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ORIGINAL PAPER

N-homocysteinylation of tau and MAP1 is increased in autopsy specimens of Alzheimer's disease and vascular dementia

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Abstract

The pathomechanisms that associate a deficit in folate and/or vitamin B12 and the subsequent hyperhomocysteinemia with pathological brain ageing are unclear. We investigated the homocysteinylation of microtubule-associated proteins (MAPs) in brains of patients with Alzheimer's disease or vascular dementia, and in rats depleted in folate and vitamin B12, *Cd320* KO mice with selective B12 brain deficiency and H19-7 neuroprogenitors lacking folate. Compared with controls, N-homocysteinylated tau and MAP1 were increased and accumulated in protein aggregates and tangles in the cortex, hippocampus and cerebellum of patients and animals. N-homocysteinylation dissociated tau and MAPs from β-tubulin, and MS analysis showed that it targets lysine residues critical for their binding to β-tubulin. N-homocysteinylation increased in rats exposed to vitamin B12 and folate deficit during gestation and lactation and remained significantly higher when they became 450 days-old, despite returning to normal diet at weaning, compared with controls. It was correlated with plasma homocysteine (Hcy) and brain expression of methionine tRNAsynthetase (MARS), the enzyme required for the synthesis of Hcy–thiolactone, the substrate of N-homocysteinylation. Experimental inactivation of MARS prevented the N-homocysteinylation of tau and MAP1, and the dissociation of tau and MAP1 from β-tubulin and PSD95 in cultured neuroprogenitors. In conclusion, increased N-homocysteinylation of tau and MAP1 is a mechanism of brain ageing that depends on Hcy concentration and expression of MARS enzyme. Its irreversibility and cumulative occurrence throughout life may explain why B12 and folate supplementation of the elderly has limited effects, if any, to prevent pathological brain ageing and cognitive decline.
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Keywords: homocysteine; microtubule-associated proteins; tau; folate; vitamin B12; aging; Alzheimer-type dementia

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No conflicts of interest were declared.

Introduction

Alzheimer's disease (AD) is the most common form of dementia; its incidence doubles every 5 years after 65 years of age. Abnormal tau lesions, including accumulation of hyperphosphorylated and aggregated tau protein in neurofibrillary tangles, play a crucial role in the pathomechanisms of the preclinical phase [1]. The plasma level of homocysteine (Hcy) is a marker closely associated with age, AD and vascular dementia (VD) [2–4]. Furthermore, deficits in vitamin B12 and folate (B9) can produce hyperhomocysteinemia through their role in Hcy conversion to methionine by methionine synthase [2]. Hyperhomocysteinemia is a predictor of the onset and severity of AD [4,5] and of aging-related cognitive decline [6–8]. It is also associated with white matter hyperintensities and brain atrophy [8,9].

The role of B vitamin supplements to lower Hcy is well established [10]. However, most interventional trials with folate/B12 supplementation have failed to find a benefit of Hcy-lowering on cognitive decline [10–14]. Recently, B-vitamin treatment was shown to slow brain shrinkage [2] and cognitive decline [15] in patients with raised Hcy at baseline.

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High concentrations of Hcy have been shown to exert harmful effects on neurons through reversible mechanisms such as oxidative stress, endoplasmic reticulum stress, excitotoxicity and epigenomic mechanisms [16–18]. Hcy can produce cumulative effects by two mechanisms demonstrated in various models [19–23]. One is the reversible S-homocysteinylation of protein cysteine residues, and the other is the more stable N-homocysteinylation of lysine residues by Hcy–thiolactone (HcyTh), a compound produced by methionine tRNAsynthetase (MARS) [24].

Tau is the major component of microtubule-associated proteins (MAPs) in neurons [25,26]. MAPs stabilize microtubules, a function that is reduced by their increased phosphorylation and/or impaired degradation. Despite the clinical and experimental evidence of the link of folate/B12 deficiency and hyperhomocysteinemia with tau hyperphosphorylation [10,26], nothing is known on the homocysteinylation of MAPs. We addressed this issue by investigating the homocysteinylation of tau and other MAPs in brains of patients with AD and other dementia, and in complementary animal and cell models. We demonstrated that tau, MAP1, and MAP4 are homocysteinylated and accumulate in protein aggregates in brains of patients as well as in experimental models. This process leads to impaired functional interactions of MAPs with partner proteins that may affect synaptic plasticity and cognitive functions.

Materials and methods

Further details can be found in supplementary material, Supplementary materials and methods.

Human samples

Fixed, paraffin-embedded blocks of postmortem brain tissues were obtained from the neuropathology centers of the University Hospital Centers from Lille and Montpellier (France) and the Oxford Project to Investigate Memory and Ageing (OPTIMA, Oxford, UK). OPTIMA is part of the Brains for Dementia Research Initiative (BDR), a UK network of five centers for brain collection, financed by The Alzheimer's Research Trust and the Alzheimer's Society. Brain samples from French centers came from four Alzheimer patients with Braak V/VI stage and definite CERAD, and four age-paired cognitively intact control subjects with Braak 0–II and negative CERAD. Brains of the OPTIMA collection originated from three patients with VD, eight with definite CERAD AD, three with undetermined dementia and three controls. The clinical and neuropsychological evaluation was done every year for at least 3 years. They were classified in four groups according to Braak staging [27] and dementia: control subjects with Braak I/II and normal brain ($n = 3$), patients with VD and Braak I/II ($n = 3$), patients with intermediate likelihood for the diagnosis of AD and Braak III/IV ($n = 3$) and CERAD Alzheimer patients with Braak V/VI ($n = 8$). Consent for autopsy was obtained in accordance with local institutional review board requirements. The median of the delay between death and autopsy was 25 h. A standard procedure was used for processing the tissues, with fixation in 10% formalin for at least 4 weeks. PMI were similar for controls and dementia cases. Blocks were used for immunohistochemistry and 'proximity ligation' assays (Duolink®). Cerebrospinal fluid (CSF) samples were from the University Hospital Center of Montpellier.

Animal experiments

In vivo experiments were performed on a validated animal model of methyl donor deficiency repeatedly shown to induce hyperhomocysteinemia [23,28,29]. They were conducted in compliance with the international guidelines for the care and use of laboratory animals, and were approved by the local University Research Ethics Board. Wistar rats (Charles River, l'Arbresle, France) were maintained under standard conditions, on a 12 h light/dark cycle, with food and water available *ad libitum*.

For gestational deficiency, adult females were fed 1 month before mating either a standard diet (Maintenance diet M20, Scientific Animal Food and Engineering, Villemoisson-sur-Orge, France) or a methyl donor deficient (MDD) low-choline diet (119 versus 1780 mg/kg) lacking folate and B12 (Special Diet Service, Saint-Gratien, France) [23,28]. Methionine content (~0.4%) was similar in both diets. Fetuses were collected at embryonic day 20 (E20), and pups were studied at postnatal day 21 (21d) or kept under standard conditions until 450 days of age.

Adult deficiency was tested in 6 month-old female rats. They received either the normal diet or the MDD diet for 4 months. We additionally used a transgenic mouse model with ablation of the transcobalamin receptor required for vitamin B12 uptake in the brain (*Cd320* KO) [30]. Further details are presented in supplementary material, Supplementary materials and methods.

Animals were euthanized by excess isoflurane, and their brains were rapidly dissected before freezing in liquid nitrogen. For immunochemistry, brains were fixed in 4% paraformaldehyde (24–48 h) at 4 °C, dehydrated and embedded in paraffin.

Behavioral evaluation was performed on rat pups. A homing test was conducted as described previously [31] to monitor basic learning functions. An Aquatic Multiple T-Maze was used to evaluate learning performances in adults, and consisted of a modified Morris Water Maze [32]. The two major parameters recorded were the escape latency and the number of errors during a trial (see supplementary materials, Supplementary materials and methods).

Cell cultures

To allow selective mechanistic analyses, we used cultured hippocampal neuroprogenitor cells. H19-7/IGF-IR

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cell line (ATCC# CRL-2526) was grown as described [20], and methyl donor deficiency was induced by using DMEM lacking B9 (Invitrogen, Cergy-Pontoise, France), as in previous studies [20]. In some experiments, to mimic endogenous Hcy accumulation, cells were treated with 100 µM HcyTh (Sigma-Aldrich, Saint-Quentin Fallavier, France), the substrate for homocysteinylation through MARS enzyme.

Immunohistological analyses

Brain sections from the CA1 hippocampal layer, prefrontal cortex, and the junction of molecular layer and Purkinje cells at the third lobe of the cerebellum were analyzed. In addition, cells cultured on poly-L-lysine-precoated glass coverslips were washed, fixed with 4% paraformaldehyde, and permeabilized with 0.1% Triton X-100 (Sigma-Aldrich), as reported by Akchiche *et al* [20]. Nonspecific binding sites were blocked in phosphate-buffered saline containing 1% bovine serum albumin and incubation was performed overnight with a primary antibody (see supplementary material, Table S1). Immunoreactivity was assessed in the presence of an appropriate secondary anti-IgG antibody conjugated to AlexaFluor for 1 h at 25 °C (Life Technologies, Saint-Aubin, France). Immunofluorescence visualization, image acquisition (×20 and ×60 magnification) and unbiased cell counts in randomly selected fields were performed with a BX51WI microscope (Olympus, Rungis, France) coupled to a ProgRes MF cool camera (Jenoptik, Lordil, Nancy, France) or a confocal microscope (Nikon Instruments, Champigny-sur-Marne, France) and analyzed by Cell® software (Brighton, United Kingdom). Autofluorescence was extremely low and diffuse in both AD and non-AD samples. It was evaluated, including in control experiments without primary antibody or secondary antibody. Moreover, autofluorescence was limited by using very short exposure times.

Protein interactions

To assess Hcy interaction with proteins, coimmunoprecipitation experiments were performed by means of a Pierce® Co-Immunoprecipitation kit (Thermo Fisher Scientific, Illkirch, France) [20]. Hcy coimmunoprecipitation was analyzed by mass spectrometry (MS), as described by Ren *et al* [33]. Further details are reported in Supplementary materials and methods.

In addition, the proximity ligation assay (Duolink® *in situ* PLA™ reagents, Olink Bioscience, Angers, France) was used to depict and quantify protein interactions [20] (see supplementary material, Supplementary materials and methods).

Western blotting

Following protein extraction with RIPA buffer, western blotting analyses were performed by standard procedure with chemiluminescence using ECL system (Bio-Rad, Marnes-la-Coquette, France), as previously detailed [23]. Primary antibodies are listed in supplementary material, Table S1. Glyceraldehyde-3-phosphate dehydrogenase was used as an internal standard. Polyvinylidenedifluoride membranes were incubated for 1 h at room temperature with the corresponding horseradish peroxidase-conjugated preadsorbed secondary antibody (Santa Cruz, Nanterre, France). All experiments were performed in triplicate.

Small interfering RNA and cell transfection

MARS expression in cell cultures was silenced using small interfering RNA (siRNA) (see supplementary material, Supplementary materials and methods).

Statistical analyses

Data were analyzed with Statview 5 software for Windows (SAS Institute, Berkley, CA, USA). They were compared by using ANOVA with Fisher's test. P values <0.05 were considered to indicate significance.

Results

Homocysteinylation of tau and MAPs is higher in patients with AD or VD than in age-paired controls, and promotes the dissociation of tau and MAPs from beta-tubulin

Immunohistological examination of human brains from the French neuropathological centers showed an accumulation of Hcy and its increased colabeling with tau in cortex, hippocampus and cerebellum in brains from Alzheimer patients compared with those from age-paired control subjects (Figure 1A,B). This colocalization, suggestive of tau homocysteinylation, was also observed in aggregates of tau protein and in tangles. Tau/Hcy, Tau/β-tubulin, and MARS/Hcy interactions were studied using the proximity ligation assay (Duolink®) in tissue sections. Interaction of Hcy with tau was significantly higher in brain tissues from Alzheimer patients than in those of controls (Figure 1C). Conversely, there was a significant decrease in the interaction between tau and its partner protein β-tubulin in the three brain areas analyzed from Alzheimer patients. These changes were accompanied by a two-fold increase in the coimmunodetection of MARS and Hcy by Duolink® in tissues of Alzheimer patients. We confirmed the homocysteinylation of tau by its coimmunoprecipitation with anti-Hcy and anti-tau in the CSF of Alzheimer patients (Figure 1D). Tau homocysteinylation was also observed in the CSF of control subjects, but to a lesser extent. This homocysteinylation was more elevated in CSF samples from patients with Hcy level in the highest quartile (>2.0 µM), compared to those with a concentration in the lowest quartile (<1.0 µM). Treatment with mercaptoethanol did not remove homocysteinyl residues, indicating that homocysteinylation did not involve disulfide bonds.

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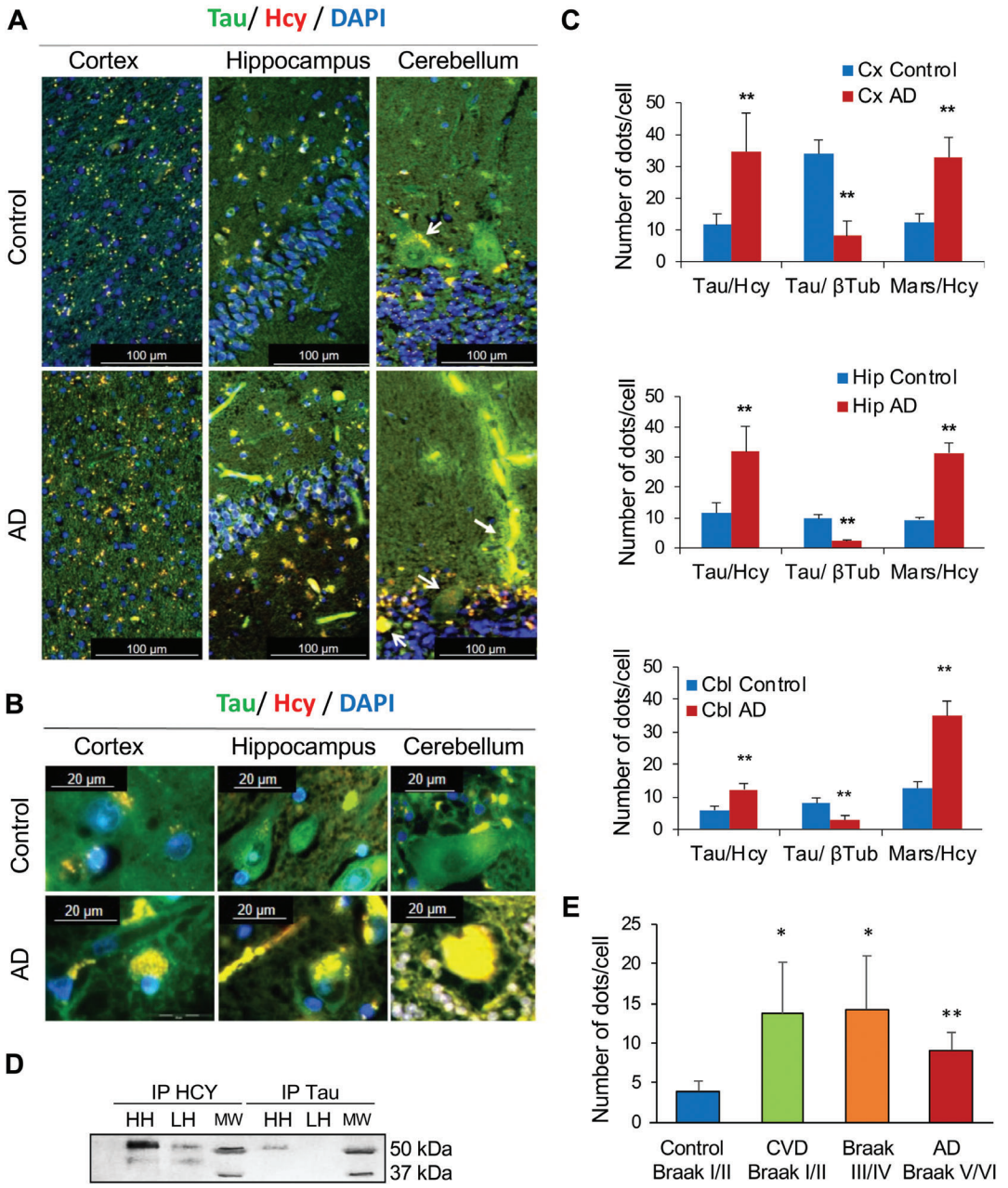


Figure 1. Tau homocysteinylation and its interaction with β-tubulin in human brain sections and CSF. (A and B) Immunohistochemical analysis at x20 (A) and x60 (B) magnification of tau and Hcy in cortex, hippocampus and cerebellum tissue sections from AD cases and age-paired controls from French neuropathological centers. Cell nuclei were counterstained by DAPI. (C) Quantification of *in situ* interactions between Tau/Hcy, Tau/β-tubulin, and MARS/Hcy monitored by the Duolink® assay in cortex (Cx), hippocampus (Hip) and cerebellum (Cbl) tissue sections from control ($n = 4$) and AD ($n = 4$) brains. Pictures were analyzed by the BlobFinder freeware. Statistically significant difference between control and AD: $^{**}p < 0.01$. (D) Tau homocysteinylation in the CSF of AD patients with high (HH) or low (LH) homocysteinemia as shown by Hcy immuno-precipitation and tau immunoprecipitation followed by Hcy immunodetection. Experiments were performed in triplicate. (E) Oxford OPTIMA collection: quantification of *in situ* interactions between tau and Hcy monitored by the Duolink® assay in hippocampus tissue sections from the brain of control subjects (Control Braak I/II, $n = 3$), patients with cerebrovascular disease (VD Braak I/II, $n = 3$), patients with neurofibrillary degeneration (Braak III/IV, $n = 3$) and AD (AD, Braak V/VI, $n = 8$). Statistically significant difference from control: $^{*}p < 0.05$, $^{**}p < 0.01$.

Increased homocysteinylation was more pronounced for the highest molecular size isoforms (Figure 1D).

We additionally examined the hippocampus in four sets of brains issued from the OPTIMA collection: patients with either VD (Braak I/II), undetermined dementia (Braak III/IV) or AD (Braak V/VI), and control subjects (Braak I/II). The main characteristics of the corresponding patients are summarized in supplementary material, Table S2. As compared with controls, interaction between Hcy and tau was increased in the hippocampus of patients with VD, undetermined dementia and AD (Figure 1E). It is noteworthy that homocysteinylation of tau and MAP1a in AD Braak V/VI patients was underestimated as Hcy interactions with other proteins could not be counted by the software as individual dots in protein aggregates and tangles (see

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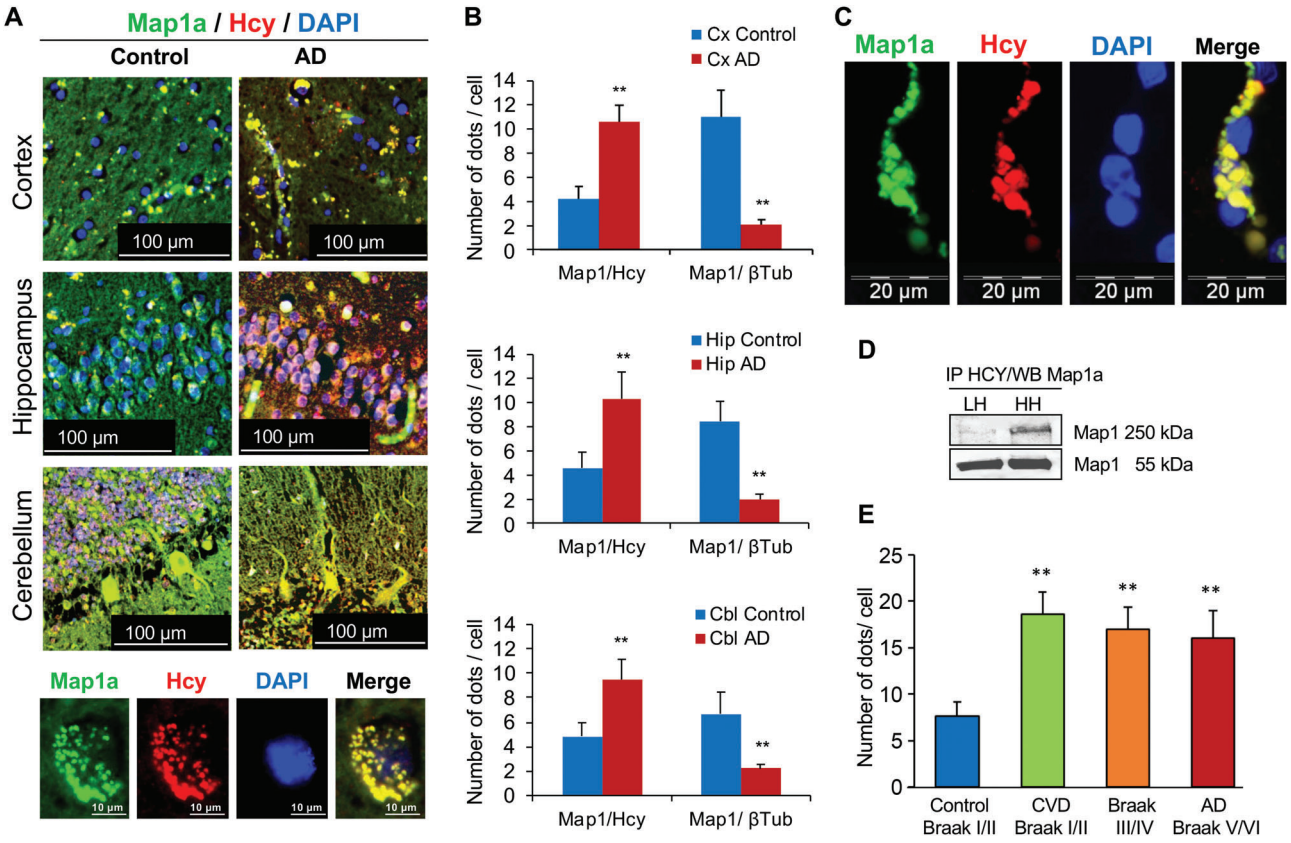


Figure 2. MAP1a homocysteinylation and its interaction with β-tubulin in human brain sections and CSF. (A) Immunohistochemical analysis of MAP1a and Hcy in cortex, hippocampus and cerebellum tissue sections from AD cases and age-paired controls from French neuropathological centers. Note cell colocalization of the two proteins, and the presence of aggregates (lower panel at higher magnification). Cell nuclei were counterstained by DAPI. (B) Quantification of *in situ* interactions between MAP1a/Hcy, and MAP1a/β-tubulin monitored by the Duolink® assay in cortex (Cx), hippocampus (Hip) and cerebellum (Cbl) tissue sections from control ($n=4$) and AD ($n=4$) brains. Statistically significant difference between control and AD: $^{**}p<0.01$. (C) Accumulation of MAP1a/Hcy coaggregates in neurofibrillary tangles. (D) MAP1a homocysteinylation in the CSF of AD patients with high (HH) or low (LH) homocysteinemia as shown by Hcy immuno-precipitation followed by MAP1 Western blot immunodetection (IP Hcy/ WB MAP1). Experiments were performed in triplicate. (E) Oxford OPTIMA collection: quantification of *in situ* interactions between tau and Hcy monitored by the Duolink® assay in hippocampus tissue sections from the brain of control subjects (Control Braak I/II, $n=3$), patients with cerebrovascular disease (VD Braak I/II, $n=3$), patients with neurofibrillary degeneration (Braak III/IV, $n=3$) and AD (AD, Braak V/VI, $n=8$). Statistically significant difference from control: $^{**}p<0.01$.

supplementary material, Figure S1). Taken together, these data showed a hyperhomocysteinylation of tau in the three dementia groups.

Higher homocysteinylation of MAP1a was also observed in Alzheimer brain tissues, compared with controls. The colocalization of Hcy labeling with MAP1a was shown by immunohistochemistry (Figures 2A, see supplementary material, Figure S2) and by Duolink® (Figure 2B) in the three areas studied. It was also present in protein aggregates and tangles (Figure 2C). As for tau, increased homocysteinylation of MAP1a was associated with a decreased interaction with β-tubulin (Figure 2B). It was confirmed by coimmunoprecipitation with anti-Hcy and anti-MAP1a in the CSF of Alzheimer patients (Figure 2D). Homocysteinylation of MAP1a was quantitatively greater in patients with the highest Hcy level. The profile of MAP1a/Hcy interaction was similar to the tau/Hcy interaction profile in hippocampus tissue sections of patients from the OPTIMA collection (Figure 2E).

Homocysteinylation of tau and MAP1 is correlated to increased Hcy concentration in animal models

We studied homocysteinylation of tau and MAPs in animal models that are known to display significant hyperhomocysteinemia, that is adult rats subjected to a diet deficient in folate and vitamin B12 (Figures 3A,B, see supplementary material, S3), rats born to mothers deficient in vitamin B12 and folate during gestation and lactation (Figure 3C–G, see supplementary material, Figure S3) and the *Cd320* KO mouse model lacking brain transcobalamin receptors (Figure 4). We observed a higher homocysteinylation of tau and MAP1a in CA1 hippocampal cells from 6 month-old rats fed a B vitamin-deficient diet, compared with rats fed a control diet (Figure 3A,B). This was consistent with homocysteinemia (30.7 ± 7.6 µM in deficient rats versus 6.1 ± 0.2 µM in controls). Homocysteinylation of tau and MAP1a was associated with their decreased interaction with β-tubulin (Figure 3B), as found in human brains.

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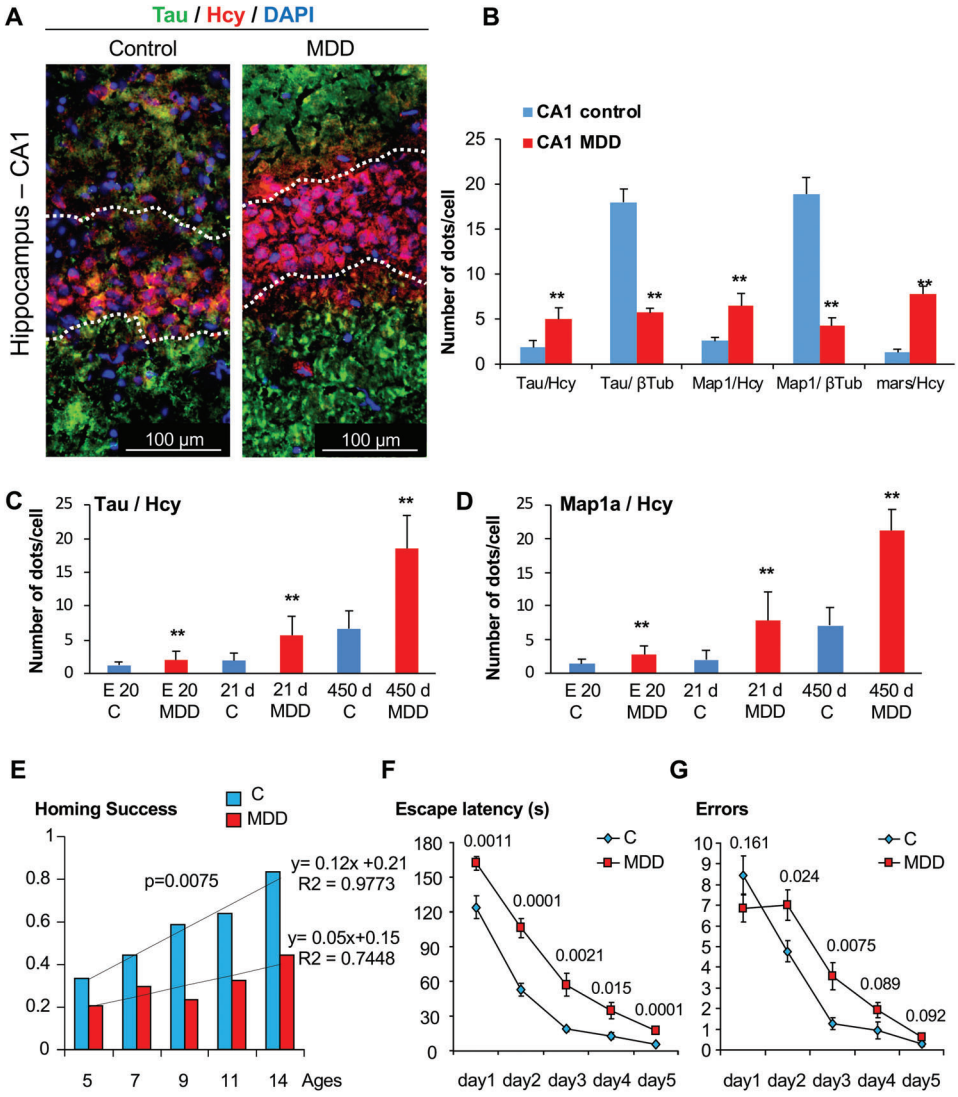


Figure 3. Tau and MAP1a homocysteinylation and their interaction with β-tubulin in rat models of methyl donor (folate and vitamin B12) deficiency. (A) 6-month-old rats fed a diet deficient in folate and vitamin B12: Immunohistochemical analysis of tau and Hcy in the hippocampal CA1 cell layer (delineated by white lines) of control and MDD rats showing evidence of cell colocalization of the two proteins, and occurrence of tau aggregates. Cell nuclei were counterstained by DAPI. (B) Quantification of *in situ* interactions between tau/Hcy, tau/β-tubulin, MAP1a/Hcy, MAP1a/β-tubulin and MARS/Hcy monitored by the Duolink® assay in the hippocampal CA1 cell layer of 6 month-old control ($n = 6$) and MDD ($n = 6$) rats. Experiments were performed in triplicate. Statistically significant difference between control and MDD: $^{**}p < 0.01$. (C) rats born to deficient dams: quantification of in situ interactions between tau/Hcy monitored by the Duolink® assay in the hippocampal CA1 cell layer of rat embryos (E20), pups (21d) and elderly born to control ($n = 4$) and MDD ($n = 4$) dams. Experiments were performed in triplicate. Statistically significant difference between control and MDD: $^{**}p < 0.01$. (D) Quantification of *in situ* interactions between MAP1a/Hcy monitored by the Duolink® assay in the hippocampal CA1 cell layer of rat embryos (E20), pups (21d) and elderly born to control ($n = 4$) and MDD ($n = 4$) dams. Experiments were performed in triplicate. Statistically significant difference between control and MDD: $^{**}p < 0.01$. (E) Evaluation of the postnatal learning function (hippocampus- and nonhippocampus-dependent) in pups. The homing success represents the ability of pups to go back to their home-cage in the T-maze (C = 33, MDD = 35). (F) and (G) Evaluation of hippocampal learning function over a 5 day-session in young rats (40–44 days of age). The escape latency (F) and the number of errors (G) committed (means±sem, C = 22; MDD = 29, ANOVA) are the main parameters reflecting spatial learning in the multiple T-maze.

These data were confirmed in cortex and cerebellum (see supplementary material, Figure S4).

Folate/vitamin B12 deficit and associated hyperhomocysteinemia produce brain accumulation of homocysteinylated tau and MAP1 throughout life in animal models

We studied brain tissues of rat progeny from dams deficient in vitamin B12 and folate during gestation and lactation. Thereafter, animals received a normal diet after weaning (21d). At this stage, animals showed significantly increased homocysteinemia compared to controls (31.0 ± 3.6 versus 6.4 ± 0.2 µM). Homocysteinemia of 450 day-old rats previously deficient was in the same order of magnitude than that recorded in matched controls (7.4 ± 0.2 µM versus 6.5 ± 0.2 µM). Homocysteinylation increased according to age and was dramatically higher in brain tissues of animals born to deficient mothers compared to those born to

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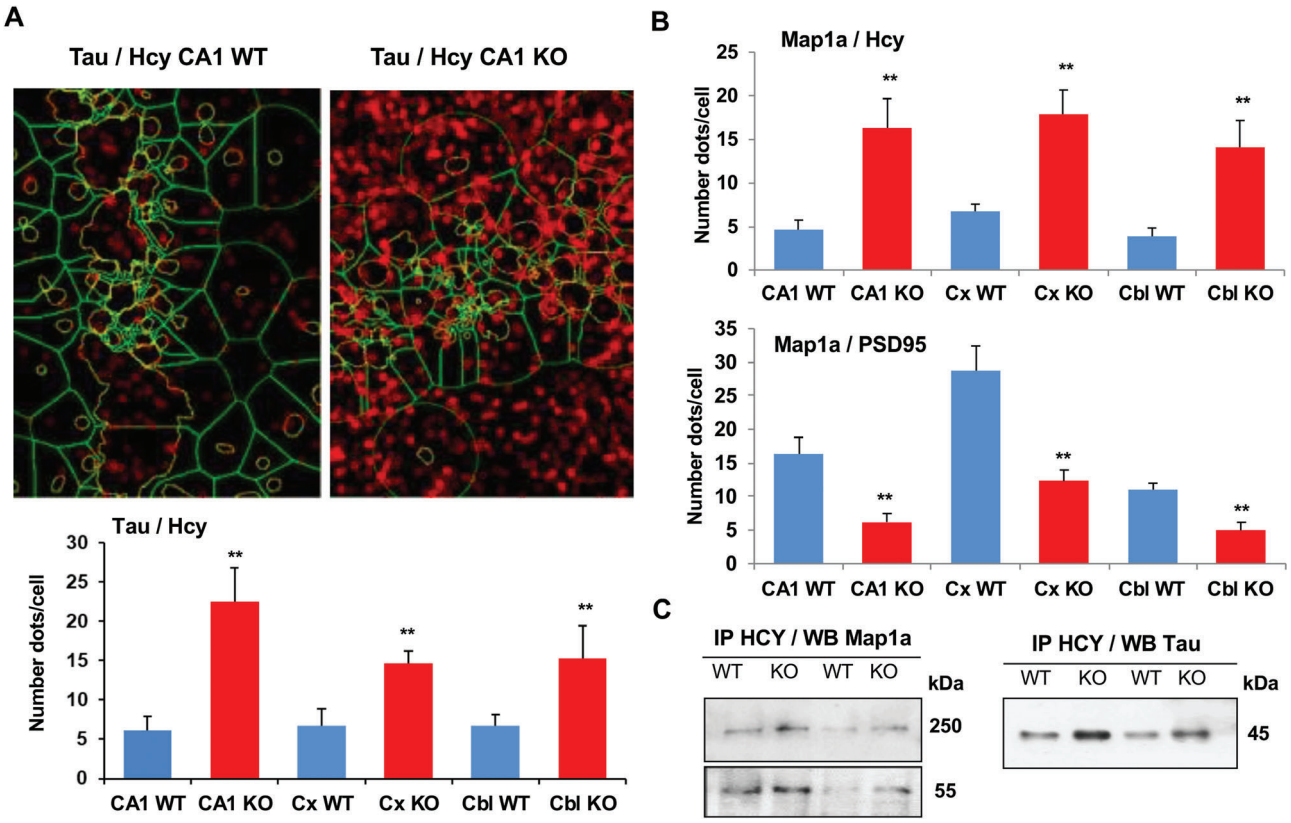


Figure 4. Tau and MAP1a homocysteinylation and their interaction with β-tubulin in brains of *Cd320* KO mice with selective cerebral deficit in vitamin B12. (A) Quantification of *in situ* interactions between Tau/Hcy monitored by the Duolink® assay in the hippocampal CA1 cell layer, in the cortex (Cx) and cerebellum (Cbl) of Wild Type (WT, $n = 6$) and *Cd320* KO (KO, $n = 6$) rats. Pictures show computational reconstitution of Tau/Hcy interaction dots in the hippocampus analyzed by the BlobFinder freeware. Experiments were performed in triplicate. Statistically significant difference between control and MDD: $^{**}p < 0.01$. (B) Quantification of *in situ* interactions between MAP1/Hcy and between MAP1/PSD95 monitored by the Duolink® assay in the hippocampal CA1 cell layer, in the cortex (Cx) and cerebellum (Cbl) of Wild Type (WT, $n = 6$) and CD320 KO (KO, $n = 6$) rats. Experiments were performed in triplicate. Statistically significant difference between control and MDD: $^{**}p < 0.01$. (C) Tau and MAP1a homocysteinylation in the hippocampus of wild type (WT) or *Cd320 KO* (KO) mice as shown by Hcy immuno-precipitation followed by Tau and MAP1 immunodetection (IP Hcy/ WB tau and IP Hcy/ WB MAP1a), respectively. Experiments were performed in triplicate.

controls at the three stages studied, namely E20, 21d, and 450d (Figure 3C, D, and supplementary material, Figure S4). Homocysteinylation of tau and MAP1a was ten times higher in animals at 450d compared to E20 embryos. These data suggest that homocysteinylation is a cumulative mechanism that starts in utero and increases dramatically during aging.

At the behavioral level, the homing test showed a poorer acquisition capacity in deficient pups compared to controls, with a smaller linear improvement score represented by the slope of learning curve (Figure 3E). In young adults, deficient rats needed more trials than controls to reach similar performances (Figure 3F,G). As a whole, these data show alterations of learning capacities related to both hippocampal and non-hippocampal functions in pups born to deficient dams, as previously documented [28,34].

We studied the *Cd320* KO mouse as a specific model of increased Hcy produced by a specific deficit of vitamin B12 in the brain. The ablation of the transcobalamin receptor produces a 85% decrease of B12 brain concentration and a five-fold increase of Hcy concentration in the hippocampus [30]. Tau homocysteinylation was increased in the CA1 layer of the hippocampus, cortex and cerebellum, compared to the wild-type mice (Figure 4A). We also showed an increase of MAP1a homocysteinylation mirrored by a decreased interaction between MAP1a and its partner protein, the postsynaptic density protein PSD95, in the three brain areas (Figure 4B). The data were confirmed by immunoprecipitation experiments (Figure 4C).

Homocysteinylation of tau and MAPs corresponds to N-homocysteinylation that depends on MARS enzyme activity

We used H19-7 neuronal progenitor cells to evaluate whether the increased homocysteinylation of tau and MAPs produced by folate deficiency is a N-homocysteinylation that depends on MARS activity. We studied these cells at 6 and 13 h after induction of their differentiation, as previously described [20]. We observed an increased expression of both 68 and 45 kDa isoforms of tau and phospho-tau in methyl donor deficiency condition (Figure 5A). Homocysteinylation was demonstrated at 13 h after induction

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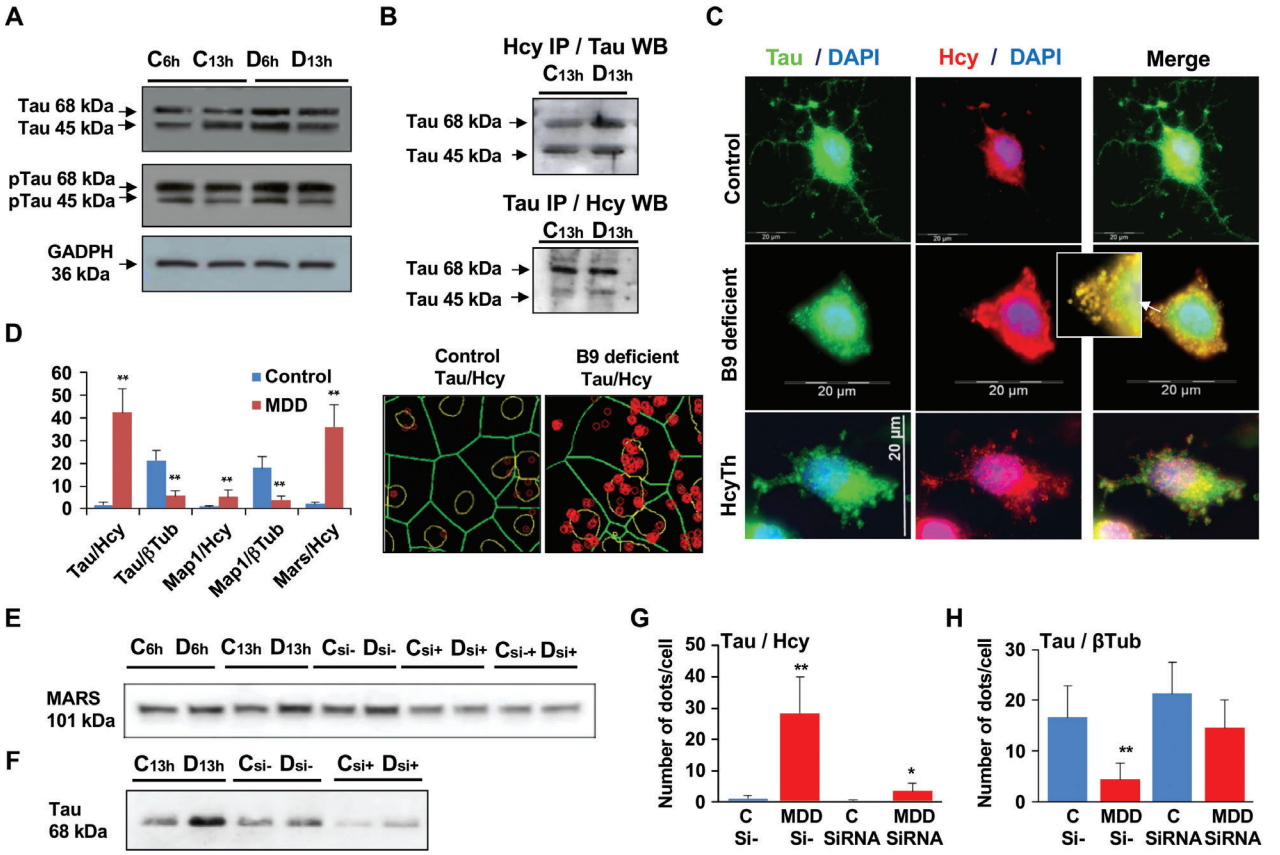


Figure 5. Effects of folate deficiency and MARS expression on N-homocysteinylation of tau and its interaction with β-tubulin in H19-7 neuroprogenitors. (A) Expression of tau and its phosphorylated forms assessed by Western blot in control (C) and folate-deficient (D) H19-7 cells at 6 h and 13 h after induction of differentiation ($n = 5$). (B) Tau homocysteinylation in 13 h-differentiated (13 h) control (C) and deficient (D) cells as shown by tau immunoprecipitation followed by Hcy immunodetection (Tau IP/Hcy WB) ($n = 3$). (C) Immunohistochemical analysis of tau and Hcy in control and folate-deficient H19-7 cells showing colocalization of the two proteins and accumulation of aggregates (insert at higher magnification). HcyTh was used as a control to mimic Hcy accumulation. Note similar effect on tau aggregation. (D) Quantification of *in situ* interactions between Tau/Hcy, Tau/β-tubulin, MAP1/Hcy, MAP1/β-tubulin, and MARS/Hcy monitored by the Duolink® assay in control (C) and folate-deficient (MDD) cells. Experiments were performed in triplicate. Statistically significant difference between control and MDD: $^{**}p < 0.01$. Pictures show tau/Hcy interaction dots analyzed by the BlobFinder freeware. (E) Effects of silencing MARS in differentiating H19-7 cells on the expression levels of MARS itself in control (C) and folate-deficient (D) cells at 6 h and 13 h after induction of differentiation (si- = non-targeting siRNA, si + = MARS targeted siRNA). Expression patterns are representative of three separate series of Western blots. (F) Effects of silencing MARS on the homocysteinylation of Tau shown by Tau immunoprecipitation followed by Hcy immunodetection in control (C) and folate-deficient (D) cells at 13 h after induction of differentiation (si− = non-targeting siRNA, si + = MARS targeted siRNA). Expression patterns are representative of three separate series of Western blots. (G) Effects of silencing MARS on the homocysteinylation of Tau monitored by the Duolink® assay in control (C) and deficient H19-7 cell (MDD) at 13 h after induction of differentiation (si- = non-targeting siRNA, siRNA = MARS targeted siRNA). Experiments were performed in triplicate. Statistically significant difference between control and MDD: $^{*}p < 0.05$ and $^{**}p < 0.01$. (H) Effects of silencing MARS on the functional interaction between Tau and β-Tubulin monitored by the Duolink® assay in control (C) and deficient (MDD) cells at 13 h after induction of differentiation (si− = non-targeting siRNA, siRNA = MARS targeted siRNA). Experiments were performed in triplicate. Statistically significant difference between control and MDD: $^{**}p < 0.01$.

of differentiation, either by immunoprecipitation with anti-Hcy antibody followed by anti-tau immunodetection or by immunoprecipitation with anti-tau antibody followed by immunodetection with anti-Hcy antibody (Figure 5B). As previously reported in human brains, we observed the colocalization of tau/Hcy and MAP1/Hcy in protein aggregates by immunohistochemistry in cells cultured in B9-depleted medium, but also in cells treated with HcyTh, the substrate of N-homocysteinylation (Figure 5C). The addition of HcyTh produced effects similar to those of the culture medium lacking folate. Duolink® assay confirmed the increased interaction of tau with Hcy in B9-depleted cells (Figure 5D). Cells grown in medium deficient in folate exhibited an increased colocalization of MARS enzyme with Hcy (Figures 5D, see supplementary material, Figure S5B) and increased expression of MARS (Figure 5E). Silencing MARS protein expression by transfecting cells with siRNA (Figure 5E) resulted in a significant decrease in the level of homocysteinylated tau, in Western blot analysis of immunoprecipitated tau (Figure 5F). Duolink® assay showed a marked decrease of tau homocysteinylation in B9-deficient cells transfected with MARS siRNA, as compared to control cells (Figure 5G). Consistently, experimentally impaired expression of MARS resulted in the restoration of the

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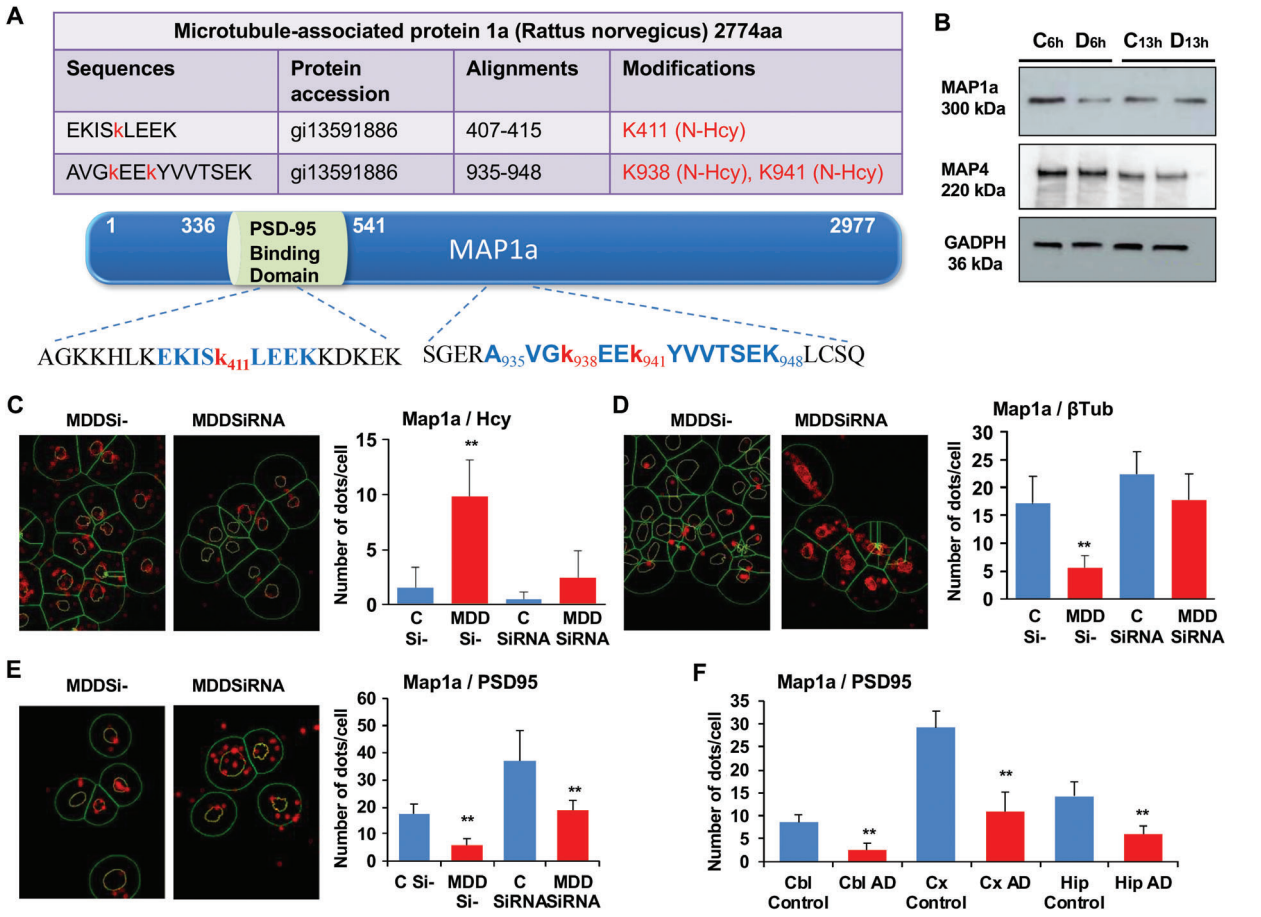


Figure 6. Effects of folate deficiency and MARS expression on N-homocysteinylation of MAPs and their interaction with β-tubulin in H19-7 neuroprogenitors. (A) MAP1 homocysteinylation sites identified by MS. Homocysteinylated lysine is shown in red. (B) Expression of MAP1a and MAP4 assessed by Western blot in control (C) and folate-deficient (D) cells at 6 h and 13 h after induction of differentiation ($n = 3$). (C) Effects of silencing MARS on the homocysteinylation of MAP1 monitored by the Duolink® assay in control (C) and deficient H19-7 cell (MDD) at 13 h after induction of differentiation (si- = non-targeting siRNA, siRNA = MARS targeted siRNA). Pictures show the computational reconstitution of Map1a/Hcy interaction (red dots) after analyses by the BlobFinder freeware. Experiments were performed in triplicate. Statistically significant difference between control and MDD: $^{*}p < 0.05$ and $^{**}p < 0.01$. (D) Effects of silencing MARS on the functional interaction between MAP1 and β-tubulin monitored by the Duolink® assay in control (C) and deficient (MDD) cells at 13 h after induction of differentiation (si− = non-targeting siRNA, siRNA = MARS targeted siRNA). Experiments were performed in triplicate. Statistically significant difference between control and MDD: $^{**}p < 0.01$. (E) Effects of silencing MARS on the functional interaction between MAP1 and PSD95 monitored by the Duolink® assay in control (C) and deficient (MDD) cells at 13 h after induction of differentiation (si− = non-targeting siRNA, siRNA = MARS targeted siRNA). Experiments were performed in triplicate. Statistically significant difference between control and MDD: $^{**}p < 0.01$. (F) Quantification of *in situ* interactions between MAP1/PSD95 monitored by the Duolink® assay in cortex, hippocampus and cerebellum tissue sections from control ($n = 4$) and AD ($n = 4$) brains. Pictures were analyzed by the BlobFinder freeware. One red dot is representative of one protein interaction. Experiments were performed in triplicate. Statistically significant difference between control and AD: $^{**}p < 0.01$.

interaction between tau and β-tubulin in transfected deficient cells compared to controls (Figure 5H).

We observed similar results with other MAPs (Figures 6, see supplementary material, Figure S5). MAP1a and MAP4 showed different expression patterns during neuronal differentiation (Figure 6B); MAP1a expression increased in control cells at 13 h, whereas MAP4 decreased. B9 deficiency led to decreased MAP1a and MAP4 expression in 13 h-differentiated cells. The Duolink® assay showed a marked decrease of MAP1a homocysteinylation (Figure 6C), along with a restored interaction of MAP1a with β-tubulin (Figure 6D) in deficient cells transfected with MARS siRNA, compared to those transfected with an empty plasmid. We also investigated the functional consequences of N-homocysteinylation on the interaction between MAP1a and PSD95 in H19-7 cells and human brains. We found a significantly lower interaction in folate-deficient cells (Figure 6E) and in Alzheimer brain tissues (Figure 6F). The decreased interaction of MAP1a with PSD95 was reversed in deficient cells transfected with MARS siRNA (Figure 6E).

N-homocysteinylation targets lysine residues of MAPs that are critical for their interactions with β-tubulin and PSD95

To identify the N-homocysteinylated lysine residues that are involved in the interaction between MAPs

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and β-tubulin, we analyzed by MS the peptides from tau and MAPs immunoprecipitated with anti-Hcy antibody. We identified the N-homocysteinylation of one lysine residue (K411) of the 407–415 peptide and two lysine residues (K938 and K941) of the 935–948 peptide of MAP1a (Figure 6A). The K411 lysine residue is located in the PSD95 binding domain of MAP1a. This domain is highly conserved and the 407–415 peptide has a 100% sequence identity between rat and human MAP1. We also identified the N-homocysteinylation of lysine residues in peptides from MAP4 and MAP1b (see supplementary material, Figure S5C).

Discussion

Brain autopsy specimens from AD patients have characteristic lesions of pretangles and neurofibrillary tangles produced by tau hyperphosphorylation and aggregation in selected types of neurons [35]. Additional posttranslational changes of MAPs may also contribute to pathological brain ageing. Our study suggests that N-homocysteinylation of tau, MAP1a and MAP4 can be one of them. We showed an increased N-homocysteinylation of tau and other MAPs in the brains of patients with AD or VD and in animal models with elevated Hcy. This increased homocysteinylation was a cumulative and irreversible mechanism that impaired the functional interactions of MAPs with their partner proteins such as β-tubulin and the postsynaptic density protein PSD95.

Homocysteinlylation of MAPs resulted from the irreversible N-homocysteinylation of lysine residues rather than from the reversible S-homocysteinylation of cysteine residues. Our data are consistent with N-homocysteinylation reported *in vitro* [22,36,37]. Silencing MARS expression using siRNA prevented homocysteinylation of tau and MAP1a in our cell model, while treatment with mercaptoethanol did not remove homocysteinyl residues, indicating that disulfide bonds were not involved in the homocysteinylation process [24]. We confirmed the N-homocysteinylation of lysine residues of MAPs by MS analyses.

N-homocysteinylation of tau and other MAPs was an age-related cumulative mechanism that started during fetal development in our rat model. We observed an increased N-homocysteinylation of tau and MAP1a in E20 rat fetuses from dams deficient in methyl donor micronutrients. This increased N-homocysteinylation was not reversed by giving a normal diet after weaning, and was still present at day 450, in association with permanent cognitive deficits [28].

Microtubules play a central role in the cytoskeleton and intracellular transport of synaptic vesicles in neurons [25,38]. The MAPs, including tau, bind transiently to β-tubulin and stabilize microtubules in axons and dendrites [38,39]. N-homocysteinylation targeted lysine residues that are critical for the binding of MAPs with β-tubulin and PSD95. MAPs interact with the neuronal cytoskeleton through highly conserved microtubule-binding repeats of their C-terminal part. The positive charge of lysine residues of microtubule-binding repeats interacts with the negatively charged C-terminal part of β-tubulin in a sequence-specific fashion [40]. N-homocysteinylation of MAPs neutralizes the positive charges of lysine residues of microtubule-binding repeats. It targets the lysine 280 located in a microtubule-binding repeat that plays a critical role in these interactions [41]. It targets also another lysine residue that belongs to a conserved KXGS motif of the C-terminal microtubule binding repeat shared by all the MAPs, including tau, MAP1a, MAP1b, and MAP4. This may abrogate the ability of tau and other MAPs to stabilize the microtubules [36,42]. MAP1a is localized at excitatory synapses, where it interacts with PSD95 and influences the recruitment of *N*-methyl-D-aspartate (NMDA) receptors at the excitatory synapses involved in learning and memory [43]. The N-homocysteinylation of lysine in the microtubule-binding domain of MAP1b may alter its interaction with GABAc receptors and glutamate receptors (NMDA, AMPA and GluR) and the subsequent localization of these proteins to the dendritic spine (see supplementary material, Figure S6). This suggests therefore that the cumulative N-homocysteinylation of MAPs might affect synaptic plasticity and cognitive functions, as observed in our animal model.

In addition to phosphorylation, it has been suggested that posttranslational modifications of tau such as nitrosation, acetylation, glycosylation and glycation could be also involved in the protein aggregates and tangles observed in AD or other tauopathies [44]. The detection of homocysteinylated tau and MAP1 in protein aggregates and tangles in our patients suggests also an influence of N-homocysteinylation. We observed a decreased binding of N-homocysteinylated tau to β-tubulin not only in AD, but also in VD, a pathology without neurofibrillary tangles [45,46]. These data indicate that N-homocysteinylation may play a role in the dissociation of tau and MAPs from β-tubulin, but not in the formation of tangles.

N-homocysteinylation of tau and other MAPs was the highest in the CSF of patients who displayed the highest Hcy concentration. Consistently, experimental models with a noticeable increase of Hcy, such as rats deficient in folate and vitamin B12, *Cd320* KO mice and H19-7 cells cultured without folate, led to dramatically higher N-homocysteinylation of tau and other MAPs compared to respective controls. This suggests a direct relationship between Hcy cell concentration and N-homocysteinylation of tau and MAPs, and thus a direct influence of the status in methyl donors, folate and vitamin B12.

Our data are consistent with a study that evaluated the association between plasma Hcy and autopsy brain pathological changes in patients with Alzheimer and cerebrovascular pathology [47]. It was shown that elevated Hcy in adults aged > 85 years may contribute to increased neurofibrillary tangles burden. This association seemed to be more pronounced in case of

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cerebrovascular pathology [47]. The irreversible and age-related cumulative N-homocysteinylation may explain the inconclusive results in population studies that have evaluated the effects of B vitamin supplementation on cognitive decline and dementia. A recent meta-analysis of randomized controlled trials of folate and B vitamin supplementation in patients with AD or dementia concluded that supplementation does not translate into cognitive improvement despite the reduction of homocysteinemia [48,49]. In contrast, a placebo-controlled trial showed the beneficial effect of B vitamin treatment on brain atrophy and on cognition [50] in subjects with high plasma omega-3 fatty acids. A recent review of 263 eligible studies pointed out promising signals for vitamin B12 plus folic acid supplementation to prevent Alzheimer-type dementia [51]. In contrast to studies performed in the elderly, the intake of vitamins B through young adulthood is associated with better cognitive function in midlife [49], at a stage where the lesions are limited to the formation of pretangles [1,44]. However, the start and accumulation of Alzheimer-type neuropathology may affect different age groups at different time points and to a different extent [52]. Whether a low plasma concentration of Hcy ensured by adequate supply in vitamin B12 and folate before aging could limit the long-term and cumulative N-homocysteinylation of tau and other MAPs is therefore uncertain and needs further investigation.

In conclusion, increased N-homocysteinylation of tau and other MAPs is an irreversible and cumulative age-related mechanism that could participate to pathological brain ageing, which depends on Hcy concentration and expression of MARS enzyme. It is associated with a decreased interaction of MAPs with their partner proteins, contributing to cognitive decline. Our data may explain why B12 and folate supplementation of the elderly has limited effects, if any, to prevent pathological brain ageing.

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Author contributions statement

CBP and JLG designed and coordinated the study, interpreted the data and wrote the paper. CBP performed experiments. JLD participated in the study design, interpreted the data and wrote the paper. ADS, BS and SL provided brain samples, interpreted the data and revised the paper. EQ and JS provided the *Cd320* KO mouse model, participated in the study design, interpreted the data and revised the paper. DH and RU performed cell and animal experiments, analyzed and interpreted the related data, and revised the manuscript. GP performed analyses of animal behavior, analyzed and interpreted the related data, and revised the manuscript. RMGR performed CSF analyses, analyzed and interpreted the related data, and revised the manuscript. All authors discussed the results and commented on the manuscript.

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SUPPLEMENTARY MATERIAL

Supplementary materials and methods

Figure S1. Illustration of tau/Hcy Duolink® interaction in brains of patients from the Oxford OPTIMA collection

Figure S2. Immunohistochemical colocalization of Hcy with tau and MAP1a in cortex of control and Alzheimer patients

Figure S3. Illustrations of Hcy interactions with tau and MAP1a in rat models of methyl donor deficiency as shown by the Duolink® assay

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Figure S4. Homocysteinylation of tau and MAP1a in rat models of methyl donor deficiency
Figure S5. Interactions of MAPs and MARS enzyme with Hcy in H19-7 neuronal cells
Figure S6. Schematic representation of the process of N-homocysteinylation of MAPs
Table S1. Primary antibodies used in immunohistochemical and western blotting studies
Table S2. Main characteristics of the subjects and brain samples issued from human cohorts

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