Biochimica et Biophysica Acta 1852 (2015) 2630–2644

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbadis

The increased potassium intake improves cognitive performance and attenuates histopathological markers in a model of Alzheimer's disease

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ARTICLE INFO

Article history:
Received 5 July 2015
Received in revised form 3 September 2015
Accepted 16 September 2015
Available online 21 September 2015

Keywords:
Alzheimer's disease
Potassium intake
Hypertension
Synaptic dysfunction

ABSTRACT

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by hallmarks that include an accumulation of amyloid-β peptide (Aβ), inflammation, oxidative stress and synaptic dysfunction, which lead to a decrease in cognitive function. To date, the onset and progression of AD have been associated with pathologies such as hypertension and diabetes. Hypertension, a disease with a high incidence worldwide, is characterized by a chronic increase in blood pressure. Interestingly, this disease has a close relationship to the eating behavior of patients because high Na^+ intake is a significant risk factor for hypertension. In fact, a decrease in Na^+ consumption, along with an increase in K^+ intake, is a primary non-pharmacological approach to preventing hypertension. In the present work, we examined whether an increase in K^+ intake affects the expression of certain neuropathological markers or the cognitive performance of a murine model of AD. We observed that an increase in K^+ intake leads to a change in the aggregation pattern of the Aβ peptide, a partial decrease in some epitopes of tau phosphorylation and improvement in the cognitive performance. The recovery in cognitive performance was correlated with a significant improvement in the generation of long-term potentiation. We also observed a decrease in markers related to inflammation and oxidative stress such as glial fibrillary acidic protein (GFAP), interleukin 6 (IL-6) and 4-hydroxynonenal (4-HNE). Together, our data support the idea that changes in diet, such as an increase in K^+ intake, may be important in the prevention of AD onset as a non-pharmacological therapy.

1. Introduction

Alzheimer's disease is the most common cause of cognitive impairment. This neurological disorder is characterized by the presence of neurofibrillary tangles, senile plaques (composed by the amyloid-β) and synaptic dysfunction. Other features of AD include oxidative stress, inflammation, Ca^{+2} imbalance, and disruption of signaling pathways [1–3]. In recent years, several clinical studies have proposed what may be an important factor in the progression of AD [4,5]. In fact, calorie restriction, the Mediterranean diet and drinking red wine, rich in polyphenols have been reported to attenuate AD deterioration of spatial memory, delay the onset of dementia and reduce amyloid neuropathology [5–7]. Epidemiological studies also suggest a relationship between AD and systemic pathologies such as metabolic syndrome, obesity and type II diabetes [5]. A common factor in these AD-related pathologies is a chronic increase in blood pressure. Hypertension is defined as persistent systolic blood pressure ≥ 140 mm Hg and diastolic blood pressure ≤ 90 mm Hg. Overall, approximately 20% of the population worldwide has hypertension, and its incidence is increasing [8]. Hypertension is considered a risk factor for several diseases, including hemorrhagic and ischemic stroke [9]. Furthermore, several studies have reported that blood pressure is increased in AD patients, even decades before the onset of the disease [5]. Furthermore, hypertension during midlife has been associated with an increase in the number of senile plaques in the neocortex and hippocampus and an augmentation of neurofibrillary tangles in the hippocampus. Therefore, prevention of hypertension may attenuate the onset or progression of AD [5,10–12].

It has been reported that increase in K^+ intake is beneficial for blood pressure and has no adverse effects [13–18]. In addition, several antihypertensive agents have shown some prevention of cognitive decline, diminishing the appearance of dementia related to AD in approximately 50% of study subjects [19]. In other reports, diuretics specifically, potassium-sparing diuretics to treat high blood pressure had an effect on dementia. These diuretics are associated with an important reduction of AD risk, and it is the antihypertensive treatment that has the

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http://dx.doi.org/10.1016/j.bbadis.2015.09.009
0925-4439/

greatest effect [20]. To study the effect of hypertension prevention on the onset and progression of AD, we studied the effect of a diet enriched in K^+. In a typical recommended diet, dietary intake of K^+ is at least 120 mmol/day (≈4.7 g/day); however, in the Western diet, Na^+ consumption is high and K^+ consumption is less than half of the recommended amount [14,21,22]. This low K^+ consumption has been associated with diseases such as hypertension, stroke and cardiovascular diseases [13,21,23].

In the present work, we used an animal model of AD: 4-month-old transgenic APPswe/PSEN1 mice. The mice received 2% potassium in their water for 8 weeks. Four-month-old animals were chosen because at that age neither significant cognitive loss nor brain Aβ plaques are detectable compared with 6-month-old mice (age at the end of treatment), which exhibit high brain Aβ content and memory loss [24–26]. We observed that treatment with 2% potassium significantly improved cognitive performance, mainly in learning and memory, suggesting a recovery in hippocampal activity. The improvement in cognitive performance was correlated with an increase in LTP generation and a decrease in inflammatory markers such us IL-6 and GFAP and the oxidative stress marker 4-HNE. Together, these results suggest that an increase in K^+ intake results in the prevention of cognitive alterations and oxidative stress in the early stages of AD in a mouse model.

2. Methods

2.1. Animals and treatment

Male APPswe/PS1dE9 (4-month-old) and C57BL/6 (6-month-old) mice were used in this study. APP/PS1 animals co-express a Swedish (K594M/N595L) mutation of a chimeric mouse/human APP (Mo/HuAPP695swe) together with the human exon-9-deleted variant of PS1 (PS1-dE9); these mice secrete elevated amounts of human Aβ peptide. This strain was obtained from The Jackson Laboratory (Bar Harbor, ME, USA). The animals (wild type and APP) were separated into two groups (17 animals by group); one group served as the control, the other group was supplemented with 2% potassium chloride in the drinking water [27]. The duration of the treatment was 8 weeks, including the behavioral tests. Mice were fed ad libitum. They were housed at the Animal House Facility of the Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile. After treatment the mice were used in the following order: 9 animals for the cognitive test and blood analysis, 5 animals for electrophysiology experiments and 3 animals for immunoblotting and immunofluorescence analysis.

2.2. Biochemical analysis

Blood was collected from the tail vein after 6 h of fasting and then centrifuged at 2500 × *g* for 10 min at room temperature to obtain serum samples. Glucose levels were measured using the hexoquinase/G-6-PDH method in the Architect Analyzer (Abbott Laboratories, Abbott Park, IL, USA), and insulin levels were measured via chemiluminescence (Beckman Coulter); in both cases, the manufacturers' instructions were followed. Triglyceride and cholesterol were assayed enzymatically in the Architect c8000 (Abbott Laboratories, USA).

2.3. Thioflavin-S (Th-S) staining

To detect amyloid formation, Th-S staining was developed using brain slices mounted on gelatin-coated slides as previously described [28,29]. Slices were dehydrated and rehydrated in xylene and ethanol baths, followed by incubation in distilled water for 10 min. Next, slices were immersed in Th-S solution (0.1% ThS in 70% ethanol) for 5 min and then washed twice in 70% ethanol for 30 s and cover-slipped with mounting medium in the dark.

2.4. ELISA of $A\beta_{42}$ and $A\beta_{40}$ peptides

The brain samples were extracted on ice and immediately processed as previously described [30]. Briefly, tissues were homogenized in RIPA buffer (10 mM Tris–Cl, pH 7.4, EDTA 5 mM, 1% NP-40, 1% sodium deoxycholate, and 1% SDS) supplemented with a protease inhibitor mixture (1 mM PMSF, 2 μg/mL aprotinin, 1 μg/mL pepstatin and 10 μg/mL benzamidine) and phosphatase inhibitors (25 mM NaF, 100 mM Na_3VO_4, 1 mM EDTA and 30 μM $Na_4P_2O_7$) using a Potter homogenizer and then passed sequentially through different caliber syringes. Protein samples were centrifuged at 14,000 rpm at 4 °C twice for 15 min. Then, brain Aβ levels from 50 μL of the processed samples were determined using Human Amyloid β_{40} and Amyloid β_{42} Brain ELISA (Millipore), and the absorbance of each plate was read in a spectrophotometer (Metertech).

2.5. Immunofluorescence

Immunofluorescence in brain slices was performed as described previously [31,32]. Slices were washed three times in ice-cold PBS and then permeabilized for 30 min with 0.2% Triton X-100 in PBS. After several rinses in ice-cold PBS, the samples were incubated in blocking solution (0.2% bovine serum albumin in PBS) for 1 h at room temperature followed by an overnight incubation at 4 °C with primary antibodies. After primary antibody incubation, the slices were extensively washed with PBS and then incubated with Alexa-conjugated secondary antibodies (Molecular Probes, Carlsbad, USA) for 2 h at 37 °C. The primary antibodies used were rabbit 4G8 and mouse anti-6E10 (Covance, Princeton, USA), rabbit anti-IL-6 (Abcam, Cambridge, UK), rabbit nitrotyrosine (Abcam, Cambridge, UK), rabbit 4-HNE (Abcam, Cambridge, UK), and rabbit anti-GFAP (Dako, Denmark). The nuclear stain was performed by treating the slices with Hoechst (Sigma-Aldrich, St. Louis, USA). The slices were subsequently mounted on slides using mounting medium and analyzed using a Zeiss LSM 5 Pascal confocal microscope. The images were analyzed using NIH Image J software.

2.6. Tricine-SDS PAGE

The brain samples were extracted on ice and immediately processed as previously detailed [33]. Briefly, tissues were homogenized in RIPA buffer (10 mM Tris–Cl, pH 7.4, EDTA 5 mM, 1% NP-40, 1% sodium deoxycholate, and 1% SDS) supplemented with a protease inhibitor mixture (1 mM PMSF, 2 μg/mL aprotinin, 1 μg/mL pepstatin and 10 μg/mL benzamidine) and phosphatase inhibitors (25 mM NaF, 100 mM Na_3VO_4, 1 mM EDTA and 30 μM $Na_4P_20_7$) using a Potter homogenizer and then passed sequentially through different caliber syringes. Protein samples were centrifuged at 14,000 rpm at 4 °C twice for 15 min. Then, the proteins (100 μg) were separated by electrophoresis performed in a Tris–Tricine buffer system [0.2 M Tris (pH 8.9) as an anode buffer and 0.1 M Tris, 0.1 M Tricine, 0.1% SDS (pH 8.25) as a cathode buffer] and then transferred to a PVDF membrane. The transfers were followed by incubation with the primary antibody 4G8 (Covance) and anti-mouse IgG peroxidase conjugated antibody (Pierce, USA) and developed using an ECL kit (Western Lightning Plus ECL, PerkinElmer).

2.7. Immunoblotting

The hippocampus was removed from each brain and homogenized in RIPA buffer (50 mM, Tris–Cl, pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 1% SDS) supplemented with a protease inhibitor cocktail (Sigma-Aldrich P8340) and phosphatase inhibitors (50 mM NaF, 1 mM Na_3VO_4 and 30 μM $Na_4P_20_7$) using a Potter homogenizer and then passed sequentially through different caliber syringes. Protein samples were centrifuged at 14,000 rpm at 4 °C twice for 15 min. Protein concentration was determined using the Bicinchoninic Acid (BCA) Protein Assay Kit (Pierce Biotechnology, Rockford, USA). Samples of

hippocampus (20 μg) were resolved by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane. Proteins were detected by incubation with a primary antibody followed by a secondary anti-goat peroxidase-conjugated antibody (Pierce) and developed using an enhanced chemiluminescence (ECL) kit (Western Lightning Plus ECL, PerkinElmer). The primary antibodies used were mouse anti-PHF-1 (Sigma-Aldrich); rabbit anti-tau (phospho-T231), rabbit anti-tau (phosphor S235) and mouse anti-tau from Abcam; mouse anti-tau AT8 (Thermo-Scientific) and rabbit anti-β-actin (Sigma-Aldrich), rabbit anti-IL-6 (Abcam, Cambridge, UK) and rabbit anti-GFAP (Dako, Denmark).

We thank Dr. Peter Davies (Department of Pathology, Albert Einstein College of Medicine, NY, USA) for his kind gift of the mouse anti-tau antibody against epitope PHF-1.

2.8. Behavioral test

2.8.1. Classical model test

The Morris Water Maze (MWM) task was performed as we have previously described [28,34]. Briefly, mice were trained in a circular pool 1.2 m in diameter (opaque water, 50 cm deep) and filled with water at 19–21 °C. For training, a submerged 9 cm platform (1 cm below the surface of water, invisible to the animal) was used with a maximum trial duration of 60 s with 10 s on the platform at the end of trials. Each animal was trained to locate the platform; testing was performed with three trials per day, and swimming was monitoring using an automatic tracking system (HVS Imagen, Hampton, UK). This system was used to measure the latency time needed to reach the platform and the time spent in each quadrant. After testing, the mouse was removed from the maze, dried and returned to its cage.

2.8.2. Memory flexibility test

This test was performed as previously described [28,34]. The conditions of the pool were the same as described for the MWM. Each animal was trained for one pseudo-random location of the platform per day, for 5 days, with a new platform location each day. Training was conducted for up to 10 trials per day, until the criterion of 3 successive trials with an escape latency of <20 s was achieved. When testing was completed, the mouse was removed from the maze, dried and returned to its cage. Animals were tested for the next location on the following day. Data were collected using a video tracking system (HVS Imagen).

2.8.3. Novel Object Recognition (NOR) task

This task takes advantage of a rodent's spontaneous preference to explore novel objects relative to familiar objects. This test is the benchmark test of recognition memory in the rodent and is dependent on the integrity of the hippocampus. The NOR paradigm was performed following the protocol described previously [35,36]. Briefly, mice were habituated to the experimental room in the experimental cages for 3 consecutive days, 30 min each day and for 1 h on the testing day. Testing took place in 25 cm × 25 cm opaque walled cages. For object familiarization, mice were allowed to explore their cage in the presence of two identical objects. Following this, animals were returned to their home cages for 1 h, followed by a 2.5 min exposure to a novel object. Mice had no observed baseline preference for the different objects. An object preference index was determined by calculating the time spent near the novel object divided by the cumulative time spent with both familiar and novel objects. Cages were routinely cleaned with ethanol following testing/habituating of mice.

2.8.4. Large open-field (LOF) test

A 120 × 120 cm transparent Plexiglas platform with 35-cm-high transparent walls was used to study locomotor and stress behavior in our mouse model because the use of a large platform is more effective than that of a small one for the study of behavior. The open field, which measured 40 × 40 cm, was defined as the "center" area of the field. Data were collected using an automatic tracking system (HVS Imagen, Hampton, UK). Each mouse was placed alone in the center of the open field, and its behavior was tracked for a 20 min session. At the end of the session, the mouse was returned to its home cage. The parameters measured included total time moving, time in the center and corner, and number of times the mouse crossed the center area of the platform [37].

2.9. Electrophysiological recording

Hippocampal slices were prepared using standard procedures as previously described [36]. Briefly, transverse slices (350 μm) of the dorsal hippocampus were cut under cold artificial cerebrospinal fluid and incubated in ACSF for 1 h at room temperature. In all experiments 10 μM picrotoxin (PTX) was included. Basal excitatory synaptic transmission was measured using an input/output curve protocol [38], which consisted of eight stimuli that ranged from 200 to 900 μA (with a 10-s interval between stimuli). LTP was generated using TBS, which consisted of 5 trains of the stimulus using an inter-train interval of 20 s.

2.10. Real-time PCR (QRT-PCR)

After Wnt3a treatment, we obtained RNA from the neuronal cultures. The RNA samples were treated how we described previously [39]. We used the following set of primers: cyclophilin: sense 5′-TGGAGATGAATCTGTAGGAGGAG-3′ and antisense 5′-TACCACATCCATGCCCTCTAGAA-3′; GFAP sense 5′-ACTCAATACGAGGCAGTGGC-3′ and antisense 5′-CTCTAGGGACTCGTTCGTGC-3′ and IL-6 sense 5′-CACTTCACAAGTCGGAGGCT-3′ and antisense 5′-CTGCAAGTGCATCATCGTTGT-3′.

2.11. Statistical analysis

The results are expressed as the mean ± standard error. Data were analyzed by one-way or two-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test; $^{*}p \le 0.05$ and $^{**}p \le 0.01$ were considered significant. Statistical analyses were performed using Prism software (GraphPad, La Jolla, CA).

3. Results

3.1. Changes in Aβ neuropathology in APP/PS1 mice supplemented with dietary potassium

To evaluate the general state of animals after the treatment, we measured several blood parameters, including glucose, insulin, cholesterol and triglycerides. We observed no significant differences between the treated and control group (Table 1). To determine whether the treatments affected the Aβ burden, we carried out the thioflavin (Th-S) staining in the cortex and hippocampus in the brains of APP/PS1 mice treated or not with K^+ (Fig. 1A, a–d). In the hippocampus, we did not observe changes in the Aβ burden in either the control or the treated group (Fig. 1A, e). However, in the cortex, we observed a 2.5-fold increase in the percentage of senile plaques in the treated group (Fig. 1A, f). Interestingly, we observed a significant change in the size of the senile plaques; treatment with potassium increased plaques smaller than 100 μm^2 (39.2 ± 0.4%) and decreased senile plaques larger than 200 μm^2 (70 ± 3%) (Fig. 1A, g). Using ELISA detection, we measured the concentrations of $A\beta_{1\text{-}42}$ and $A\beta_{1\text{-}40}$ in the hippocampus and observed a significant decrease in the concentration of $A\beta_{1\text{-}42}$ from 450 ± 45 pg/ml in the control condition to 300 ± 25 pg/ml in the treated group (Fig. 1B, a). We did not observe differences in $A\beta_{1\text{-}40}$ levels or in the $A\beta_{1\text{-}42}/A\beta_{1\text{-}40}$ ratio (Fig. 1B, b and c).

We also studied the expression of Aβ deposits, using the 6E10 antibody, which is reactive to amino acid residues 1–16 of β-amyloid [24,40]. We did not observe changes in the levels of expression after the

Table 1
Blood values after the treatment with 2% K^+.

	Glucose (mg/dL)	Insulin (μIU/mL)	Cholesterol (mg/dL)	Triglycerides (mg/dL)
Control	92 ± 12	1.3 ± 0.3	122 ± 11	100 ± 12
2% K^+	105 ± 10	0.9 ± 0.1	120 ± 11	109 ± 10

The values are expressed as the means ± SEM of n: 9 animals per group. *p < 0.05 and **p < 0.01 based on ANOVA (one-way) followed by Bonferroni post hoc analysis.

treatment with K^+ in hippocampus. However, the staining of plaques in animals supplemented with 2% potassium seemed to be less defined and more diffuse (Fig. 2A, a–b). We also observed that the immunofluorescence was present in a majority of the area in comparison with control transgenic animals, where plaques appear more compact and defined (Fig. 2A, c and d). To go further in the study of Aβ plaque conformation, we used a second antibody, 4G8, which is specific for amino acid residues 17–24 of the Aβ peptide [41,42], and observed a result similar to that using the 6E10 antibody with diffuse plaques in both

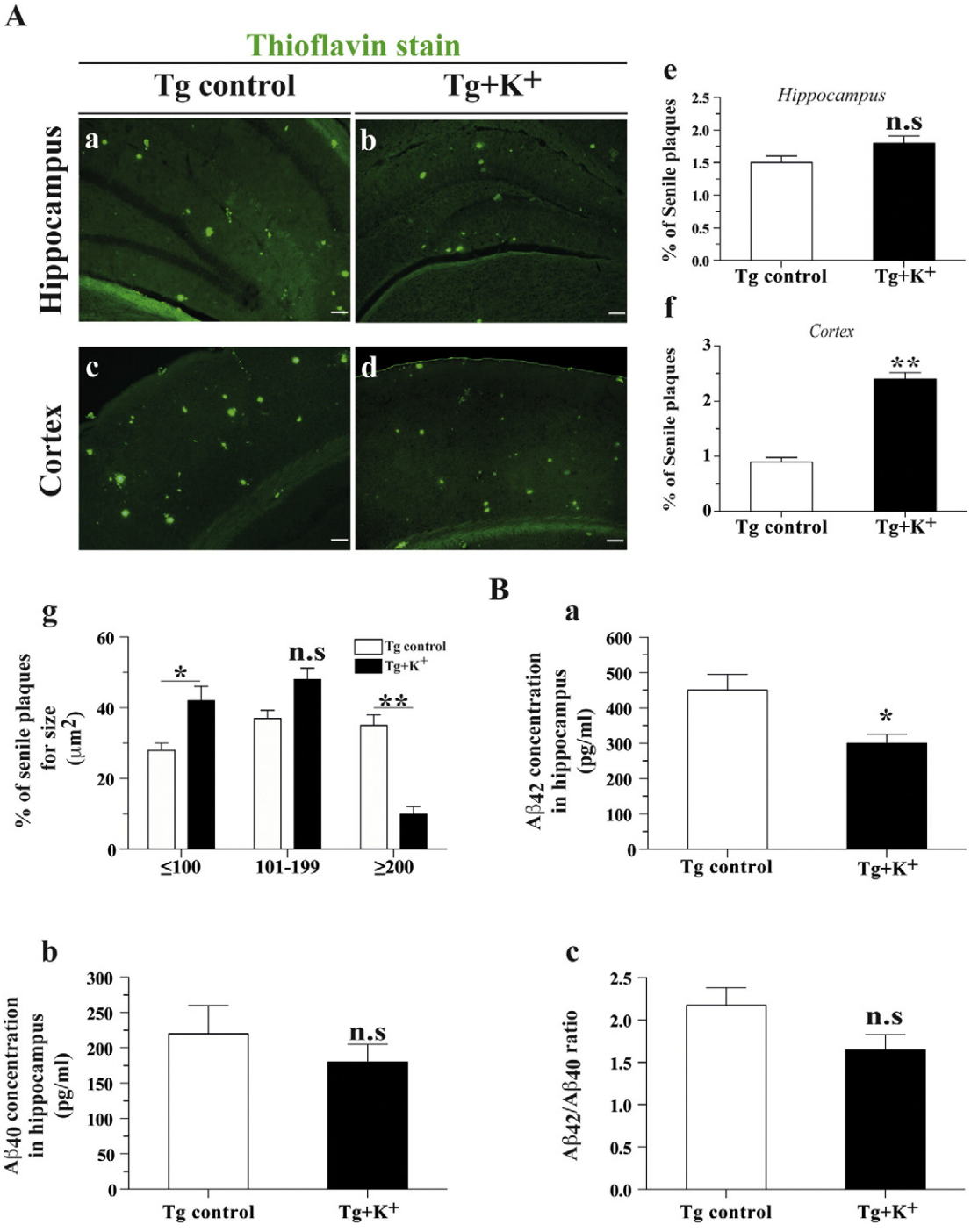

Fig. 1. Changes in Aβ neuropathology in APP/PS1 mice treated with KCl. Brain slices from APP/PS1 control mice and treated with 2% KCl were stained with Th-S to detect amyloid plaques in cortex and hippocampus. Th-S-positive area fraction for hippocampus and cortex in control and treated conditions (A, a–d). Cumulative frequency plot of amyloid plaque for control and KCl-treated APP/PS1 mice in cortex (e) and hippocampus (f), and cumulative frequency of amyloid plaque size in both conditions (g). Quantification of $A\beta_{1-42}$ levels present in hippocampal lysates of both control and KCl-treated mice by a sandwich-type ELISA assay (B, a). Quantification of $A\beta_{1-40}$ levels present in hippocampus lysate in both conditions through a sandwich-type ELISA assay (B, b). Aβ ratio ($A\beta_{42}/A\beta_{40}$) was calculated for both conditions (B, c). Data are means ± S.E.M. Significant differences were calculated by ANOVA, followed by post hoc Bonferroni's test. Asterisks indicate statistical significance of the observed differences (*p < 0.05); n = 3 per group. Scale bar: 100 μm.

the cortex and hippocampus (Fig. 2B, a and b). The size of plaques showed the similar patter than the result obtained with 6E10 antibody (Fig. 2B, c and d) Using Western blot for 4G8, we observed a decrease in the plaque size. Further studies are required to resolve this issue (Fig. 2B, e and f).

Phosphorylation of tau, particularly the appearance of the epitope PHF-1 (phosphorylated Ser-396 and Ser-404) and AT8, were analyzed in the hippocampus. We observed that treatment with 2% potassium decreased the expression of tau-PHF1 and tau-AT8 by 25% and 15%, respectively (Fig. 3a–c). However, we did not observe differences in other epitopes of tau, including tau-T231, tau-S235 and total tau [43–45] (Fig. 3d–f).

These results suggest that a diet supplemented with potassium may affect the processing or clearance of Aβ deposits, promoting the diffusion of Aβ plaques, a decrease in plaque size and a decrease in some epitopes of Tau phosphorylation.

3.2. The increase in potassium intake triggers improvement of cognitive performance

We studied the general condition of both groups and did not observe changes in blood parameters such as glucose, cholesterol and insulin. To study the general behavior of the mice, we performed the open-field test and did not find significant differences between the control and

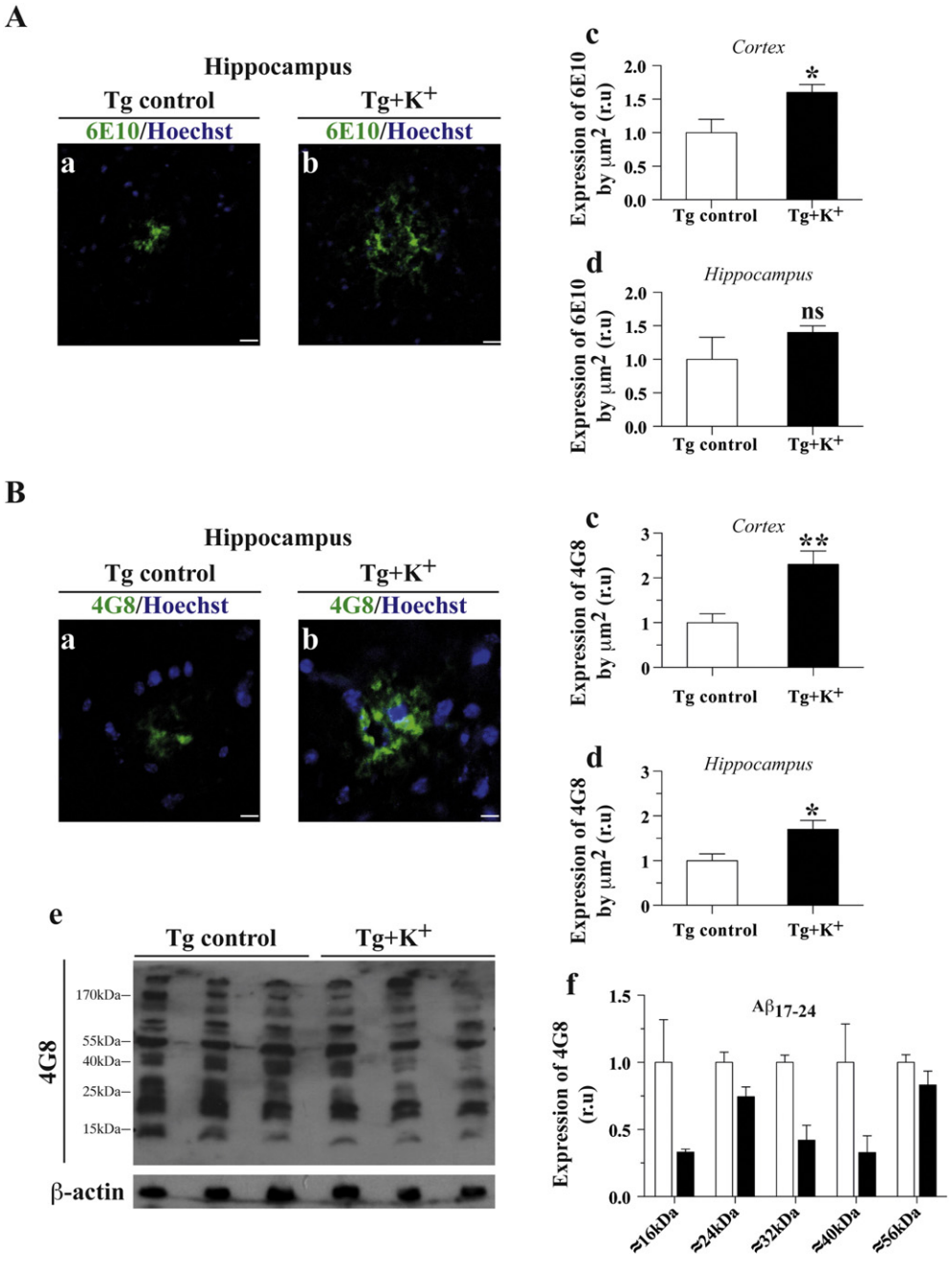

Fig. 2. Representative immunofluorescence for Aβ in APP/PS1 mice treated with KCl. Localization of Aβ deposits using 6E10 immunostaining in the cortex and hippocampus of control APP/PS1 mice and KCl-treated mice (A, a and b). Quantification of the expression of 6E10 by area (μm^2) in the cortex (A, c) and hippocampus (d). Aβ deposit detection with 4G8 immunostaining in the cortex and hippocampus of control APP/PS1 mice and KCl-treated mice (B, a and b). Quantification of 4G8 expression by area (μm^2) in the cortex and hippocampus (B, c and d). Tricine-SDS PAGE analysis of 4G8 in brain lysate of both conditions and the corresponding quantification of $A\beta_{17-42}$ levels (B, e and f). Data are means ± S.E.M. Significant differences were calculated by ANOVA, followed by post hoc Bonferroni's test. Asterisks indicate statistical significance of the observed differences (*$p < 0.05$); $n = 3$ per group. Scale bar: 50 μm.

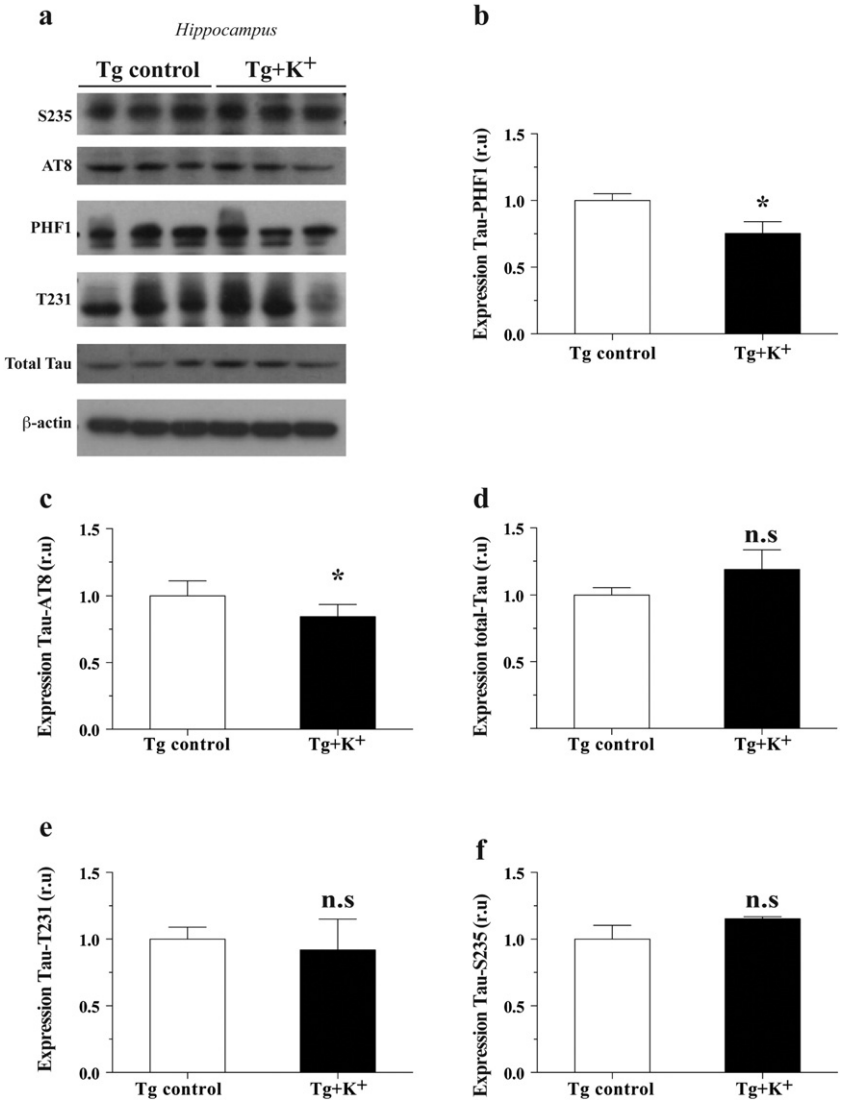

Fig. 3. Tau expression in APP/PS1 mice treated with KCl. Western blot analysis of several epitopes of tau (S235, AT8, PHF1, T231) and total tau in the hippocampus of APP/PS1 control mice and KCl-treated mice (a). Quantification of the levels of each epitope in both conditions: PHF1 (b), AT8 (c), total tau (d), T231 (e), S235 (f). Data are means ± S.E.M. Significant differences were calculated by ANOVA, followed by post hoc Bonferroni's test. Asterisks indicate statistical significance of the observed differences (*$p < 0.05$); $n = 3$ per group.

the treated groups in movement time or number of lines crossing the center of the cages. Furthermore, neither group showed differences in comparison with the wild-type mice, suggesting normal general behavior (Fig. 4a and b). Then, we performed the MWM and observed a significant change in the latency time during the consolidation phase in the last 3 days of the experiment. The wild-type animals took 23.5 ± 4.3 s to find the platform, whereas the control and treated groups took 43.5 ± 6.1 s and 30.3 ± 2.6 s, respectively (Fig. 4c). We did not observe changes in the speed of movement between the control and the treated group (Fig. 4d). Finally, we observed that after the removal of the platform, the treated group spent more time in the quadrant where the platform was previously located, therefore, the potassium intake helped to recover this cognitive function; however, it was insufficient to match the wild-type performance (Fig. 4e).

Therefore, we evaluated mice performance using a modified spatial memory paradigm associated with episodic memory (memory flexibility), a technique that is more sensitive to hippocampal dysfunction [46]. We observed that after 5 days, the treated group presented a significant difference from the control group, with the treated group taking 8 trials to reach the criteria and the control group taking more trials (14) (Fig. 4f). Finally, we studied the effect of potassium intake on the NOR test, and the results showed an improvement in the performance of the potassium-treated group in comparison with the control group (Fig. 4g). These results showed that an increase in potassium intake clearly leads to an improvement in the cognitive performance of AD mice.

3.3. The increase of K^+ intake improves hippocampal synaptic plasticity

We then examined the effects of a diet containing potassium supplement on synaptic plasticity to elucidate the potential mechanism underlying our behavioral results. Synaptic integrity was assessed using electrophysiological recordings for input–output analysis [36,47]. The field excitatory postsynaptic potential (fEPSP) showed improvement in the treated group in comparison with the control group (Fig. 5a). Additionally, the fiber volley, an indicator of axonal excitability, recovered in amplitude (Fig. 5b and c), which indicates an improvement in synaptic transmission at the presynaptic level. Synaptic plasticity was assessed by measuring LTP, which reflects persistent changes both in synaptic connectivity and in the underlying learning and memory functions. Experimentally, a set of protocols for theta burst stimulation and low-frequency stimulation (LFS) were used to induce LTP and LTD, respectively. It was not possible to induce robust LTP in the control

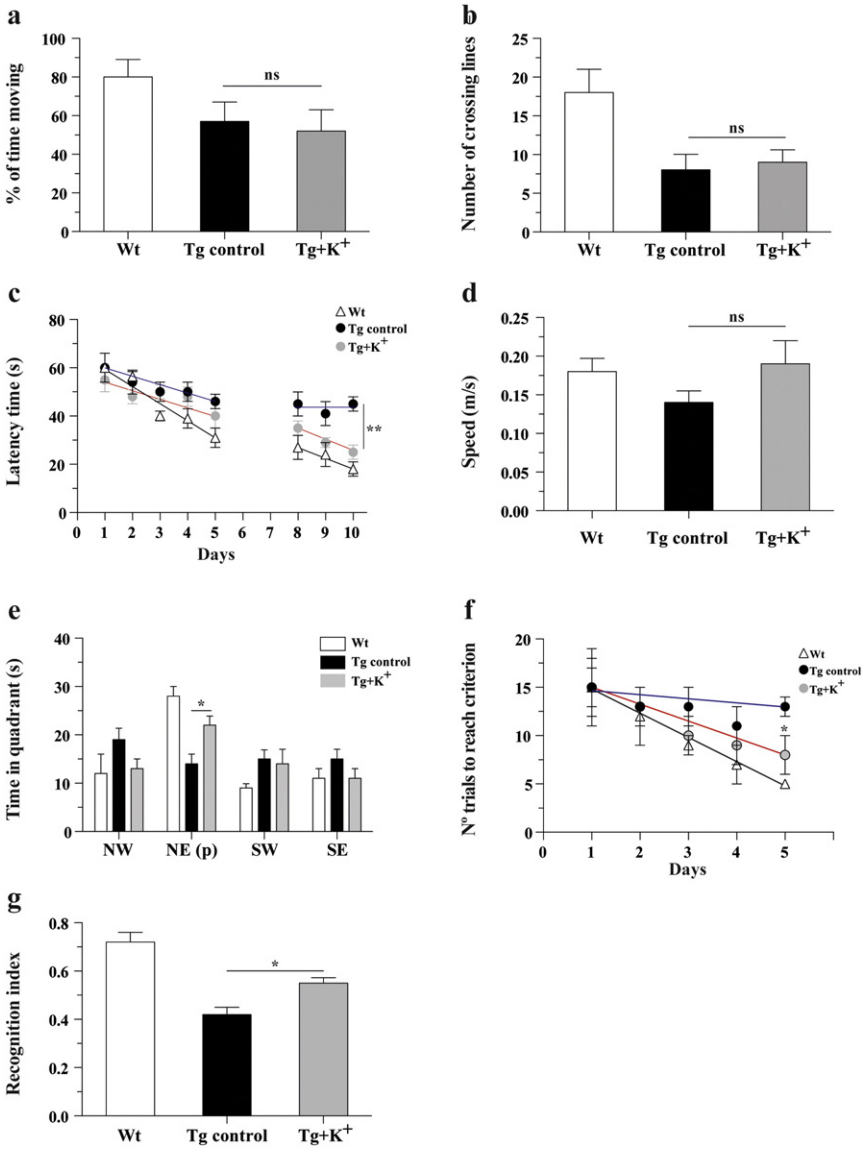

Fig. 4. Behavioral test in APP/PS1 mice treated with KCl. Behavior performance in C57bl6 control mice and APP/PS1 control or treated with 2% of KCl in the drinking water for 8 weeks. In the open-field test, exploring behavior was measured. After treatment, each experimental group was placed in the open-field apparatus, and their (a) moving time and (b) number of crossing lines were measured. In the MWM we measured (c) escape latency (time required to reach the hidden platform), (d) swimming speed and (e) the time spent in each quadrant in the absence of the platform. (f) Behavioral performance in the memory flexibility test was analyzed by assessing the number of trials needed to meet the criteria. (g) Analysis of the recognition index (time spent exploring the novel object/time spent exploring the novel and the old objects) in the memory flexibility test. Data are means ± S.E.M. Significant differences were calculated by ANOVA, followed by post hoc Bonferroni's test. Asterisks indicate significant observed differences (*$p < 0.05$); $n = 9$ per group.

group, which displayed a maximum of 1.43 ± 0.17 mV compared with a maximum of 1.99 ± 0.22 mV in the treated group (Fig. 5d). Furthermore, the induction and maintenance of LTP in the control group persisted for at least 60 min of recording, 1.07 ± 0.11 mV in the control group and 1.67 ± 0.14 mV in the treated group (Fig. 5e). These results suggest that the increase in potassium intake leads to a recovery of hippocampal synaptic transmission and plasticity.

3.4. Increased potassium intake attenuates the expression of the oxidative stress marker 4-HNE

Oxidative stress is triggered by an imbalance between oxidants and antioxidants in favor of the formation of oxidized compounds and results in damage to membranes, proteins and DNA [48]. Extensive early work demonstrated that oxidative stress plays a role in the onset of AD [49–52]. Tissue oxidative stress markers include lipid peroxidation, protein oxidation, DNA oxidation, and structurally and functionally damaged mitochondria [53,54]. We analyzed a marker of lipid peroxidation, 4-HNE. In the control group, we observed strong expression of this marker, in both the cortex (Fig. 6A, a–d) and in the hippocampus (Fig. 6A, e–h). The expression of 4-HNE decreased in the treated group by approximately 30% in the hippocampus and 50% in the cortex (Fig. 6A, i); the immunofluorescence signal was measured relative to the control group in both regions. Our data suggest that the increased potassium intake partially prevented the oxidative stress present in the APPswe/PS-1 transgenic mouse AD model.

Also, we study the expression of nitrotyrosine other marker of oxidative stress product of tyrosine nitration mediated by reactive nitrogen species such as peroxynitrite anion and nitrogen dioxide [55]. We observed a decrease in the mark only in the hippocampus and not significant difference in cortex of both groups (Fig. 6B, a–e).

3.5. Increased potassium intake decreases glial activation

Increased astrogliosis is an undisputed feature of AD brains with increased Aβ loads [56,57]. Brain sections obtained from the control

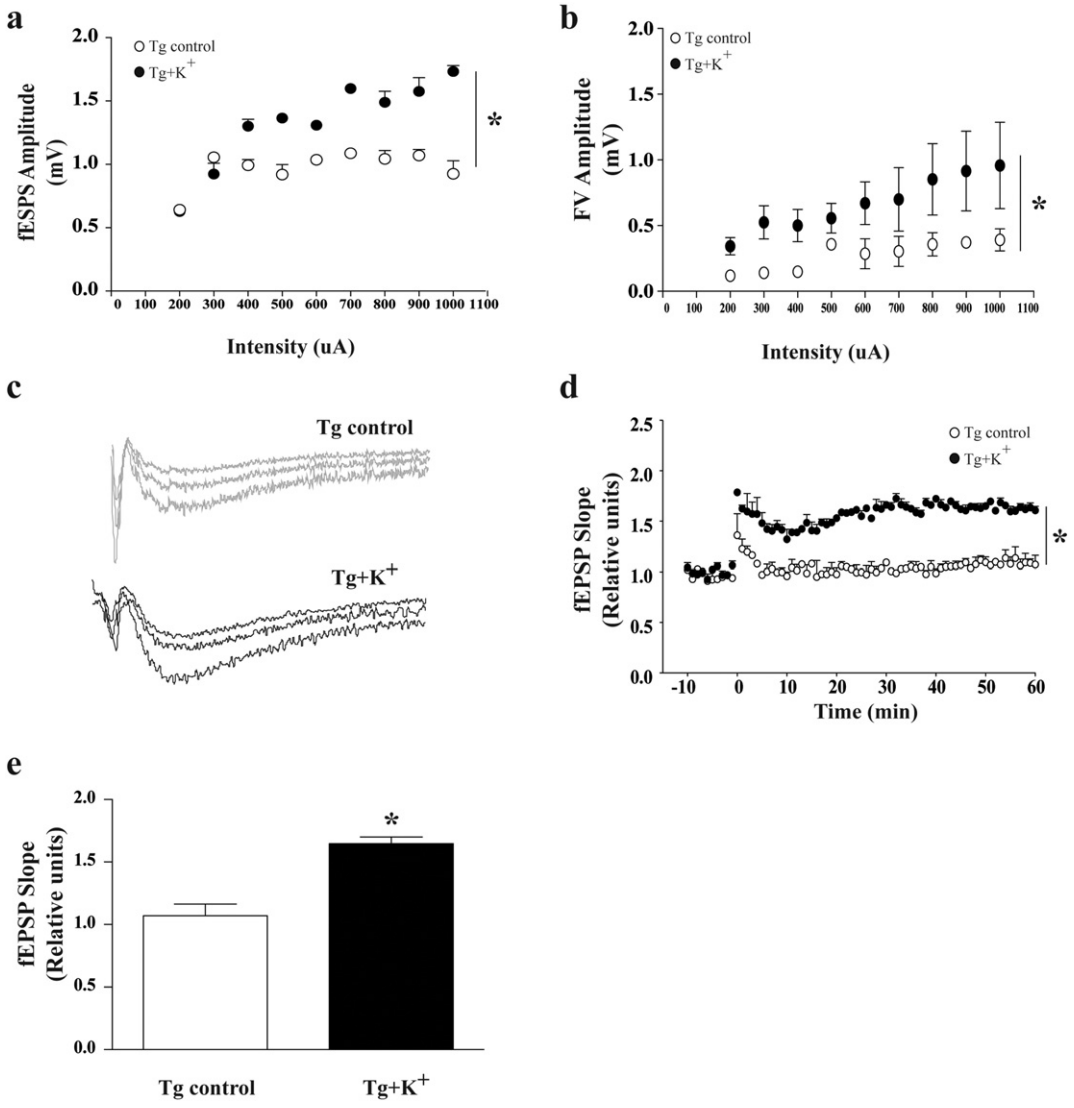

Fig. 5. Potassium effects in hippocampus excitability. Field recordings of hippocampal slices from APP/PS1 control mice and mice treated with KCl. (a–c) Synaptic integrity was evaluated via input–output analysis of the electrophysiological recordings. (d–e) LTP recording, plot of fEPSP amplitude versus time in hippocampal slices of both groups. The values are expressed as means ± SEM of 5 animals per group. *$p < 0.05$ based on ANOVA (one-way), followed by Bonferroni post hoc analysis.

transgenic group showed strong expression of GFAP in cortex (Fig. 7A, a–d) and hippocampus (Fig. 7A, e–h), while the treated group showed a decrease in the expression of the oxidative marker, mainly in hippocampus (Fig. 7, i). To support these results we performed QRT-PCR against the mRNA of GFAP and we observed a significant decrease (70%) in the levels of mRNA only in hippocampus (Fig. 7B). Also we correlated the result from QRT-PCR and immunofluorescence with Western blot and with this methodology we only observed a decrease in the levels of GFAP in the hippocampus, without changes at cortex levels (Fig. 7C, a and b). The decrease in the number of GFAP levels could be correlated with a decrease in brain inflammation mainly in hippocampus.

3.6. Increased potassium intake treatment decreases the expression of IL-6 inflammation marker

Neuroinflammation is one of the cellular and neurochemical processes that may underlie neurodegeneration [58–60]. As a prototypical cytokine with roles in the control of inflammation, IL-6 is altered in many central nervous system (CNS) diseases. IL-6 is overexpressed in the brains of AD patients and increased around amyloid plaques and in the cerebrospinal fluid (CSF) [61,62]. We measured IL-6 in the cortex (Fig. 8A, a–d) and in the hippocampus (Fig. 8A, e–h) of both the treated and control groups. A trend for decreased IL-6 staining was observed in the hippocampus of K^+-treated mice, but this difference was not significant (Fig. 8A, e–h). Treatment with 2% K^+ decreased the expression of IL-6 primarily in the cortex, while in the transgenic control we observed strong expression of this cytokine (Fig. 8A, a–d). The quantification of IL-6 signal showed a decrease in the cortex to nearly 58% (Fig. 8A, i). Using QRT-PCR we observed a decrease in the mRNA levels of IL-6 both cortex and hippocampus (Fig. 8B). Finally using Western blot we observed a decrease in the proteins of IL-6 only in cortex (Fig. 8C, a and b). These results suggest that the increase in the intake of K^+ stimulate a partial delay in the progress of inflammation.

4. Discussion

In the present work, we showed that an increase in potassium intake in early stages of AD triggers a delay in the expression of some markers of AD in a mouse model. The increased intake of K^+ led to a reduction in the level of $A\beta_{1\text{-}42}$. Additionally, diffusion of the smallest plaques and plaques increased. Furthermore, the diet decreased the tau of important antigenic sites, including PHF-1, associated with the onset of AD. The diet supplemented with K^+ stimulated and improved cognitive

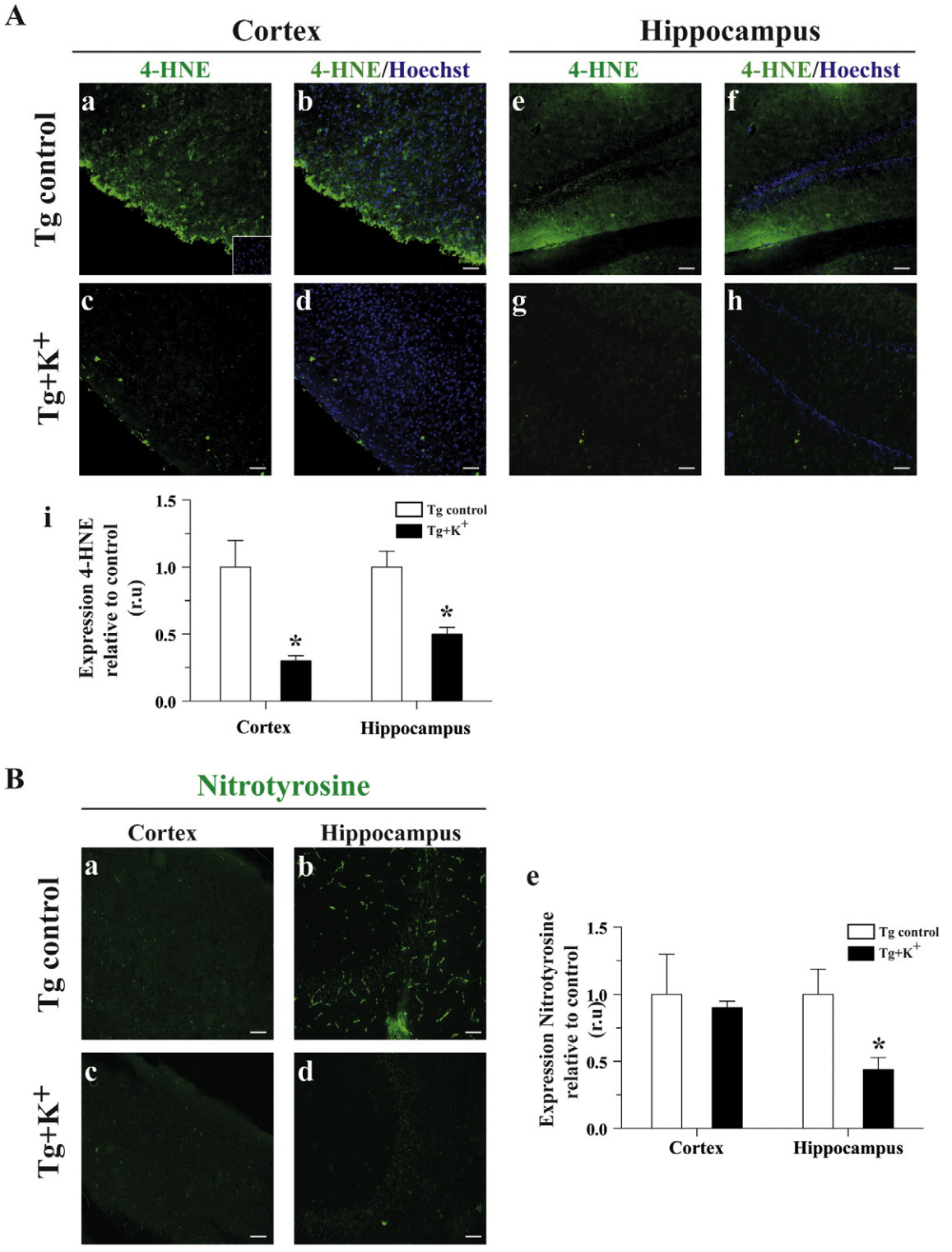

Fig. 6. 4-HNE detection in the brains of APP/PS1 mice. Representative immunofluorescence for 4-HNE in APP/PS1 control mice and mice treated with KCl. 4-HNE, green; nuclear stain Hoechst, blue. 4-HNE in hippocampus and cortex (A, a, b, e and f) of control mice and hippocampus and cortex of mice treated with KCl. (A, c, d, g and h) Quantification of the immunofluorescence signal normalized to that of the control transgenic animals (A, i). Immunofluorescence for nitrotyrosine in cortex and hippocampus (B, a-d). Quantification of signal (B, e). Data are means ± S.E.M. Significant differences were calculated by ANOVA, followed by post hoc Bonferroni's test. Asterisks indicate significant observed differences (*$p < 0.05$; ***$p < 0.001$); $n = 3$. Scale bar: 100 μm.

performance in processes related to memory and learning. Cognitive recovery was correlated with recuperation in neuronal plasticity at the level of LTP, the neurophysiological correlate of memory. Additionally, the diet enriched in K^+ induced a decrease in several histopathological AD markers, including GFAP, IL-6 and 4-HNE. Together, our data suggest that a diet rich in K^+ could serve as a preventive factor against the onset or early progression of AD (Fig. 9).

AD is the most common form of dementia associated with age, affecting approximately 36 million people worldwide. It is estimated that by 2050 the number of cases will rise to 110 million [5,63,64].

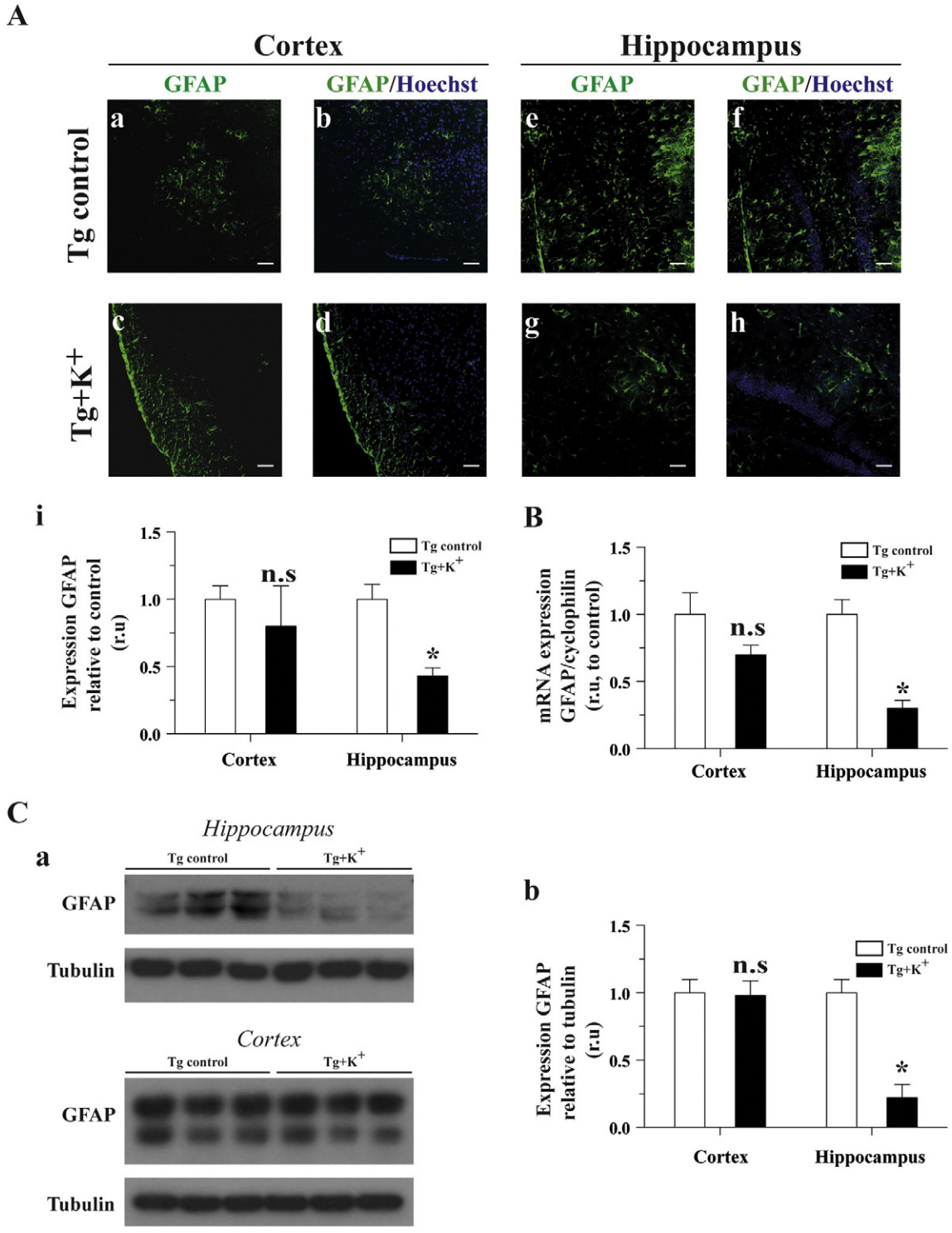

Fig. 7. GFAP detection in the brains of APP/PS1 mice. Representative immunofluorescence for GFAP in APP/PS1 control mice and mice treated with KCl. GFAP, green; nuclear stain Hoechst, blue. GFAP in the hippocampus (A, e and f) and cortex (A, a and b) of control mice and hippocampus (A, g and h) and the cortex (A, c and d) of mice treated with KCl. Quantification of the immunofluorescence signal normalized to that of the control conditions (A, i). QRT-PCR for mRNA levels of GFAP (B). Western blot against GFAP and quantification (C, a and b). Data are the means ± S.E.M. Significant differences were calculated by ANOVA, followed by post hoc Bonferroni's test. Asterisks indicate significant observed differences (*$p < 0.05$; ***$p < 0.001$); $n = 3$. Scale bar: 100 μm.

Typically, AD is characterized by several cellular dysfunctions, including the presence of extracellular Aβ aggregates, and by intracellular clusters of hyper-phosphorylated tau protein, i.e., the neurofibrillary tangles (NFTs). However, there are other markers of onset and progression of AD, such as activation of glial cells, metabolic disorders, oxidative stress and inflammation. Together, these forms of cellular dysfunction lead to the clinical manifestation of the disease [1,65–67]. Clinically, AD is characterized by a progressive loss of cognitive abilities, including memory and learning. The cognitive failure has been correlated with the loss of synapses and dendritic structures; both processes are related to the described neurodegeneration observed in AD [2, 68]. Although no cure exists for AD, and the molecular mechanisms that trigger the disease are not fully understood, several studies have suggested diverse mechanisms to explain the onset of AD. In

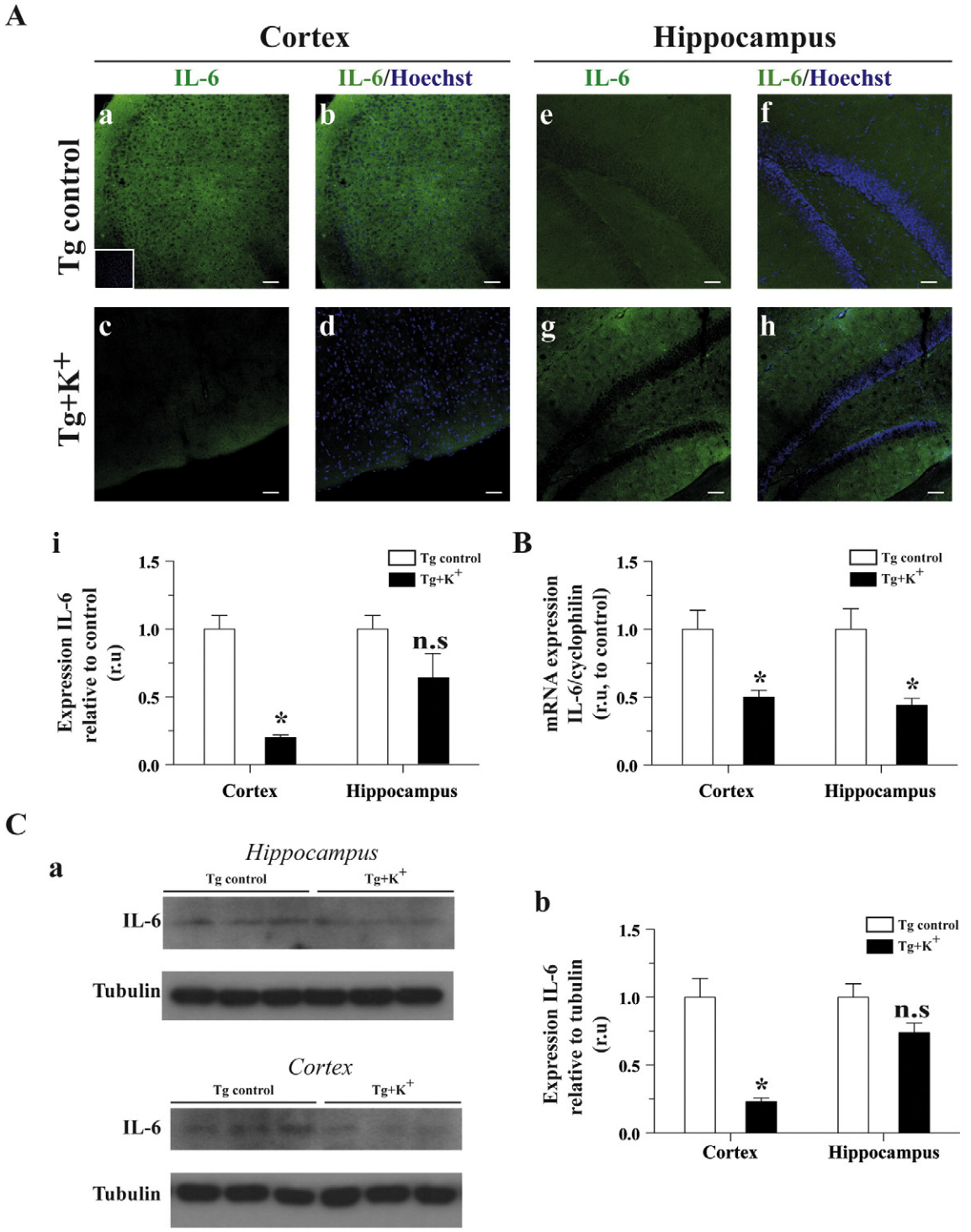

Fig. 8. IL-6 detection in the brains of APP/PS1 mice. Representative immunofluorescence for IL-6 in APP/PS1 control mice and mice treated with KCl. IL-6, green; nuclear stain Hoechst, blue. IL-6 in the hippocampus (A, e and f) and cortex (A, a and b) of control mice and the hippocampus (A, g and h) and cortex (A, c and d) of mice treated with KCl. Quantification of the immunofluorescence signal normalized to that under control conditions (A, i). QRT-PCR for mRNA levels of IL-6 (B). Western blot against IL-6 and quantification (C, a and b). Data are the means ± S.E.M. Significant differences were calculated by ANOVA, followed by post hoc Bonferroni's test. Asterisks indicate significant observed differences (*$p < 0.05$; ***$p < 0.001$); $n = 3$. Scale bar: 100 μm.

recent years, clinical studies have proposed that nutritional patterns could be an important factor in the onset and progression of AD [5]. In fact, some epidemiological studies have suggested a relationship between metabolic syndrome and AD that links unhealthy nutritional behavior and its associated diseases, such as obesity, hypertension and diabetes mellitus type 2, with the onset of dementia syndromes, including AD [69,70].

Hypertension has a direct correlation with diet; elevated intake of Na^+ stimulates an increase in blood pressure. In the Western diet, meals have high levels of Na^+ and very low levels of K^+, and it has been suggested that an increase in K^+ intake could be an efficient, non-pharmacological strategy to prevent hypertension and associated diseases [13,15,17,23]. In fact, epidemiological studies have shown that hypertension leads to dementia and cognitive decline. However,

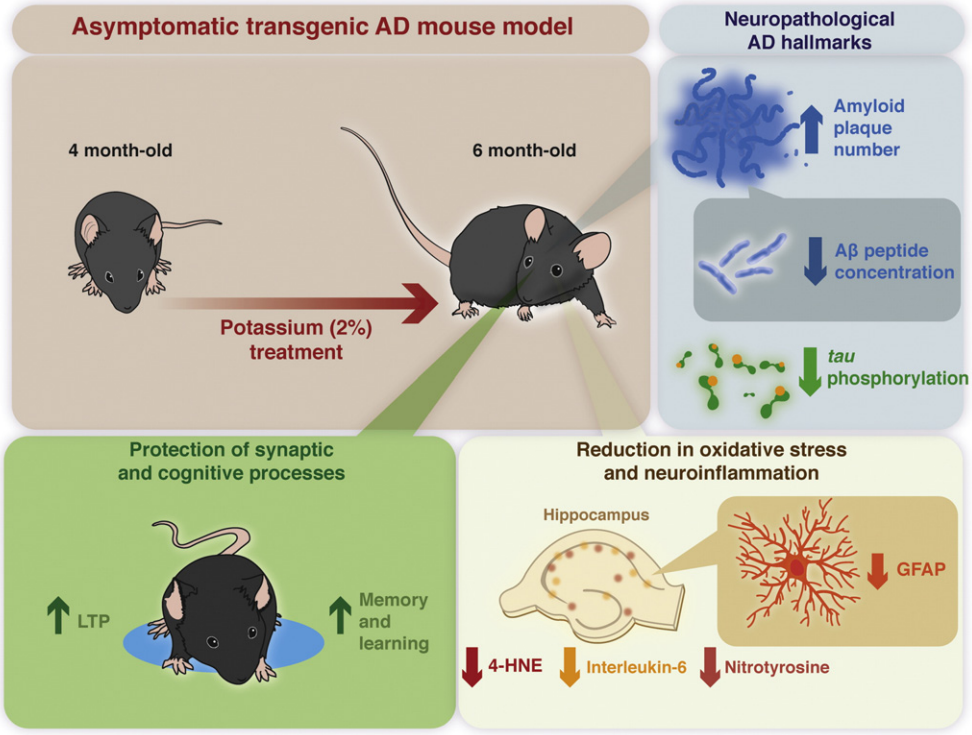

Fig. 9. Summary of the effects of K^+ intake on AD markers. The intake of 2% began at an age without the presence of AD markers. After 8 weeks, the transgenic mice without K^+ supplementation showed an increase in the numbers of Aβ, phosphorylation of tau. Additionally, we observed a decrease in cognitive performance and in neuronal plasticity. Finally, we observed an increase in the expression of the inflammation and oxidative stress markers IL-6, GFAP and 4-HNE. All the mentioned histopathological AD markers decreased in the transgenic mice supplemented with 2% K^+, suggesting a delay in the onset of AD. These results suggest that changes in diet could help delay the onset of AD.

the mechanism of this interaction is unclear [71–74]. One possible explanation of the interaction between hypertension and AD is that chronic hypertension leads to several pathological alterations, including effects on brain protein processing. In fact, in patients with hypertension, increased numbers of NFTs and amyloid plaques in post-mortem brains have been described, indicating a direct link between hypertension and AD [75,76].

In our APPswe/PS-1 transgenic model, AD pathology is established at approximately 6 months of age. We therefore treated mice in a pre-AD state by starting the treatment at 4 months of age, with the intent of learning whether the diet rich in K^+ could prevent the onset of the AD. In relation to the main brain marker of AD, Aβ aggregates, we observed an increase in both the number of small senile plaques and plaque diffusion. The size of plaques and the grade of diffusion could be related to APP processing and consequently to the generation of Aβ aggregates. In our model we studied the size of Aβ aggregates using several approaches described in literature including antibodies 4G8 and 6E10 and the thioflavin stain, with all this methodologies we observed a similar result, supporting our observation [40,42,77–79]. Therefore, the increase in small plaques triggered by 2% K^+ intake might suggest increases in APP processing to promote the elimination of the Aβ peptide [80,81]. Additionally, we observed a decrease in the concentration of $A\beta_{1\text{-}42}$, an important aspect considering that $A\beta_{1\text{-}42}$ is critical in the aggregation of the Aβ peptide. Aggregation along with the resulting formation of senile plaques, highlight the importance of $A\beta_{1\text{-}42}$ levels as an important biomarker of AD progression. In fact, the concentration of $A\beta_{1\text{-}42}$ in the cerebral spinal fluid (CSF) is a biomarker of both AD progression and Aβ metabolism in brain, primarily in the CSF [82,83].

The increase in K^+ intake also led to a decrease in two of the markers for tau hyperphosphorylation, an important finding given that the progression of AD is affected by the presence of phosphorylated tau [43,44].

The major clinical manifestation of AD is the loss of cognitive capacities. In our model, we evaluated whether the increase in K^+ intake affected this response in the early stages of AD. First, we used the MWM test to study spatial learning and memory, processes that both depend, in part, on hippocampal structure [84,85]. In the transgenic mice, we observed a significant increase in latency time, which was partially recovered in the group treated with K^+. Interestingly, this recovery occurred in the last three days of experiments, which corresponds to processes associated with the establishment of memory in the hippocampus and other brain regions [86]. To further evaluate the implications of hippocampal activity, we performed the NOR test, which is used to evaluate cognition, particularly recognition memory, in rodents. This test is based on the spontaneous tendency of rodents to spend more time exploring a novel object than a familiar one. The choice to explore the novel object reflects the use of learning and recognition memory [87]. Using this test, we observed an increase in the recognition index in the animals treated with K^+, a result that confirms a recovery of cognitive processes and also supports the data obtained in the MWM, which together suggest a recovery of hippocampal activity. To study hippocampal function and neuronal plasticity, we performed electrophysiological studies and observed that the control transgenic mice could not generate efficient LTP and also showed decreased potentiation activity, as we observed previously [28,30,36]. By contrast, the group treated with K^+ displayed partial recovery of LTP generation and an increase in hippocampus excitability. Together, these findings suggest that K^+ intake induces partial recovery in the brain function of AD model mice, primarily in processes related to learning, memory and neuronal plasticity. Finally, we studied whether the intake of K^+ affects the expression of other markers of AD progression, such as glial activation, oxidative stress and inflammation.

Glial activation and inflammatory processes have been described in several neurodegenerative diseases, including AD. Both processes have also been correlated with oxidative stress. In fact, the damage induced by oxidative stress in the hippocampus has been suggested as being critical to the failure of cognitive performance in AD patients [88–91]. In our

model, we observed that the intake of K^+ decreased expression of the oxidative stress marker 4-HNE, a marker of lipid peroxidation [92]. The decrease in the 4-HNE signal was significant in all hippocampus regions as well as in the cortex. In both regions, an increase in the expression of 4-HNE during the progression of AD has been described [93–96].

We also observed a decrease in glial activation in the group treated with K^+. Activation of astrocytes has been described in several models of neurodegenerative disease and has been postulated to represent a common alteration in AD in response to neuronal damage [97,98]. Neuronal damage in AD induced by Aβ aggregates (amyloid fibrils and Aβ oligomers) could be an important trigger for the activation of glial cells. The activation of glial cells is a well-recognized characteristic of an inflammatory response in the progression to brain damage [98–102]. The reduction in the number of cells positive for GFAP was correlated with a decrease in the inflammatory marker IL-6. The activation of glial cells induced the production of IL-6 by blood cells, including T-cells and monocytes. In brain tissue, IL-6 stimulates several processes related to protection against brain injury, including tissue remodeling, reduction of oxidative stress and inhibition of apoptosis induced by an increase in intracellular Ca^{+2} [103–105]. However, the molecular mechanisms responsible for the effects of IL-6 remain unclear.

Together, all the data suggest that an increase in the intake of K^+ could help prevent pathologies such as hypertension, which could possibly prevent or retard the onset of cognitive-related diseases such as AD (Fig. 9). The positive effects described for the K^+ diet could be an interesting non-pharmacological therapy at least to some extent, the incidence of cognitive decline and the progression of AD.

Conflict of interest statement

The authors declare that they have no competing interests.

Author contributions.

Conceived and designed the experiments: P.C., C.P.V. and N.C.I.
Performed the experiments: P.C., C.B.L., P.S., R.M.R., C. SA. and F.G.S.
Analyzed the data: P.C., C.P.V., C. SA. and N.C.I.
Contributed reagents/materials/analytical tools: C.P.V. and N.C.I.
Wrote the manuscript: P.C., C.P.V. and N.C.I.

Sources of funding

This work was supported by grants from the Basal Center of Excellence in Aging and Regeneration (CONICYT-PFB 12/2007) to N.C.I. and C.P.V., FONDECYT (no. 1120156) to N.C.I., FONDECYT (no. 11130529) to C.SA, FONDECYT (no. 1130747) to C.P.V. and postdoctoral fellowship from FONDECYT (no. 3150475) to P.C. We also thank to Sociedad Química y Minera de Chile (SQM) for special grants "The role of K^+ on hypertension and cognition" and "The role of lithium in human health and disease".

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