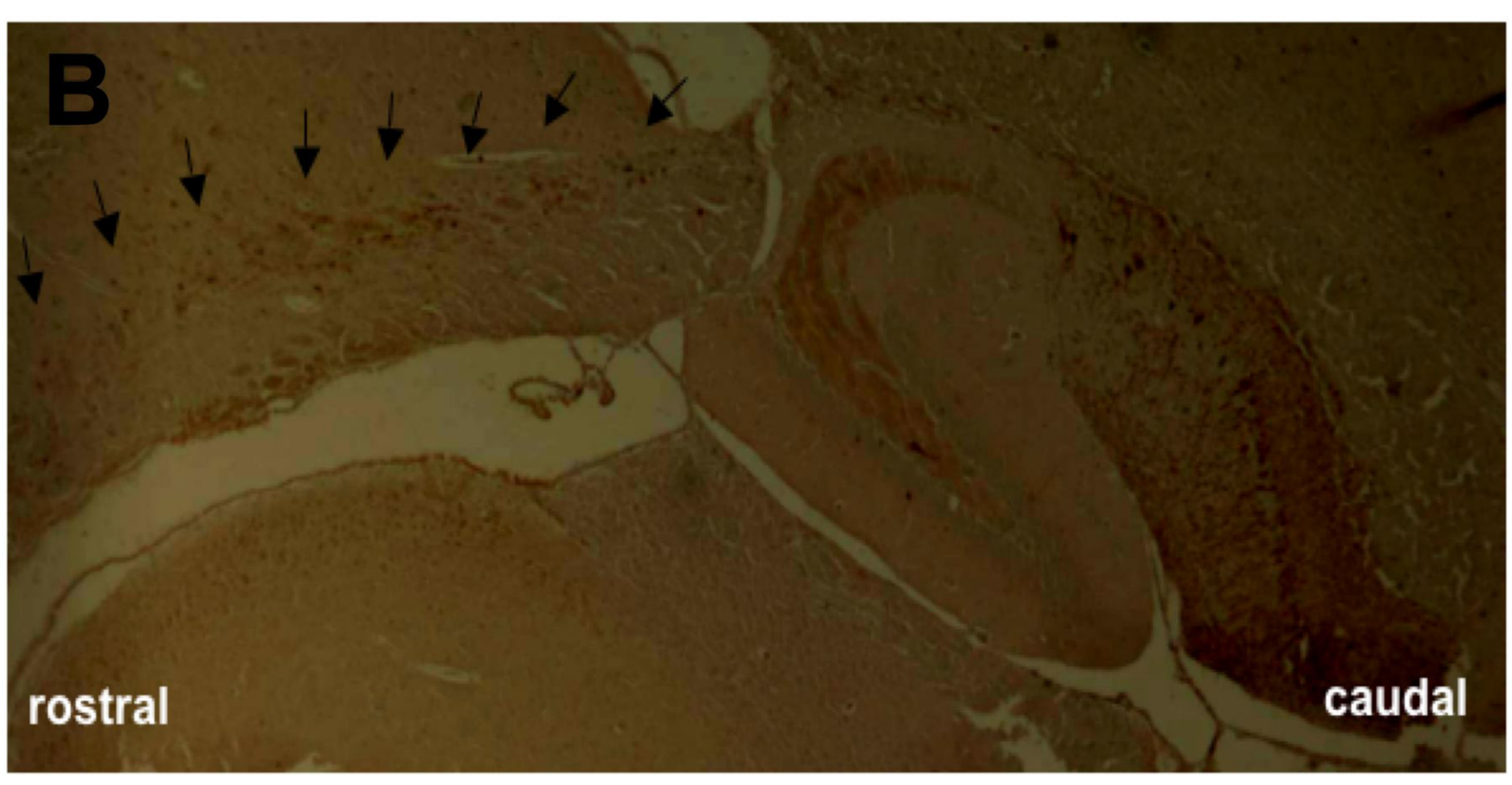
The septohippocampal pathway

A. Schematic representation of the septohippocampal pathway: connecting fibers from basal forebrain cholinergic neurons (in blue) to the hippocampus (in red). Stars indicate AT8-immunoreactivity.

Tau pathology (as visualized by AT8 antibody) in fiber connections projecting from the basal forebrain to the hippocampus (B, lateral setion of the septohippocampal pathway; C, medial section of the septohippocampal pathway).

A


hippocampus
Cholinergic neurons


B
rostral
caudal

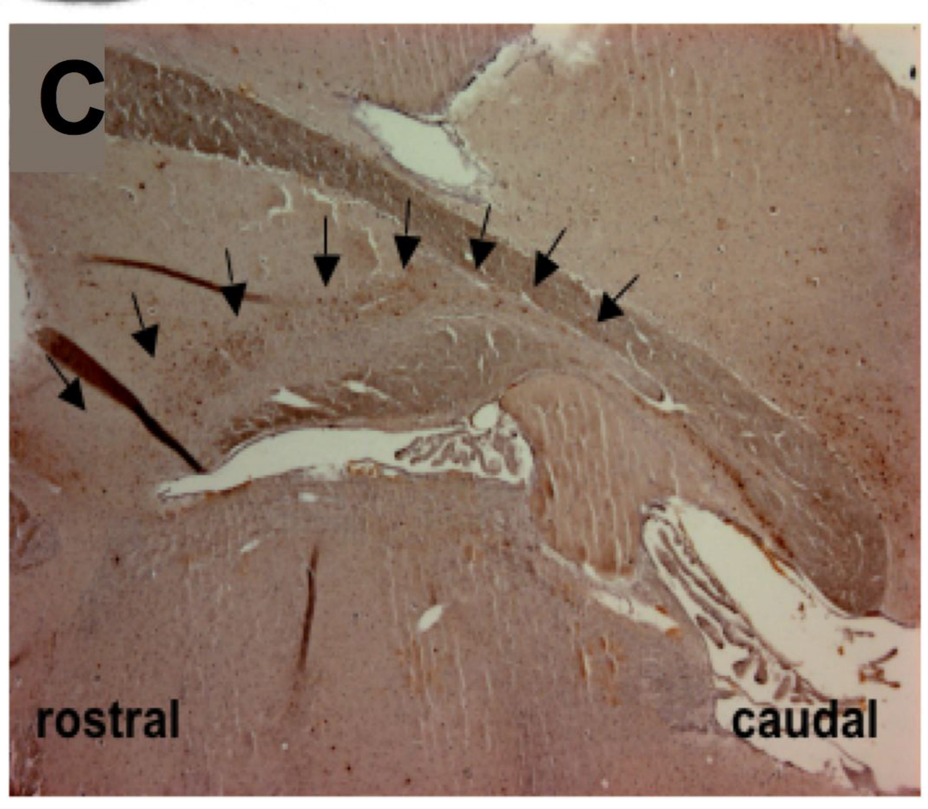
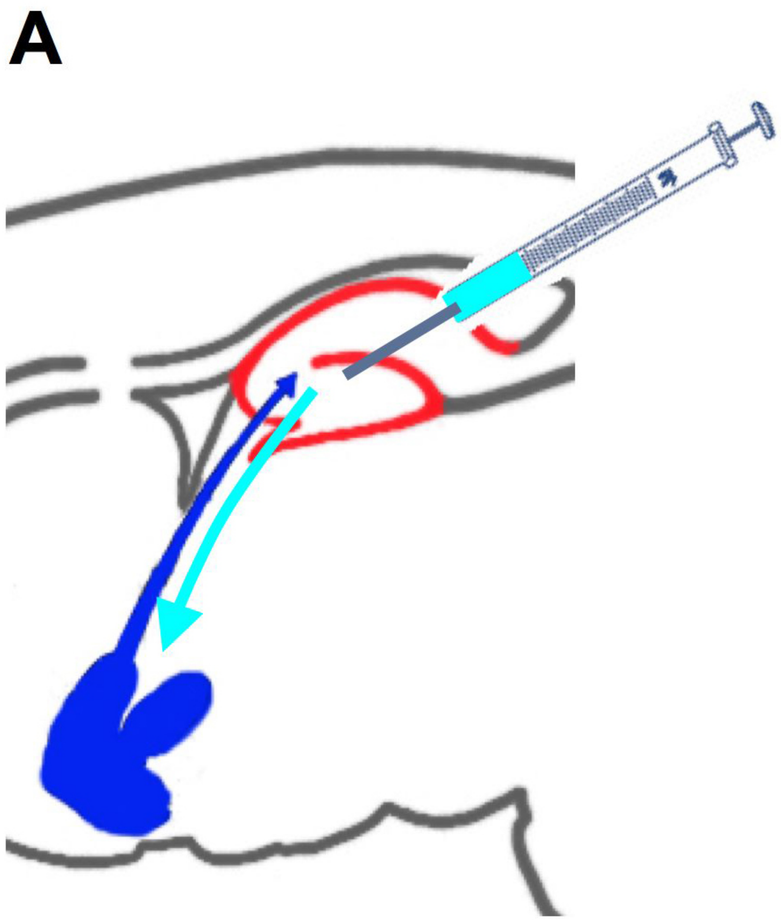

C
rostral
caudal

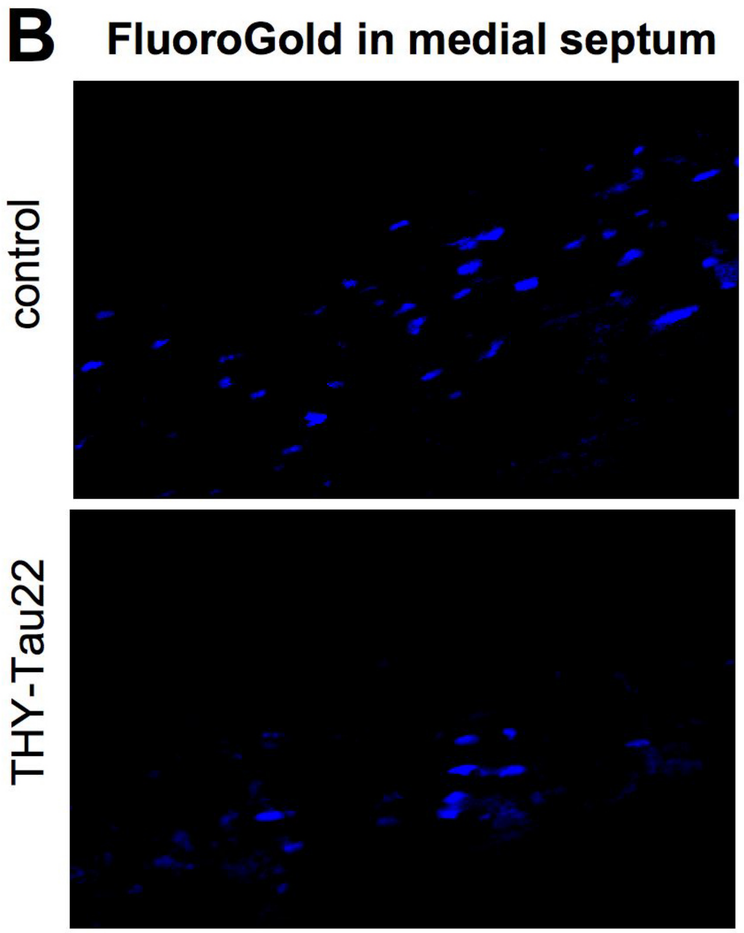
Fig. 4

Figure 5

Impairment of retrograde axonal transport in basal forebrain cholinergic neurons

The retrograde tracer FluoroGold (light blue) is injected within the hippocampus (in red) and then transported to the cell body of the basal forebrain cholinergic neurons (A). FluoroGold-labelled basal forebrain cholinergic neurons were then identified under fluorescent microscope in medial septum in both THY-Tau22 and wild-type littermate controls (B). After quantification, a fifty percent significant decrease in FluoroGold-positive neurons in THY-Tau22 was observed when compared to wild-type littermate controls (C, $p<0.05$).


A


B
FluoroGold in medial septum
control
THY-Tau22

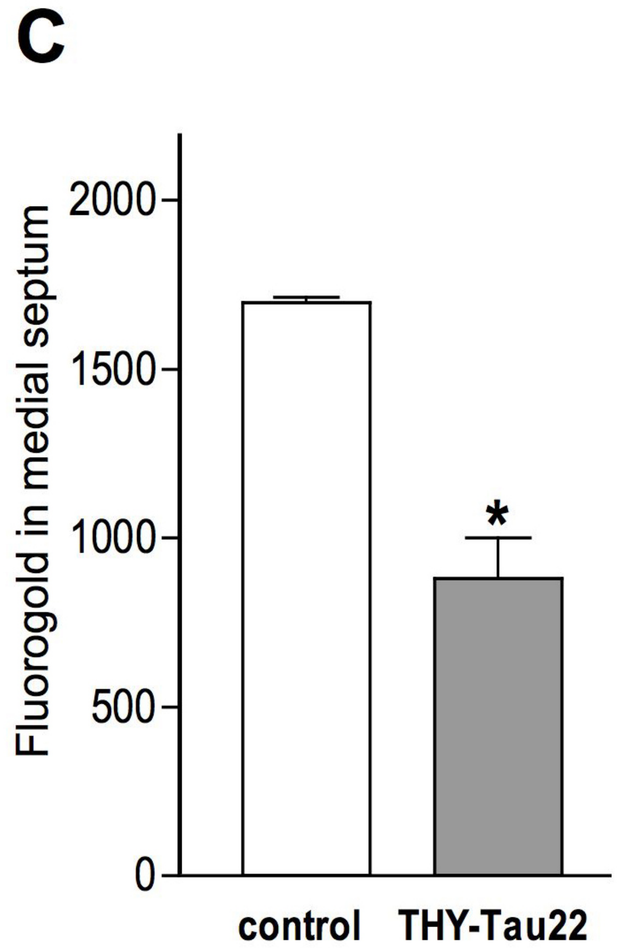

C
Fluorogold in medial septum
2000
1500
1000
500
0
*
control
THY-Tau22

Fig. 5