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Alterations of Brain Cholesterol Homeostasis in the Tg2576 Mouse Model of Alzheimer's Disease

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Abstract

Background: It is now well established that perturbations in cholesterol levels are crucial in the onset and development of Alzheimer's disease (AD). Despite this evidence, scarce information is available about prospective alterations of proteins controlling brain cholesterol homeostasis in Alzheimer's disease. Thus, the aim of this work was to analyze the protein expression of enzymes and proteins involved in brain cholesterol biosynthesis and uptake in a mouse model of AD.

Methods: Transgenic Tg2576 mouse was used as experimental model of Alzheimer's disease. Phosphorylated HMGCR, AMPK, LDLr, LRP1 and SR-B1 protein levels were assessed by Western blot in both hippocampus and brain cortex at different disease stages.

Results: HMGCR phosphorylation, AMPK and LDLr levels were significantly increased in the hippocampus of 9- and 15-month-old Tg2576 mice. At 15 months of age, hippocampal LRP1 and SR-B1 were also upregulated. On the contrary, no differences were detectable in the analyzed proteins in the brain cortex, at both stages.

Conclusion: Marked alterations of proteins regulating cholesterol homeostasis were found in the hippocampus of Tg2576, further sustaining the crucial involvement of cholesterol metabolism in AD.

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Introduction

Alzheimer's disease (AD) represents one of the most common types of dementia. Clinically, this pathology is a neurodegenerative disorder characterized by progressive and consistent cognitive impairments and memory loss.

Morphologically, AD brains display cerebral atrophy, accumulation of extracellular senile plaques, intraneuronal neurofibrillary tangles (NFTs), synaptic alterations and neuronal loss. The build-up of abnormally folded amyloid beta and tau proteins in the CNS constitutes the most peculiar pathological hallmarks of the disease [1]. NFTs stabilize microtubules in axons under physiological conditions, whereas they become hyperphosphorylated under pathological conditions. This event leads to the detachment of tau from microtubules, and induces the generation of insoluble tau aggregates, which hinder the microtubular transport system of neurons. Senile plaques are characterized by aggregates of amyloid β-peptide (Aβ). This toxic peptide is produced by the proteolytic cleavage of the amyloid precursor protein (APP). APP processing is operated by the activity of β-secretase (beta-site APP cleaving enzyme 1, BACE 1) and the γ-secretase complex. Cognitive alterations observed in AD are deeply related to the presence of Aβ aggregates and tau pathology, but the causative connection between these hallmarks and the development of clinical manifestations is not clearly elucidated [2].

During last decades, cholesterol emerged as a crucial molecule in regulating Aβ production and cognitive decline in AD [3]. Notably, amyloidogenesis appears to depend at least in part on cholesterol, as both β- and γ-secretase complexes are located in cholesterol-rich lipid rafts, and the activity of these cleavage complexes is modulated by the cellular cholesterol content [4,5]. It is also postulated that lipoprotein receptors play a key role in the pathogenesis of AD. Indeed, Low Density Lipoprotein receptor (LDLr) and LDL-receptor Related Protein 1 (LRP1) play a crucial role in maintaining the homeostatic mechanisms which controls Aβ clearance from the brain [6,7]. On the other hand, experimental data demonstrate that HMG CoA reductase (HMGCR), the key and rate-limiting enzyme of cholesterol biosynthetic pathway, is reduced when APP is overexpressed in neuronal cells [8]. Additionally, molecular and genetic investigations further highlight the strong connection between cholesterol and AD: for instance, the apolipoprotein isoform ε4 (apoE4) is considered one of the most important risk factors for late onset AD [9].

Despite this evidence, limited information is available about prospective alterations of other important proteins involved in cholesterol metabolism in AD brain. The identification of impairments in the machinery regulating cholesterol homeostasis may provide relevant information to further support the hypothesis that perturbation of lipids metabolism is responsible for AD onset and/or progression.

Thus, the aim of this study was to characterize the expression of the main proteins implicated in brain cholesterol metabolism. Tg2576 mouse has been used as transgenic experimental model of AD. Tg2576 mice express the Swedish mutation of APP (APPK670N,M671L), which causes age-dependent behavioral deficits and the concurrent deposition of neuritic amyloid plaques comparable to those seen in AD patients [10]. Protein analysis was performed in both the hippocampus and brain cortex at different stages of the disease progression. In particular, HMGCR, LDLr, LRP1 and SR-B1 were estimated in 9-month-old Tg2576 mice (when minimal histological evidence of Aβ deposition is observed) and in 15-month-old Tg2576 mice (when diffuse plaques are evident) [10].

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Materials & Method

Animals

9-month-old and 15-month-old male wild type (WT) and Tg2576 mice were housed in groups of four and maintained under controlled temperature (20±1°C), humidity (55±10%), and illumination (12/12 h light cycle with lights on at 07:30 am). Food and water were provided ad libitum. Tubes for tunneling and nesting materials (paper towels) were routinely placed in all cages as environmental enrichment. All procedures involving animal care were approved by the Italian Ministry of Health and performed in compliance with the guidelines of the Italian Ministry of Health (according to D.Lvo. 26/2014), the Directive 2010/63/EU of the European Parliament and the Council of 22 September 2010 on the protection of animals used for scientific purposes.

All efforts were made to minimize the number of animals employed for the experiments and their suffering.

Lysate preparation

9-month-old mice (six WT and six Tg) and 15-month-old mice (four WT and four Tg) were sacrificed by cervical dislocation. Subsequently, brains were rapidly removed on an ice-cold plate, and cortices and hippocampi were dissected out and collected in order to obtain total lysates. Total lysates were prepared as previously described [11]. Briefly, hippocampus and brain cortex were homogenized in lysate buffer (0.01 M Tris–HCl, 0.001 M CaCl2, 0.15 M NaCl, protease and phosphatase inhibitors, pH 7.5). Samples were solubilized by sonication and centrifuged for 10 min at 13.000 rpm, the pellet was discarded and the supernatant was transferred into microtubes. Protein concentration was estimated by using the DC™ Protein Assay (Bio-Rad Laboratories). All samples were boiled for 3 min before loading for Western blot.

Western blot

Western blot was carried out by using the already described protocol [12]. Briefly, proteins (30 µg) were resolved by 7% SDS-PAGE at 30 mA (constant current) for 60 min. Proteins were then transferred onto nitrocellulose membrane with trans-blot turbo transfer system (Bio-Rad Laboratories) for 7 min at room temperature. The nitrocellulose membrane was subsequently blocked with 5% fat-free milk in Tris-buffered saline (0.138 M NaCl, 0.027 M KCl, 0.025 M Tris-HCl, and 0.05% Tween-20, pH 6.8) at room temperature, and probed at 4°C overnight with primary antibodies followed by incubation for 1 h with horseradish peroxidase-conjugated secondary IgG antibodies (Bio-Rad Laboratories). Bound antibodies were visualized using Clarity Western ECL substrate (Bio-Rad Laboratories) and exposure to Amersham Hyperfilm ECL (GE Healthcare). Image acquisition and analysis were realized with ImageJ (National Institute of Health, Bethesda,

MD, USA) software. All samples were normalized for protein loading by using α-tubulin, chosen as housekeeping protein. Densitometric values were obtained from the ratio between arbitrary units derived by the protein band and the respective housekeeping protein.

Statistical analysis

Statistical analysis was performed by using unpaired Student's t test. All the data were expressed as as means ± SD (standard deviation). Values of $p<0.05$ were considered to indicate a significant difference. Statistical analysis was calculated through GRAPHPAD INSTAT3 (GraphPad, La Jolla, CA, USA) for Windows.

Results and Discussion

Here we wanted to uncover whether the protein network of cholesterol homeostasis was affected in two different brain areas of Tg2576 mice: hippocampus and cortex. Firstly, we analyzed HMGCR, the most important regulatory enzyme in cholesterol production. HMGCR catalyzes the key and rate-limiting step committed in cholesterol biosynthesis and, for this reason, its activity results to be tightly regulated. Indeed, the enzyme is subjected to both short-term regulation, which is achieved by phosphorylation/dephosphorylation cycles. The phosphorylation at S872 residue suppresses HMGCR catalytic activity, and AMP-activated kinase (AMPK) represents the major kinase involved in HMGCR inhibition [13]. Thus, HMGCR inhibitory phosphorylation was assessed in the hippocampus of WT and Tg2576 mice. Hippocampal HMGCR phosphorylation was strongly increased in both 9- and 15-month-old Tg2576 transgenic mice if compared to age-matched WT animals (Figure 1a, b), suggesting an overall suppression of cholesterol biosynthetic pathway in AD brains. To the best of our knowledge, this is the first report which directly shows HMGCR phosphorylation status in AD brain. Notably, HMGCR hyperphosphorylation is accompanied by a significant increase in AMPK activation, as demonstrated by its enhanced activating phosphorylation (Figure 1 a, b). AMPK dysregulation observed in this work is consistent with literature data, showing that the phosphorylation of this kinase is increased in the hippocampus of AD mouse models and AD human brains. Interestingly, AMPK activation appears to be directly dependent on Aβ production [14].

Cholesterol homoeostasis maintenance in the brain is assured by a fragile equilibrium between local biosynthesis and uptake operated by lipoprotein receptors. Among the lipoprotein receptor family members, LDLr and LRP1 are highly expressed in the brain, and participate in cholesterol metabolism and intracellular transportation. Additionally, LRP1 was shown to be involved in signal transduction [15]. Together with LDL receptor family members, the scavenger receptor SR-B1 plays a pivotal role in cholesterol homeostasis, since it regulates the uptake of HDL in both peripheral tissues and central nervous system [16]. Considering the involvement of these cholesterol receptors in the proper regulation of neuronal functions, the protein levels of LDLr, LRP1 and SR-B1 were checked. LDLr expression was significantly upregulated at both 9-month-old and 15-month-old Tg2576 mice, whereas LRP1 and SR-B1 were significantly increased only in the hippocampi of 15-month-old Tg2576 animals with respect to WT mice (Figure 2 a, b). The rise in LDLr and SR-B1 supports previous findings, which indicate that the expression levels of both proteins are increased in other AD preclinical models [17,18]. Changes in LRP1 also corroborates literature data, supporting a crucial role for this lipoprotein receptor in AD physiopathology. Indeed, it has been shown that LRP1 polymorphisms are associated with the onset of AD, and its expression is upregulated in AD tissues [19].

It is interesting to note that none of the proteins assessed in this work were significantly changed in the brain cortex of Tg2576 mice if compared to WT (Figure 3a and 3b).

Conclusion

Our results demonstrate that alterations of proteins belonging to the machinery controlling cholesterol homeostasis (HMGCR and

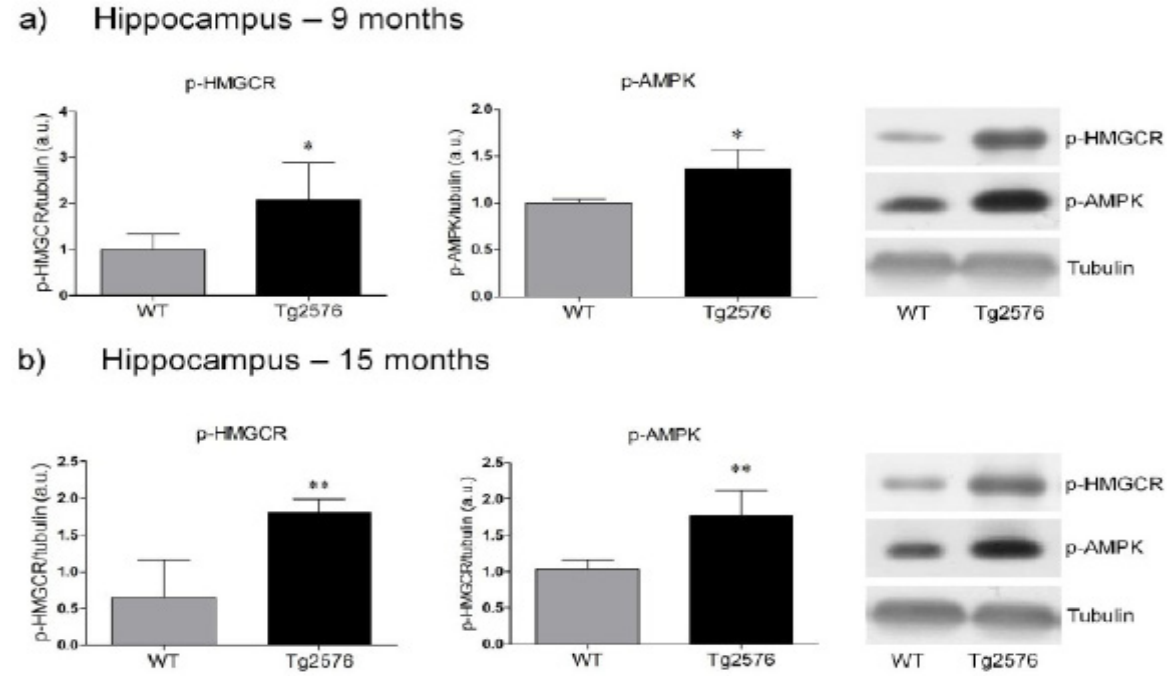

Figure 1. Densitometric analysis and representative Western blots of HMGCR phosphorylation (p-HMGCR) and AMPK phosphorylation (p-AMPK) in the hippocampus of wild type (WT) and Tg2576 mice at 9 months (a) and 15 months (b). n=6 animals per group. Data are expressed as means±SD. *p<0.05 and **p<0.01 as from unpaired t-test compared to WT.

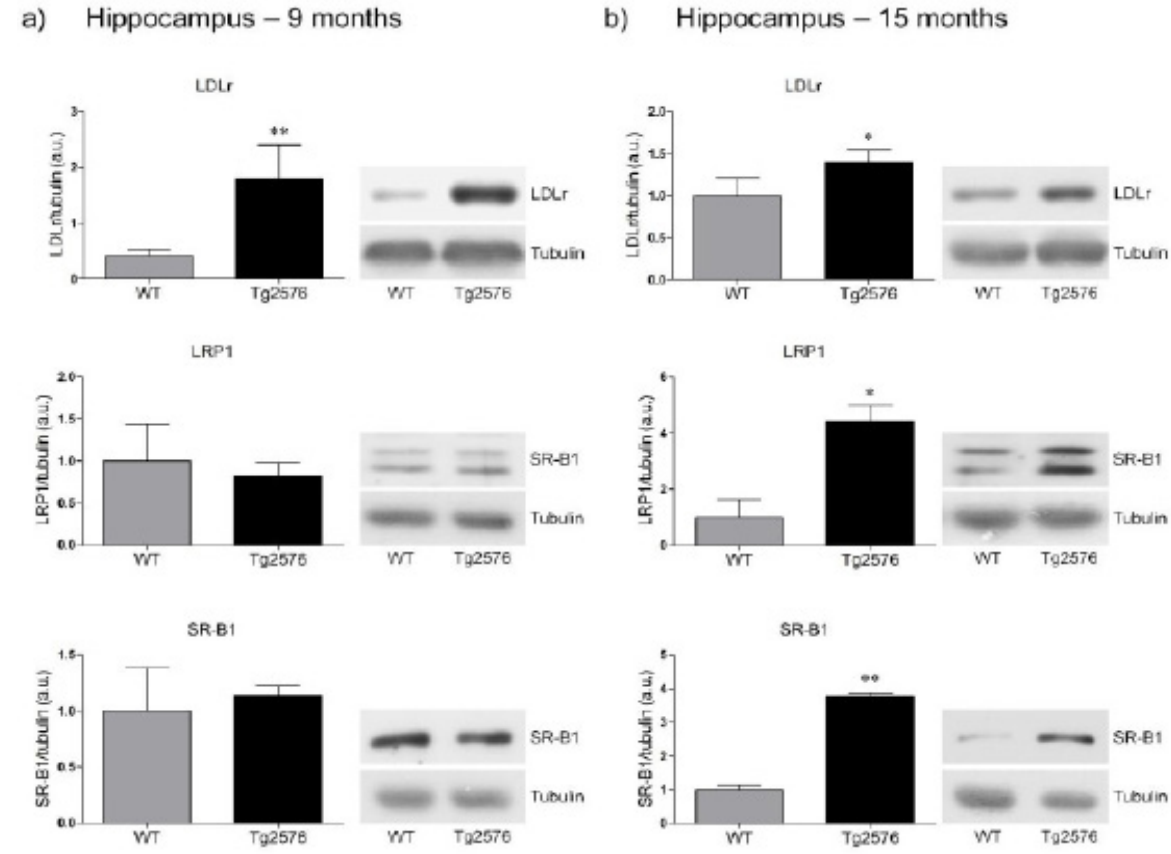

Figure 2. Densitometric analysis and representative Western blots of LDLr, LRP1 and SR-B1 in the hippocampus of wild type (WT) and Tg2576 mice at 9 months (a) and 15 months (b). n=6 animals per group. Data are expressed as means±SD. *p<0.05 and **p<0.01 as from unpaired t-test compared to WT.

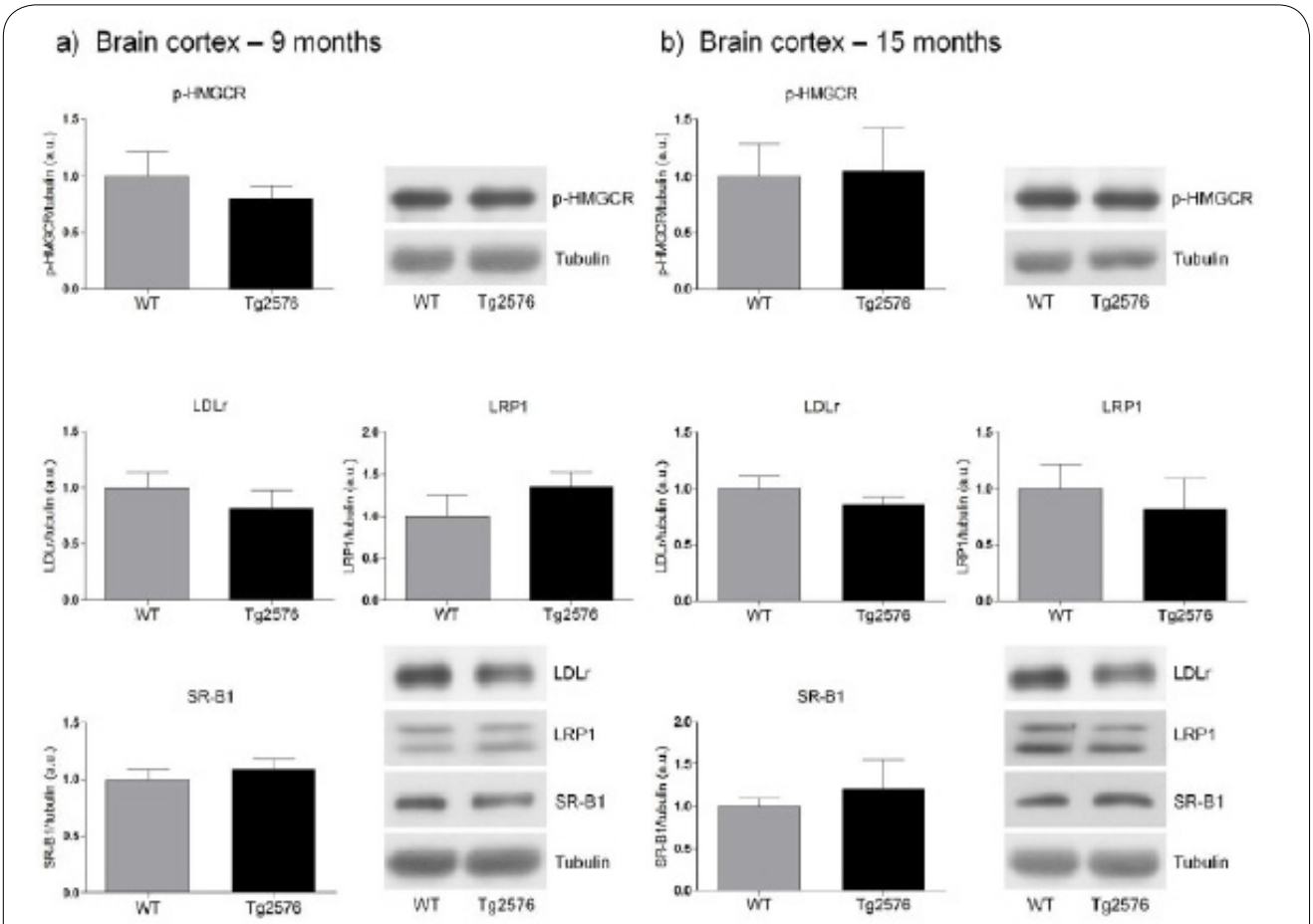

Figure 3. Densitometric analysis and representative Western blots of HMGCR phosphorylation (p-HMGCR), LDLr, LRP1 and SR-B1 in the brain cortex of wild type (WT) and Tg2576 mice at 9 months (a) and 15 months (b). n=4 animals per group. Data are expressed as means±SD. Statistical analysis was assessed by unpaired t-test.

LDLr) are already present at 9 months in the hippocampus of Tg2576, when amyloid plaque deposition is almost absent. However, exacerbations in these abnormalities occur with disease progression, as variations in LRP1 and SR-B1 levels are observable at 15 months. Notably, no changes were detected in brain cortex of both 9- and 15-month-old Tg2576 mice. It is reasonable to speculate that the strong divergences in the regional modulation of the proteins analyzed in this work can be dependent on the specific cellular context of each brain area, which respond in a different manner to the same physiopathological insult. This hypothesis is supported by an interesting report demonstrating that the administration of an LXR agonist is able to counteract the amyloid pathology in Tg2576 mice by selectively modulating cholesterol metabolism in the hippocampus [20]. In addition, differently from other brain regions, the hippocampus is especially vulnerable to damage at early stages of AD, and it is characterized by an extremely active cholesterol metabolism [13]. Thus, it could result particularly sensitive to deregulations in lipid homeostasis. In conclusion, the experimental data provided by this work indicate that AD pathology exhibits marked alterations of cholesterol metabolism in the hippocampus, suggesting that enzymes and proteins involved in this biological process may be used as novel pharmacological targets for counteracting neurodegeneration.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

VP and MS conceived and designed the study; VP coordinated the experimental plan; VP performed sample collection; MS carried out sample preparation and performed Western blot; MS analyzed the data and performed statistical analysis; MS and VP were involved in the interpretation of the data; MS wrote the manuscript; MS and VP critically discussed the results, revised the manuscript, approved its final version and qualify for authorship.

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