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Long-term pantethine treatment counteracts pathologic gene dysregulation and decreases Alzheimer's disease pathogenesis in a transgenic mouse model.

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Conflict of interest

The authors declare no financial conflict of interest that might be construed to influence the results or interpretation of the manuscript.

Abstract

Background: The low-molecular weight thiol pantethine, known as a hypolipidemic and hypocholesterolemic agent, is the major precursor of co-enzyme A. We have previously shown that pantethine treatment reduces amyloid- β ($A\beta$)-induced IL-1 β release and alleviates pathological metabolic changes in primary astrocyte cultures. These properties of pantethine prompted us to investigate its potential benefits *in vivo* in the 5XFAD (Tg) mouse model of Alzheimer's disease (AD).

Methods: 1.5-month-old Tg and wild type (WT) male mice were submitted to intraperitoneal administration of pantethine or saline control solution for 5.5 months. The effects of such treatments were investigated by performing behavioral tests and evaluating astrogliosis, microgliosis, $A\beta$ deposition and whole genome expression arrays, using RNAs extracted from the mice hippocampi.

Results: We observed that long-term pantethine treatment significantly reduced glial reactivity and $A\beta$ deposition, and abrogated behavioral alteration in Tg mice. Moreover, the transcriptomic profiles revealed that after pantethine treatment, the expression of genes differentially expressed in Tg mice, and in particular those known to be related to AD, were significantly alleviated. Most of the genes overexpressed in Tg compared to WT were involved in inflammation, complement activation and phagocytosis, were found repressed upon pantethine treatment. In contrast, pantethine restored the expression of a significant number of genes involved in the regulation of $A\beta$ processing and synaptic activities, which were down-regulated in Tg mice.

Conclusions: Altogether, our data support a beneficial role for long-term pantethine treatment in preserving CNS crucial functions altered by $A\beta$ pathogenesis in Tg mice, and highlight the potential efficiency of pantethine to alleviate AD pathology.

Keywords: Alzheimer's disease, pantethine, gene expression profiles, neuroprotection, phagocytosis, 5xFAD mice

Background

Alzheimer's disease (AD) is the most common cause of dementia worldwide. It is characterized by a progressive cognitive impairment thought to be associated with the accumulation of extracellular amyloid plaques, composed of amyloid- β peptide ($A\beta$) and intracellular neurofibrillary tangles (NFTs). NFTs result from an alteration of microtubule stabilization following the abnormal hyperphosphorylation of Tau, a microtubule-associated protein. The production of $A\beta$ is triggered by a successive proteolytic cleavage of the β -amyloid precursor protein (APP) by the β -secretase, BACE-1 (beta-site APP cleaving enzyme 1) and the γ -secretase complex. Among the different $A\beta$ peptides generated from APP, $A\beta_{42}$ is believed to play a primary role in the development of AD (1). The deposition of extracellular $A\beta$ is associated with a neuroinflammatory response and it is suggested that upon sustained $A\beta$ production, the resulting chronic inflammation contributes to AD pathology (2, 3). Therapeutic strategies based on the reduction of Tau hyperphosphorylation or $A\beta$ are under evaluation in addition to those developed to improve cognitive function (acetylcholinesterase inhibitors) and mood disturbance or to provide neuroprotection (memantine). However, to date, available treatments show only modest symptomatic effects. Moreover, epidemiological studies have suggested that longstanding use of anti-inflammatory drugs such as NSAIDs (non-steroidal anti-inflammatory drugs) can prevent or delay the development of AD (4, 5). Unfortunately, treatment of AD patients with anti-inflammatory drugs such as celecoxib or naproxen failed to demonstrate clinical benefits (6, 7). These failures illustrate our poor understanding of the sequential events of neuroinflammation in AD that, depending possibly on timing and context, can either promote protective effects or exacerbate the disease (8). Thus, in addition to their potential therapeutic efficacy, search for new modulators of inflammation can be useful in determining whether inflammation is a driving force or a bystander effect in AD.

Pantethine, a dietary low-molecular-weight thiol widely distributed in the living world and synthesized from pantothenic acid (vitamin B5) and cysteamine, is an intermediate in the production of Co-enzyme A (CoA). This activity is thought to be related to its well-known effect on lowering cholesterol synthesis (9). However, pantethine can be rapidly hydrolyzed *in vivo* to pantothenic acid and cysteamine by pantetheinase encoded by the vanin gene, and it cannot be excluded that its observed activities might result from cysteamine or pantothenic acid (10, 11). Pantethine elicits broad physiological activities involving multiple cellular pathways. It has been shown to exert neuroprotective effects and to reduce metabolic

dysfunctions (12-14), but also to decrease inflammation and mediate immune responses (15, 16). We have previously shown that pantethine is able to protect mice against cerebral malaria by preserving blood brain barrier integrity and by lowering TNF- α levels (16). More recently, we demonstrated that pantethine alleviates metabolic dysfunctions, reduces astrogliosis and IL-1 β production in primary cultured astrocytes of 5XFAD mice (17). Such beneficial effects were also observed in WT astrocytes treated with A β oligomers. These data suggest that pantethine could help maintain brain homeostasis and function during AD pathogenesis.

Such observations prompted us to explore pantethine activity *in vivo* using 5XFAD mice. This animal model bears five mutations linked to familial forms of AD and recapitulates in a few months the main features of AD. These mutations act in an additive manner to boost the production of A β peptides, particularly A β ₄₂. Though they do not present tau pathology, these mice develop cerebral amyloid plaques and gliosis as early as two months (18), along with increased levels of pro-inflammatory mediators *Il-1 β* and *Ccl2* mRNAs (19). Electrophysiological studies have detected hippocampal synaptic dysfunctions in 4- to 6-month-old 5XFAD animals, concomitant with memory deficits (18, 20-25). Neuronal death, which is absent in most AD mouse models, has been described in the cortex and subiculum in 9-month-old 5XFAD mice (18, 26). In a previous longitudinal transcriptomic study, we showed that the main processes altered in 5xFAD mice, from 4 months onwards, were related to microglial activation and inflammation (27).

To investigate the effects of pantethine on AD pathology, 1.5 month-old 5xFAD transgenic (Tg thereafter) and wild type (WT thereafter) mice were treated with pantethine for 5.5 months (see materials and methods). In line with our previous data (17), a significant decrease in gliosis and A β plaque deposits was observed in treated Tg mice compared to untreated. Treatment also improved behavior with a significant decrease of aggressiveness. Transcriptomic analyses showed that pantethine was indeed able to tune down the modulations in gene expression characteristic of the Tg mice. We present for the first time an array of evidences supporting a beneficial effect of pantethine in AD. Thus, its current use and the possibility of chronic treatment in the absence of known side effects provide the grounds to test the efficacy of pantethine in AD as a new therapeutic agent in preclinical and clinical studies.

Materials and methods

Chemical

Pantethine was purchased from Sigma-Aldrich (Saint Quentin-Fallavier, France). Purity of the preparation was ascertained using high performance liquid chromatography-coupled mass spectrometry (HPLC-LC-UV-MSD) and the purity of the compound reached 99%. Sodium Chloride (NaCl) and Phosphate Buffer Saline (PBS) were purchased from Euromedex (Souffelweyersheim, France). Proteinase inhibitors cocktail was from Millipore (Molsheim, France). All the chemical powders were of analytical grade and purchased from Sigma-Aldrich.

Animals and pantethine treatment

We used 5XFAD male transgenic mice, which overexpress two human transgenes bearing five mutations linked to familial AD: *App* (Swedish mutation K670N, M671L; Florida mutation I716V; London mutation V717I) and *Psen1* (M146L; L286V), under transcriptional control of the mouse *Thy1* promoter. These mice exhibit AD-related symptoms earlier than other AD animal models and amyloid deposition starts in the cortex and subiculum at two months of age (18). Heterozygous male 5XFAD transgenic animals (B6/SJL background) and WT controls were obtained after breeding in our animal house facility (19, 27, 28).

Animals were housed in cages in a controlled environment (22-25°C, 50% humidity and a 12-hour light/dark cycle) with free access to standard laboratory diet and water. At 1.5 month, Tg and WT mice were each separated into treated and vehicle groups (n=10 per group). Based on a previous study (13), Tg and WT treated mice were given each an intraperitoneal injection of 15 mg of pantethine diluted in saline, three times a week for 5.5 months; vehicle animals received saline injections as control. At the age of 7 months, the animals were submitted to the intrudal aggressivity test (see below) and then sacrificed. Mice were anesthetized with pentobarbital (0.36 g per kg) and transcardially perfused with 50 ml of NaCl 0.9%. Brains were microdissected and hippocampi collected for the assays. All experimental procedures were approved by the Ethics Committee of the Medical Faculty of Marseille and were carried out in accordance with the guidelines published in the European Communities Council Directive of November 24, 1986 (86/609/EEC). All efforts were made to reduce animal suffering and the number of mice.

Total RNA isolation and extraction

Hippocampi from each experimental group (n=3 per group) were quickly snap-frozen in liquid nitrogen and stored at -80°C until use. Total RNA was extracted using the RNeasy Mini kit (Qiagen, Courtaboeuf, France) according to the manufacturer's instruction. RNA concentration was determined using a nanodrop 2000 spectrophotometer (ThermoFisher Scientific, Villebon sur Yvette, France) and RNA integrity assessed on an Agilent 2100 Bioanalyzer (Agilent Technologies, Les Ulis, France).

Microarray assay

The microarray assay included four different experimental conditions: hippocampi from WT and Tg mice vehicle or treated with pantethine (n=3 per group). A total of 12 biological samples were therefore processed for the microarray experiment. The mouse WG-6 expression BeadChip (Illumina Inc., Cambridge, UK), comprising 47,232 transcripts was used to generate gene expression profiles and hybridized to cRNAs, which were synthesized, amplified and purified using the Illumina TotalPrep RNA Amplification Kit (Ambion, Foster City, CA, USA) following manufacturer's recommendations. Briefly, 200 ng of RNA were reverse transcribed. After second strand synthesis, the cDNA was transcribed *in vitro* and cRNA labelled with biotin-16-UTP. Labelled probe hybridization to the Beadchips was carried out using Illumina's protocol. The Beadchips were scanned on the Illumina IScan using Illumina IScan image data acquisition software. Illumina GenomeStudio software and LumiR (www.bioconductor.org) were used for preliminary data analysis, data normalization and quality controls. Microarray statistical analyses were performed using the Biometric Research Branch array tools ([http://linus.nci.nih.gov/BRB-Array Tools.html](http://linus.nci.nih.gov/BRB-Array_Tools.html)) and the Multi Experiment Viewer software (MeV4.0; (29)). Expression values were filtered for a *p* value \leq 0.05. Functional analysis was performed using the text-mining based software, PredictSearch (27). Microarray data are available in the Array Express database [www.ebi.ac.uk/arrayexpress] under accession number E-MTAB-6772.

Real-time quantitative PCR (qPCR)

Following microarray analysis, the expression levels of some selected genes were further validated using qPCR technique. Total RNA samples (1 µg) extracted from the hippocampi of all animals (n=3) in each group were subjected to reverse-transcription reactions to synthesize cDNA using oligodT, RNase-out and M-MLV RT enzyme (ThermoFisher Scientific) according to the manufacturer's instructions. Two genes, *Gfap* and *Aif1*, related to astrocytic and microglial activation, respectively, were selected for pre-validation of samples, and one

housekeeping gene, *Gapdh*, was used for sample normalization. Real-time qPCR experiments were carried out with the 7500 Fast Real-Time PCR system (Applied Biosystems, Thermo Fisher Scientific), using TaqMan Fast Universal PCR Master Mix (2X) and the following TaqMan Gene Expression probes were used: *Gfap*, Mm01253033_m1; *Aif1*, Mm00479862_g1; *Il-1 β* , Mm01336199_m1; *Csf1r*, Mm01266652_m1; *Tlr2*, Mm01213946_g1; *Cyba*, Mm00514478_m1; *Tnf- α* , Mm00443258_m1 and *Gapdh*, Mm99999915_g1. We used 7.5 ng of previously prepared cDNA and samples were run in triplicates. Relative expression levels were determined according to the $\Delta\Delta C_t$ method where the expression level of the mRNA of interest is given by $2^{-\Delta\Delta C_t}$ where $\Delta\Delta C_t = \Delta C_t \text{ target mRNA} - \Delta C_t \text{ reference mRNA (Gapdh)}$ in the same sample (19).

ELISA assay

The cortices of mice brains (n=3 for each condition) were homogenized in 100 mM PBS containing 2 mM NaF, 2.5 mM $\text{Na}_4\text{P}_2\text{O}_7$, 1 mM Na_3VO_4 , 1% Triton X-100, pH 7.2, supplemented with 1% protease inhibitor cocktail and centrifuged at 12,000 x g for 20 min at 4°C. Protein concentration was quantified by using the Bio-Rad DC™ protein assay kit following the manufacturer's instructions (Bio-Rad, Marnes-La-Coquette, France). The levels of IL-1 β were analyzed by ELISA assay (Peprotech, Neuilly-sur-Seine, France) according to the manufacturer's instructions.

Immunostaining, image analysis and quantification

Anesthetized mice were transcardially perfused with 50 ml of NaCl 0.9%, followed by 50 ml of AntigenFix solution (Diapath, MM France, Brignais, France). Coronal sections of 30 μm were obtained using a vibratome (Microm HM650V, Thermo Fisher Scientific), and stored at -20°C in cryoprotectant containing 30% glycerol, 30% ethylene glycol and 40% in phosphate buffer 0.5 M, pH 7.4. Free-floating sections were first permeabilized and blocked for 1 h at room temperature using a solution of PBS 1X, 0.1% Triton X-100 and 3% BSA. Sections were then incubated overnight at 4°C with primary antibodies: mouse monoclonal anti-A β 6E10 (1/300; Covance, Ozyme, Saint-Quentin en Yvelines, France), rabbit polyclonal anti-IBA1 (1/300; Wako, Sobiota, Montbonnot, France), Rabbit polyclonal anti-GFAP (1/300, Dakocytomation, France) followed by an incubation with appropriate secondary antibodies: AlexaFluor®488-goat anti-rabbit (1/800) or AlexaFluor®568-goat anti-mouse (1/800) (Thermo Fisher Scientific) for 1 h at room temperature. Nuclei were stained with Hoechst (0.5

$\mu\text{g/mL}$, Thermo Fisher Scientific). Omission of the primary antibody was used as control. Sections were mounted using Prolong Gold Antifading reagent on Superfrost glass slides (Thermo Fisher Scientific). Images were taken and processed using a confocal microscope (LSM700) and Zen software (Zeiss, Jena, Germany), as previously described (24, 30). Densitometric analysis of immunostained sections was performed using ImageJ software on 3 hippocampal sections from 3 animals for each experimental group. The area of GFAP⁺ astrocytes and IBA1⁺ microglia was determined. The same threshold of fluorescence was applied across the experimental groups and data were normalized to the values in the WT group. Extracellular amyloid deposits were blindly scored in the three brain sections per animal and expressed as number of plaques per mm^2 of brain surface.

Intrudal aggressivity test

The effect of pantethine on Tg male mice aggressiveness was evaluated with the resident-intruder paradigm according to a previously published method (31), and modified according to our experimental situation. The animals to be tested, treated or not with pantethine (n=10 per group) were maintained in their individual resident cage in order to mimic the natural pattern of wild animals that defend their territory. The intruders were WT male mice (n=10) from the same genetic background, but not included in this protocol. For the test, a WT animal was introduced in the cage of a Tg and their behavior were monitored with a video camera for 180 sec. The following behavioral elements were scored: 1) latency: the time between the introduction of the intruder and the first threat or attack; 2) number of threats that are generally in the form of frenetic tail agitation; 3) number of assaults. Threats and assaults were exclusively facts of the Tg resident as the WT intruder always behaved passively. In the case of violent assault, the animals were immediately separated and the test ended. The offense intensity was estimated on a scale of 0 to 5. The latency time was set at 180 sec when no reaction, threat or attack was noticed. The tests were done 3 times on 3 consecutive days under normal light, starting at 10 am. From one test to another, the intruders were circularly permuted. Intrudal aggressivity experiments were performed at least three times for each animal.

Statistical analysis

All the data were analyzed with Kaleida Graph or Prism softwares and the values represent the means \pm SEM of the indicated number of independent experiments/animals. ANOVA followed by a Fisher's LSD *post-hoc* test was used for multiple comparisons. For the

aggressiveness analysis we used a non-parametric Mann-Whitney test and results are represented as median and interquartile range. Only p values less than 0.05 were considered significant. The p levels $p < 0.05$, $p < 0.01$ and $p < 0.001$ are denoted with asterisks *, ** and ***, respectively.

Results

Pantethine reduces gliosis and amyloid plaque number

The 5XFAD mouse model of AD is characterized by an increase in glial reactivity and amyloid plaque number as aging progresses. At first, immunohistological analyses of brain sections at the hippocampal level in 7-month-old Tg and WT mice (Fig. 1A) supported our previous results on cultured astrocytes (17). Indeed, in sections immunostained with anti-GFAP and anti-IBA1 antibodies, Tg mice consistently exhibited a higher signal (10-fold and 20-fold increase, respectively) than WT mice (Fig. 1A, B). Pantethine treatment reduced this signal significantly in Tg animals, with an 80% and 40% reduction for GFAP and IBA1, respectively. Interestingly, pantethine reduced GFAP staining nearly to WT levels (Fig. 1B). Similarly, staining with A β -specific antibodies showed that the numerous amyloid deposits observed in Tg mice were dramatically reduced by 85% upon pantethine treatment (Fig. 1C). These results indicated that pantethine was able to reduce significantly glial reactivity as well as the number of amyloid plaques in Tg mice.

Pantethine reduces 5XFAD aggressive behavior

AD pathology leads to cognitive impairment and behavioral alterations linked to amyloid-mediated neuroinflammation and neurodegeneration. Although altered cognition has been well characterized in a variety of transgenic models, it has been reported that disturbance in hippocampal learning performance can be detected using the Morris water maze (MWM) in male 5XFAD not before 6 to 9 months of age (23, 32). Alternatively, social withdrawal and aggressiveness have been clearly documented not only in AD patients (33-35), but also in different AD mouse models (34, 36-38), and was proposed to be useful in screening putative drug treatments for AD (36). For these reasons, we chose to evaluate the potential benefit of pantethine on the behavior of 7 month-old AD mice rather than on cognitive performances. Thus, we performed an intrudal aggressive test with treated and untreated Tg mice in the presence of WT animals. When comparing untreated and treated 5XFAD mice (Fig. 2), we observed a significant difference between these two groups, not only in offense intensity (Fig. 2A), but also in attack latency (Fig. 2B). Most (8 out of 10) vehicle-treated Tg animals react aggressively to the presence of WT mice, while only 4 out of 10 treated animals exhibit such a behavior (Fig. 2A). Moreover, pantethine treated animals present a longer latency period (150s) between the attacks (Fig. 2B), confirming that pantethine treatment is able to modulate the aggressive attitude of Tg mice.

Pantethine moderates the dysregulation of gene expression observed in 5XFAD mice

cDNA microarrays were performed to investigate whether the reduced astroglial and microglial activation, as well as the positive impact on behavior observed after pantethine treatment, could be related to a modulation of the expression of genes involved in these processes. The selection of genes in Tg animals presenting a reduced expression upon pantethine treatment (filtered for a threshold ≤ 0.6) led to the identification of 47 specific genes (Fig. 3, left panel). Interestingly, 91% of these genes (gene set 1) were expressed at higher levels in Tg mice compared to WT. Similarly, 89% of the top 47 genes (gene set 2) up-regulated in pantethine-treated Tg animals when compared to untreated Tg mice (filtered for a threshold ≥ 1.5), were down-regulated in Tg when compared to WT (Fig. 3, right panel). Noteworthy, pantethine had very little if no effect at all on the expression of most of these genes in WT animals (Fig. 3). It must be noted that none of the genes encoding proteins directly associated with the APP/A β metabolism (e.g., *Bace-1*, *App*, or γ -secretase members) were modulated between vehicle and pantethine-treated Tg mice (data not shown).

A similar tendency was confirmed when we analyzed the genes modulated in Tg mice after pantethine treatment in relation to all genes differentially expressed in Tg compared to WT (Fig. 4). Among the 154 genes down-regulated in Tg, 80% (124 genes) were up-regulated by pantethine in Tg while none were further repressed. On the other hand, out of the 362 genes whose expression was upregulated in Tg versus WT, only 43 genes (12%) showed a reduced expression upon pantethine treatment (Fig. 4). These observations suggested that pantethine was able to counteract the modulation of gene expression in 5XFAD mice and this inhibitory effect could account for the preservation by pantethine of physiological functions normally altered in these mice.

Inhibitory effect of pantethine on the expression of 5XFAD dysregulated genes in inflammation and related activities

At first, to investigate which processes could be targeted by pantethine, we focused our analysis on genes whose up-regulation was statistically significant in Tg compared to WT and that presented a lower expression in pantethine-treated Tg animals. A significant number of these genes (Table 1) were reported in our previous temporal gene profiling study (27) and are known to be related to glial activation and neuroinflammation in an AD context. Figure 5 shows the relationships between these genes and related AD activities identified through a bibliographic search using PredictSearch software (27, 39). Indeed, most of these activities

highlight the role of the selected genes in astroglial activation (*Gfap*, *S100a6*), microglial activation (*Aif1/Iba1*, *CD68*, *Hexb2*) and phagocytosis (*Cd14*, *Trem2*, *Tyrobp*, *Csf1r*, *C1qa*, *C1qb*, *C1qc*, *C4a*, and *C4b*).

Similarly to allograft inflammatory factor 1/*Iba1* (AIF1), CD68 is considered a marker of phagocytic microglia. *Cd63*, encoding a marker of exosomes, and *Lgals3* (galectin 3), were found to be co-expressed in 5XFAD mice in A β plaque-associated microglia (40), cells that are considered as primary contributors to neuroinflammation in AD pathology.

Furthermore, the reduced expression of *Gfap*, a marker of astroglial activation, suggested that the effects of pantethine were not restricted to microglia. Indeed, *S100a6*, that encodes a Ca²⁺/Zn²⁺ binding protein, was reported to be overexpressed in astrocytes of AD patients and AD mouse models (41). In contrast to astrocytes of white matter where all S100A6 proteins were expressed homogeneously, almost all S100A6 immunoreactive cells within the gray matter were concentrated in astrocytes surrounding the A β deposits.

Genes encoding TLR2, TLR7, and CD14 were upregulated in the cortex of distinct transgenic murine models of various neuroinflammatory diseases, including AD (42). It must be noted that the TLR-mediated microglial response has both beneficial roles in stimulating phagocytosis as well as detrimental roles in the A β -stimulated release of neurotoxic products (43). TLR2 is a known primary receptor for A β peptide to trigger neuroinflammation (44, 45). Its co-receptor, CD14, is a critical regulator of the microglial inflammatory response that modulates A β deposition (46).

In addition to other inflammatory markers, complement activation products are known to be markedly upregulated in AD brains in response to soluble and insoluble amyloid peptides (47, 48). Their activation, detected at the early stages of amyloid deposition, coincides with the clinical expression of AD (49, 50). The encoded products are components of both the alternative phagocytic pathway (AP), triggered by bacterial membranes, and the classical phagocytic pathway (CP), initiated when C1Q binds to immune complexes (48, 51, 52). The expression of these components in transgenic mice might also be considered as part of a protective response. For instance, C1Q was shown to protect cultured primary neurons against A β (53), and C3 deficiency in AD transgenic mice was reported to elicit an acceleration of plaque deposition and neurodegeneration compared to WT (54).

Other genes revealed by our analyses such as *Csf1r*, *Trem2* and *Tyrobp* (Table 2) have been reported to be involved in phagocytosis (Fig. 5). In addition to the classical phagocytic receptors FCGR3 and CD68, CSF1R (colony stimulating factor 1 receptor) as well as CD14,

contribute to A β phagocytosis (55, 56). In a recent report (57), the role of CSF1R was determined as being crucial for the accumulation of intraneuronal amyloid peptides and the consequent formation of neuritic plaques. Here, we confirmed that the expression of *Csf1r* was increased in Tg compared to WT and significantly decreased upon pantethine treatment (Table 2). Furthermore, pantethine reduced the expression of *Trem2*, which presented the highest fold change (10-fold) within the set of Tg overexpressed genes (Table 1), along with the gene encoding its interactor, *Tyrobp*. *Trem2* is highly expressed in microglia and the *Trem2/Tyrobp* pathway activates phagocytosis of apoptotic neurons as well as A β in cultured cells (58). While this pathway inhibits TLR-induced inflammation (59), it was reported that, in a mouse model of AD, *Trem2* deficiency reduced pro-inflammatory cytokines (IL-1 β and IL-6) as well as astrogliosis (60). According to this observation, in AD mice, the reduced expression of *Trem2* in response to pantethine at a particular inflammatory stage might contribute to hamper the production of cytokines in *Trem2* expressing cells.

AIF1 binds to and polymerizes F-actin, and regulates RAC1 activity (61). RAC1 participates in the activation of the NADPH oxidase (NOX) complex leading to production of superoxide (Fig. 5). Among the genes encoding the different subunits of this complex, only the overexpression of *Cyba* in Tg (cytochrome b-245 alpha gene/p22phox) was drastically reduced in pantethine-treated Tg as shown in Table 1. CYBA is considered as a critical component of the membrane-bound oxidase of phagocytes that generates superoxide. A β is known to act on NOX in both hippocampal microglial and astrocyte cells to produce neurotoxic superoxide (62, 63), through the interaction of the NOX complex with phosphatidylinositol-3-phosphate (PI3P). Production of PI3P requires activation of RAC2, a member of the Ras superfamily of small guanosine triphosphate (GTP)-metabolizing proteins, VAV (vav guanine exchange factor) and FCG receptor ligation (64). Among these factors, Tg overexpression of *Vav1* was indeed lowered by pantethine (Table 1). Thus, the inhibitory effect of pantethine on the up-regulation of *Cyba*, and *Vav1* genes in transgenic mice supported a reduced activity of the NOX complex and therefore an ongoing decreased production of superoxide.

On the other hand, we found that some of the genes up-regulated in Tg and lowered by pantethine (Table 1) are known to be involved in neuroprotection, such as *Lyz* and *Hexb* (Fig. 5). For instance, rescuing *Hexb* expression in *Hexb*^{-/-} mice, which was detected exclusively in microglia (65), reduces neuroinflammation and can attenuate behavioral deficits (66).

As previously reported (27), we also observed an increased expression of *Grn* (granulin) in Tg that was reduced by pantethine (Table 1). GRNs are a family of secreted peptides generated by proteolytic release from progranulin. Interestingly, mutations in the *Grn* gene cause frontotemporal lobar degeneration and may contribute to AD (67).

Regarding genes of the TGF family, such as *Tgfb1* and *Tgfb2*, studies in transgenic animal models suggest that TGF- β might primarily contribute to AD pathogenesis by influencing A β production and deposition, resulting in brain microvasculature damage (68). Moreover, TGF- β was shown to act in synergy with IL-6 to turn on the expression of *Gfap* (69). In contrast, several studies report evidence for neuroprotective features of TGF- β 1 (70, 71). Indeed, it was reported that reduced TGF- β signaling leads to neurodegeneration and results in increased levels of secreted A β (72).

Overall, these data confirmed an inhibitory effect of pantethine on genes involved in inflammation. Thus, reducing inflammation might in turn abrogate its downstream induced activities including NO production, phagocytosis and as a consequence the need of neuroprotection. However, we cannot exclude that pantethine acts further upstream by interfering with A β production, which, in the long term, will tune down inflammatory processes. Of note, despite the overexpression of inflammatory markers in the 5XFAD mice, no changes were observed in our arrays for the expression of pro-inflammatory genes such as *Il-1 β* or *Tnf- α* . To validate the transcriptional data, RT-qPCR analyses were performed on genes differentially expressed in Tg when compared to WT and the effect of pantethine was evaluated. As shown in Fig. 6A, expression of *Gfap*, *Aif1* and *Tlr2* was increased in Tg compared to WT and this increase was significantly reduced by pantethine treatment in Tg but not in WT. Furthermore, while the absence of modulated expression was confirmed for *Tnf- α* (Fig. 6A), we observed a significantly higher expression of *Il-1 β* in Tg when compared to WT that was decreased by pantethine (Fig. 6B). Such a change in *Il-1 β* expression was also confirmed at the protein level (Fig. 6C) with a two-fold increase in Tg compared to WT, an increase that was completely abrogated by pantethine.

Pantethine reduces the expression of underexpressed genes in 5XFAD mice involved in neurotransmission and A β production

For this analysis, we only considered genes filtered for a threshold ≤ 0.6 in Tg compared to WT animals (Table 2) that were underexpressed in our previous transcriptomic study on hippocampi of either 6- or 9-month-old 5XFAD mice (27). Decreased expression of *Syn2*

(synapsin 2) was indeed observed at the earliest clinically detectable stage of AD (73) and significantly down-regulated in the hippocampus of the 3xTg-AD mice compared to the WT mice (74). It encodes a neuron-specific phosphoprotein involved in the regulation of neurotransmitter release and synaptogenesis (Fig. 7) that binds to small synaptic vesicles in the presynaptic nerve terminal (75). Noteworthy, as pantethine, ginsenoside Rg1, a drug shown to improve behavioral abnormalities in AD, restores its expression in the hippocampus of 3xTg-AD mice (74).

Reduced *Gria2* (glutamate ionotropic receptor AMPA type subunit 2) expression was observed also at 12 months in the hippocampus in 3xTg-AD mice (76). Its encoded product belongs to a family of glutamate receptors, the main excitatory neurotransmitter at many synapses in the CNS, and functions as ligand-activated cation channels. It was found that *Gria2* is subjected to RNA editing, a process shown to be essential for brain function (77), and defective *Gria2* editing was observed in the hippocampus of AD patients (78).

Several other selected genes encoded proteins involved in neurotransmission (Fig. 7). DGKG, a member of the diacylglycerol kinase family belongs to a class of enzymes that catalyze the ATP-dependent conversion of diacylglycerol (DAG1) to phosphatidic acid (PtdOH). It is well documented that the coordinated regulation of these two lipid second messengers is particularly important in the nervous system where it modulates a variety of neurological functions. Indeed, several reports support roles for these enzymes in neuronal spine density, myelination, synaptic activity, neuronal plasticity, epileptogenesis and neurotransmitter release (reviewed in (79)). *Kcnma1* (potassium calcium-activated channel subfamily M alpha 1) encodes the pore-forming alpha subunit of the MaxiK channels that play a role in neuronal excitability (80). NRXN1 (neurexin 1) is a single-pass type 1 membrane protein that binds neuroligins to form a Ca^{2+} -dependent complex at synapses in the CNS required for neurotransmission (81). Heterozygous mutations in *NRXN1* were found to impair synaptic functions in human neurons (82). Calcium voltage-gated channels such as CACNA2D1 have an important role in neurotransmission and genomic aberrations of its gene were found in patients with epilepsy and intellectual disability (83).

In addition, pantethine up-regulation of several genes suggested a possible inhibitory effect of pantethine on APP processing and A β production (Table 3, Fig. 7), with the exception of *Grm5* (metabotropic glutamate receptor 5). Indeed, genetic deletion of this gene inhibits A β production of oligomers and amyloid plaques, thus reducing cognitive impairment and pathogenesis in APP/PS1 Δ E9, another AD mice model (84). In contrast, restored expression

of *Apba2*, *Appbp2* and *Gpd2* should decrease A β toxicity. *Apba2* (amyloid beta precursor protein family A member 2) encodes a neuronal adapter protein interacting with APP. This interaction stabilizes APP and inhibits production of proteolytic APP fragments including A β (85-89). Furthermore, it was shown that APBA2-mediated reduction in cerebral A β was associated with normalization of both cognition and *in vivo* long-term potentiation in an AD transgenic mice model (90). The protein encoded by *Appbp2* (Amyloid beta precursor protein binding protein 2) interacts with microtubules and is functionally associated with APP transport and/or processing (91).

On the other hand, the restored expression of *Gpd2*, glycerol-3-phosphate dehydrogenase 2, can be related to the effect of pantethine on glycolysis since GPD2 is involved in maintaining a high rate of this process (92). Thus, restoring glycolysis, whose impairment was shown to promote an increase in A β aggregation and internalization (93), might be part of the beneficial effect of pantethine. Altogether, these data supported that pantethine maintained the constitutive expression of genes underexpressed in Tg to preserve proper CNS functions.

Discussion

Although the clinical impact of pantethine remains to be proven, we presented here some lines of evidence in favor of a beneficial effect of pantethine in AD. Our behavioral test suggested that pantethine treatment reduced aggressiveness (Fig. 2). Such an effect was supported by the decrease of astrogliosis and microgliosis as well as a reduction of the number of amyloid plaques (Fig. 1). While it is well admitted that AD starts with A β deposition, the precise process that leads to neurodegeneration, is still unknown. A β aggregation triggers neuroinflammation that is accompanied with an increase of ROS production resulting in an oxidative stress. Despite the ability of inflammation to increase phagocytosis for A β clearance, it is thought that its chronicity can impact negatively the protective activities, and restoration of CNS functions cannot be anymore achieved.

The inhibition of pantethine on *Il-1 β* expression seen in primary cultures of astrocytes upon treatment with A β oligomers (17) was confirmed *in vivo* in the present work at both mRNA and protein levels (Fig. 6B and C). Such a decrease might suggest a direct effect of the drug on the signaling pathway leading to *Il-1 β* expression. For instance, the nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) is one of the factors involved in the transcriptional induction of cytokines such as *Il-1 β* and IL-1 β -induced inflammatory genes (94). Reduced activity of NF- κ B by blocking IL-1 β signaling was described to alleviate

cognitive deficits, attenuate Tau pathology, and partly decrease certain fibrillar and oligomeric forms of A β (95). At least part of the mechanism leading to *Il-1 β* expression requires the translocation of NF- κ B to the nucleus that can be achieved after binding of A β_{42} to TLR2. Although the mechanism of action of pantethine is not yet elucidated, it was shown that pantethine alters lipid composition and cholesterol content of membrane rafts (96). Such an alteration with methyl- β -cyclodextrin was reported to decrease the levels of nuclear translocation of NF- κ B p65, phosphorylated ERK1/2 and TLR4 (97, 98). It is therefore possible that through its effects on lipid rafts as prime subcellular regions where amyloidogenic APP processing takes place (99, 100), pantethine affects NF- κ B activation, thus preventing the transcription of inflammatory genes. However, on one hand, neuroinflammation and IL-1 β contribute to neurodegeneration, a hallmark of AD pathology, on the other hand, IL-1 β overexpression in a transgenic AD model was reported to trigger beneficial effects (101). Indeed, crossing the APP/PS1 AD mouse model with transgenic mice that specifically overexpress *Il-1 β* (APP/PS1-IL-1 β) in the hippocampus led to a reduction of amyloid pathology (101). It was suggested that reduced amyloid plaques and therefore the beneficial effect of IL-1 β resulted from the enhancement of microglial phagocytosis. Recently, mapping the immune cell subsets in AD onset and progression (102) led to the identification of a potential microglia protective-type associated with neurodegenerative diseases (DAM). This subset of cells, which was also detected in *post-mortem* human brain, is characterized by higher expression levels of genes involved in phagocytic and lipid metabolism pathways. This DAM activation requires a mechanistically coupled temporal event that is initiated in a TREM2-independent pathway, followed by activation of the TREM2-dependent program. As suggested in APP/PS1-IL-1 β transgenic mice, a beneficial role of this DAM population was related to its phagocytic activity that should trigger A β clearance. However, despite a reduction of plaques, it was shown that neuronal loss induced by low concentration of A β (not leading to neuronal apoptosis) was mediated by primary phagocytosis of neurons by microglia (103). These observations should provide an explanation for the failure of therapeutic assays based on A β vaccination that did not alleviate cognitive deficits (104). We can hypothesize that pantethine through its anti-inflammatory effect might hamper the phagocytic activity (illustrated by the reduced expression of *Tyrobp* and *Trem2*) to a level where healthy neurons are preserved, while formation of amyloid plaques is reduced.

Although pantethine treatment may be preventive, according to the lipid raft hypothesis, we cannot exclude that it acts only in an AD context. Preliminary results (data not shown) on 4-month-old mice failed to detect any change following pantethine treatment in the expression of genes that were modulated in Tg compared to WT. At this time point, neither *Aif1*, *Gfap*, *Cd68*, *Cd14*, *Lyz*, *Cyba*, *Grn*, *S100a6*, *Tyrobp*, *Trem2* nor genes encoding complement factors, whose expression was increased in Tg, were affected by pantethine. Moreover, selecting the genes modulated by pantethine in WT did not lead to the identification of processes that can be supported by a significant number of genes.

In addition, another feature that could help understand the beneficial effect of pantethine is related to its role in the production of CoA. Inborn errors of CoA biosynthesis have been implicated in neurodegeneration with brain iron accumulation (NBIA), a group of rare neurodegenerative disorders, suggesting pantethine as suitable treatment for affected individuals (105). Moreover, glucose-derived pyruvate is a principal source of acetyl-CoA in all brain cells through pyruvate dehydrogenase complex (PDHC) activity. Pantethine could therefore compensate the decrease of PDHC activity observed in AD (106) that is responsible for cholinergic deficits and loss of cognitive functions.

On the other hand, it is possible that the effects observed in the 5XFAD result from pantethine metabolites. Pantethine can be converted to its monomeric form (pantetheine), which is metabolized by pantetheinase to generate pantothenic acid (vitamin B5) and cysteamine, known to have direct anti-oxidant effects (107-109). Cysteamine can cross the blood brain barrier (110) and was shown to induce the release of BDNF (brain derived-neurotrophic factor), inhibiting neuronal loss in Huntington's disease (111). Cysteamine is highly reactive and its oxidation leads to form cystamine. It has been shown that cystamine can activate the activity of NRF2, a crucial transcriptional factor involved in the anti-oxidant response both in cell cultures and in brain tissue (112). Interestingly, although cystamine inhibits the γ -glutamyl synthase that consequently blocks ROS scavenging, it reduces transglutaminase activity known to be associated with protein aggregates in AD brain and to trigger A β oligomerization and aggregation (113). Noteworthy, no side effects of pantethine were reported (114, 115). Thus, in case of clear clinical benefits of pantethine treatment, determining whether pantethine itself or one of its metabolites is responsible should not be a barrier to its use.

Conclusions

The present study demonstrated the efficiency of pantethine to reverse AD features such as astrogliosis, microgliosis, A β deposition and to AD-associated aggressive behavior. These changes were accompanied by the reversion of the overexpression of several genes in Tg mice related to inflammation/phagocytosis (summarized in figure 8). Since neuroinflammation and phagocytosis are crucial events in the course of AD, the various effects of pantethine or one of its metabolites (cysteamine, cystamine, pantothenic acid) on these processes are of importance. Blocking the signaling pathways that lead to either A β deposition, inflammation or oxidative stress or the vicious cycle that amplifies these alterations will have several downstream consequences that altogether can alleviate AD pathology. It will decrease: i) APP processing that will in turn reduce A β deposition; ii) the NF- κ B signaling pathway involved in the transcriptional activation of inflammatory genes and in particular those involved in phagocytosis such as TREM2. Ultimately, the reduced expression of these genes will impact the generation of the highly phagocytic DAM population and consequently might protect healthy neurons from phagocytosis and therefore neuronal loss.

Investigating more deeply the mechanism that sustain the effects of pantethine in 5XFAD mice overtime should determine whether pantethine treatment is preventive or curative. In addition to test depicting mood changes (i.e., aggressiveness), cognitive evaluations will have to be performed in order to provide further confirmation of the beneficial therapeutic effect of pantethine treatment. Based on our results, pantethine, provided routinely as a dietary supplement, could be considered as a serious therapeutic option for preventing, slowing or halting AD progression.

Declarations

Ethics approval and consent to participate

All experimental procedures were approved by the Ethics Committee of the Medical Faculty of Marseille and were carried out in accordance with the guidelines published in the European Communities Council Directive of November 24, 1986 (86/609/EEC). All efforts were made to reduce animal suffering and the number of mice.

Availability of data and materials

The microarray datasets generated in the present study are available in the Array Express database [www.ebi.ac.uk/arrayexpress] under accession number E-MTAB-6772.

Competing interests

The authors declare no financial conflict of interest that might be construed to influence the results or interpretation of the manuscript.

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Author contributions

KB, MvGB, DS, SR, MK, BG, MdR and PB performed experiments, collected, analyzed and interpreted data. WC performed the microarray experiment. KB, BG, MdR and PB wrote the manuscript. MdR and PB supervised the project.

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Figures, tables, legends

Figure 1: Effects of pantethine on astroglia and microglia reactivity in WT and Tg mice.

A) Representative epifluorescence microphotographs showing changes in astroglia and microglia reactivity with GFAP and Iba1 immunostaining, respectively, and amyloid plaques (anti-A β 6E10 immunostaining) in the hippocampus (Hc) of untreated or pantethine-treated WT and Tg animals, counterstained with nuclear marker Hoechst (blue). Scale bar 100 μ M. Quantification of changes (B) in GFAP, IBA1 immunostaining and (C) number of A β plaques measured as the immunostained area/total area of the hippocampus. Values are the mean \pm SEM of three brains (3 slices/brain) per group. * p <0.05; ** p <0.01; *** p <0.005 when comparing WT+P, Tg and Tg+P groups vs WT, ANOVA followed by *post hoc* Fisher LSD test for (B) and Student *t* test for (C).

Figure 2: Effects of pantethine on the aggressive behavior of 5XFAD mice. Quantification of the number of attacks (A) and latency to attacks (B) of untreated (Tg, black circles) and pantethine-treated (Tg+P, black squares) Tg mice towards WT male mice intruders. Values of ten animals are represented as median with interquartile range (done in triplicate with a different intruder as described in Materials and Methods). * p <0.05, Mann-Whitney test.

Figure 3: Effects of pantethine on genes differentially expressed in Tg and WT mice.

Expression values were filtered for p values <0.05 as provided by Illumina technology. Fold changes were deduced from the ratios (Tg+P/Tg, Tg/WT, WT+P/WT) between the four different groups and means were calculated. Selecting a fold change \leq 0.6 leads to 47 genes repressed by pantethine (Gene set 1 in left panel). A similar number of the top pantethine-induced genes (Gene set 2 in right panel) selected for a fold change \geq 1.5 is displayed. All the repressed or induced fold changes values are colored in white or in dark gray, respectively while unmodulated genes are indicated in light gray.

Figure 4: Gene distribution in untreated and treated Tg mice. Genes were numbered and selected for a fold change \geq 1.5 (up-regulated genes) and for a fold change \leq 0.6 (down-regulated genes) indicated by an up and a down arrow, respectively. Only genes with expression values exhibiting p values <0.05 as provided by Illumina technology were considered.

Figure 5: Schematic representation of functional networks involving genes whose overexpression in Tg was repressed by pantethine. Table of legends is provided (at the top right of the figure). Modulated genes are within a gray box and reduced expression by pantethine is indicated by P- in superscript.

Table 1 : Pantethine reverses the overexpression of genes involved in neuroinflammation in Tg. Values represent the ratio of the indicated comparison between groups (three animals per group and normalized to WT). $p^* < 0.05$; $**p < 0.01$ and $***p < 0.005$. ANOVA followed by *post hoc* Fisher LSD test.

| Genes | Tg/WT | Tg+P/Tg | Tg+P/WT | WT+P/WT |
|--------|--------|---------|---------|---------|
| Aif1 | 2.5*** | 0.6*** | 1.5 | 1 |
| Cd68 | 4.5** | 0.5** | 2.1 | 0.9 |
| Tlr2 | 2.7** | 0.6** | 1.6 | 1 |
| Tlr7 | 1.9** | 0.7* | 1.3 | 1 |
| Cd14 | 2.2*** | 0.7** | 1.4 | 0.9 |
| Gfap | 5.1** | 0.5** | 2.6* | 1.3 |
| S100a6 | 2.8** | 0.5** | 1.3 | 1 |
| Lyz | 10** | 0.4** | 3.7 | 0.9 |
| Hexb | 3.1** | 0.6* | 1.9 | 1.1 |
| Grn | 2.7*** | 0.6** | 1.5 | 1 |
| Tgfb1 | 3** | 0.6* | 1.5 | 1 |
| Cd63 | 1.8* | 0.5** | 0.9 | 1.1 |
| Lgals3 | 1.9* | 0.6* | 1 | 1 |

Table 2 : Pantethine reverses the overexpression of genes involved in phagocytosis in Tg. Values represent the ratio of the indicated comparison between groups (three animals per group and normalized to WT). $p^* < 0.05$; $**p < 0.01$ and $***p < 0.005$. ANOVA followed by *post hoc* Fisher LSD test.

| Genes | Tg/WT | Tg+P/Tg | Tg+P/WT | WT+P/WT |
|-------|-------|---------|---------|---------|
|-------|-------|---------|---------|---------|

| | | | | |
|--------|---------|---------|------|-----|
| C1qa | 4.8* | 0.5* | 1.8 | 1 |
| C1qb | 4.2** | 0.5* | 2.2 | 1 |
| C1qc | 4.6** | 0.5* | 1.9 | 1 |
| C4a | 6.8**** | 0.3**** | 2.2 | 1.3 |
| C4b | 8.1**** | 0.3**** | 2.8 | 1.5 |
| Csf1r | 2.7**** | 0.6** | 1.5* | 1.1 |
| Trem2 | 9.5**** | 0.4**** | 3.4 | 1.2 |
| Tyrobp | 6**** | 0.4**** | 2.5* | 1 |
| Cyba | 3.9**** | 0.5**** | 1.7 | 0.9 |
| Vav1 | 2.4**** | 0.7** | 1.6* | 1.1 |

Figure 6: Expression analyses of inflammatory genes. RT-qPCR analyses of inflammatory genes *Gfap*, *Aif1*, *Csf1r*, *Tlr2*, *Cyba* and *Tnf- α* (A) and *Il-1 β* (B). Values are the mean \pm SEM of three mice per group normalized to WT. (C) Quantification of IL-1 β levels in the hippocampus by ELISA. Values are the mean \pm SEM of three mice per group. * p <0.05; ** p <0.01; *** p <0.005 when comparing WT+P, Tg and Tg+P groups vs WT, ANOVA followed by *post hoc* Fisher LSD test.

Table 3: Down-regulated genes in Tg whose expression was reversed by pantethine. Values represent the ratio of the indicated comparison between groups (three animals per group and normalized to WT). p *<0.05; ** p <0.01 and *** p <0.005. ANOVA followed by *post hoc* Fisher LSD test.

| Genes | Tg/WT | Tg+P/Tg | Tg+P/WT | WT+P/WT |
|--------|---------|---------|---------|---------|
| Syn2 | 0.3**** | 3.1**** | 0.9 | 0.9 |
| Gria2 | 0.5**** | 2.1** | 1 | 1.3 |
| Apba2 | 0.5* | 2* | 1.1 | 1 |
| Appbp2 | 0.5**** | 1.7** | 0.7* | 0.8 |
| Grm5 | 0.3** | 3.7** | 0.9 | 1 |
| Gpd2 | 0.4**** | 2.2** | 0.9 | 0.9 |
| Dgkg | 0.5** | 1.8** | 0.9 | 0.9 |

| | | | | |
|----------|--------|-------|-----|-----|
| Kcnma1 | 0.4*** | 2.1** | 0.8 | 0.8 |
| Nrxn1 | 0.5* | 2** | 0.9 | 1 |
| Cacna2d1 | 0.5* | 2.5** | 1.1 | 1.2 |

Figure 7: Schematic representation of functional networks involving genes whose down-regulation in Tg was reversed by pantethine. Table of legends is indicated in Figure 6. Modulated genes are within a gray box and induced expression by pantethine is indicated by P+ in superscript.

Figure 8: Schematic representation of pantethine inhibitory activity (P in red) in AD as evidenced in this study. P followed by a question mark (?) means that the activity remains to be demonstrated. P with + or – in superscript indicates either an up-regulation or a down-regulation of the genes in Tg in response to pantethine treatment, respectively, when compared to untreated Tg.