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ORIGINAL ARTICLE

In vivo effects of APP are not exacerbated by BACE2 co-overexpression: behavioural characterization of a double transgenic mouse model

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Abstract Down syndrome, the most common genetic disorder leading to mental retardation, is caused by the presence of all or part of an extra copy of chromosome 21. At relatively early ages, Down syndrome patients develop progressive formation and extracellular aggregation of amyloid-β peptide, considered as one of the causal factors for the pathogenesis of Alzheimer's disease. This neuropathological hallmark has been attributed to the overexpression of *APP* but could also be contributed by other HSA21 genes. *BACE2* maps to HSA21 and is homologous to *BACE1*, a β-secretase involved in the amyloidogenic pathway of APP proteolysis, and thus it has been hypothesized that the co-overexpression of both genes could contribute to Alzheimer's like neuropathology present in Down syndrome. The aim of the present study has been to analyse the impact of the co-overexpression of *BACE2* and *APP*, using a double transgenic mouse model. Double transgenic mice did not present any neurological or sensorimotor alterations, nor genotype-dependent anxiety-like behaviour or age-associated cognitive dysfunction. Interestingly, TgBACE2-APP mice showed deregulation of BACE2 expression levels that were significantly increased with respect to single TgBACE2 mice. Co-overexpression of *BACE2* and *APP* did not increase amyloid-β peptide concentration in brain. Our results suggest that the in vivo effects of *APP* are not exacerbated by *BACE2* co-overexpression but may have some protective effects in specific behavioural and cognitive domains in transgenic mice.

Keywords Down syndrome · Alzheimer disease · BACE2 · APP · Chromosome 21

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Introduction

Down syndrome (DS) or trisomy 21 is a clinically heterogeneous disorder which shows developmental delay, mental retardation and Alzheimer's disease (AD) type neuropathology by age 30–40 years (Wisniewski et al. 1985; Holtzman et al. 1996; Lott and Dierssen 2010). However, the precise mechanism(s) by which trisomy 21 leads to either developmental or the early-onset of AD-like neuropathology remains to be elucidated. Extracellular aggregation of amyloid-β (Aβ) peptide, one of the causal factors for the pathogenesis of AD (Yan et al. 2001), is common in DS patients at relatively early ages and has been attributed to the overexpression of amyloid precursor protein (*APP*) (Murphy et al. 1990), a large type I transmembrane glycoprotein precursor that maps to human chromosome 21 (HSA21).

Aβ peptide is generated by the endoproteolytic processing of APP, when a β-secretase cleaves APP to generate APPsβ, a soluble N-terminal fragment, and a C-terminal fragment (C99). This C99 fragment is cleaved by a γ-secretase to form the mature Aβ peptide comprising 39–42 amino acids (Mattson 2004). An increased accumulation of the C99 fragment in the brain of DS individuals has been observed (Busciglio et al. 2002; Sun et al. 2006), suggesting that abnormal processing at the APP β-site might be involved in DS. It has been shown that the β site APP cleaving enzyme 1 (BACE1) protease is one of principal enzyme in this pathway (Hussain et al. 1999; Vassar et al. 1999; Sambamurti et al. 2004). Since *BACE1* has a paralogous gene in vertebrates, *BACE2*, which in humans maps to HSA21 at 21q22.3 in the DS critical region (Acquati et al. 2000; Solans et al. 2000; Cheon et al. 2008), it has been speculated that BACE2 co-overexpression with APP would promote the early appearance of amyloid plaques in DS patients.

However, there has been an intense debate about BACE2 function. Some in vitro studies showed that BACE2 cleaves APP at the β-secretase site (Farzan et al. 2000; Hussain et al. 2000), but others showed that cleaves at the α-secretase site (Yan et al. 2001; Basi et al. 2003) or at a novel site named θ-secretase site (Sun et al. 2006). Recently, in an in vivo study we showed that BACE2 is not involved in the amyloidogenic pathway, cognitive dysfunction or cholinergic degeneration observed in elderly people with DS. However, we observed increased anxiety-like behaviour along with increased numbers of noradrenergic neurons in the locus coeruleus of TgBACE2 mice (Azkona et al. 2010).

The aim of the present study has been to analyse the impact of the co-overexpression of *BACE2* and *APP* along lifetime using a double transgenic mouse model (TgBACE2-APP).

Materials and methods

Animals

To generate transgenic mice co-overexpressing *BACE2* and *APP*, we crossed heterozygous TgBACE2 females (previously characterized, see Azkona et al. 2010) with heterozygous TgAPP males (Lamb et al. 1993), both generated on C57BL/6J × SJL F1 (B6/SJLF1/J) genetic background. Wild-type, TgBACE2, TgAPP and TgBACE2-APP littermates were housed in standard macrolon cages (4–5 animals per cage, 40 × 25 × 20 cm) with freely available food and water in standard environmental conditions (constant humidity and temperature of 22 ± 1°C) and a 12-h light/dark cycle (lights on at 7:00 a.m.). All animal procedures were approved by the local ethical committee (CEEA-PRBB), and met the guidelines of the local (law 32/2007) and European regulations (EU directive no. 86/609, EU decree 2001-486) and the Standards for Use of Laboratory Animals no. A5388-01 (NIH). The CRG is authorized to work with genetically modified organisms (A/ES/05/I-13 and A/ES/05/14) and the experimenters hold the official accreditation (law 32/2007).

Western blotting

Four adult animals (4 months) per genotype were killed, and brains rapidly removed. Tissues were homogenized in lysis buffer (10 mM HEPES pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1 mM $MgCl_2$, phosphate-buffered saline (PBS) 0.2% Triton X-100 and a protease inhibitor cocktail (Roche, Mannheim, Germany). After clearance of the lysates by centrifugation (1,400×g, 20 min at 4°C), protein quantification was performed following the BCA Protein Assay Reagent (Pierce, Rockford, IL, USA) protocol. Western blot analysis was performed using 50 µg of protein resolved on a 10% SDS-PAGE and electro-blotting onto nitrocellulose membranes (Hybond-C, Amersham Pharmacia Biotech, Freiburg, Germany). Membranes were blocked with 5% non-fat dry milk in Tris-buffered saline including 0.1% Tween-20 (TBS-T) and incubated with the primary antibodies in 5% non-fat dry milk in TBS-T overnight at 4°C. The following primary antibodies were used: goat anti-Bace2 (1:500; D-20; Santa Cruz, Heidelberg, Germany), rabbit anti-App (N-Terminal, 1:1,000; Sigma, Saint Louis, MO, USA), and anti-actin (1:2,000; Sigma, St Louis, MO, USA). Incubation with horseradish peroxidase (HRP)-conjugated anti-goat, anti-rabbit or anti-mouse IgG (Pierce, Rockford, IL, USA), followed by enhanced chemiluminescence (ECL, Pierce, Rockford, IL, USA) assay allowed for protein detection. Quantification was made by densitometric analysis of non-saturated films (Quantity One image software).

Quantification of A$\beta_{1–40}$ and A$\beta_{1–42}$

A$\beta_{1–40}$ and A$\beta_{1–42}$ peptide levels were determined using an enzyme-like immunoadsorbent assay (ELISA; Covance, Dedham, MA, USA) using soluble extracts from whole brain, cerebral cortex and hippocampus of four wild-type and four TgBACE2-APP mice per genotype and age, and following the manufacturer's instructions.

Preweaning behaviour

Fifty-one animals of the four genotypes (wild type $n = 12$, TgBACE2 $n = 13$, TgAPP $n = 10$ and TgBACE2-APP $n = 16$), from different litters were studied. All the

pregnant dams were allowed to deliver spontaneously. The day of delivery was designated as PD1 of age of the neonates (erroneous estimates on time of birth = ±6 h). On delivery, the litter size of each dam was recorded and each pup was checked for gross abnormalities. The pups were individually marked with India ink at PD1 and were nursed by their natural dams until weaning. During the testing protocol whole litters were separated from the dams and maintained for 30 min in a warmed environment. Neurobehavioral development on PD1-21 was assessed by daily testing of the pups as previously described (Dierssen et al. 2002; Altafaj et al. 2001).

Behavioural analysis in adult and old animals

To check behavioural genotype-associated changes at two different ages, we studied adult (4 months) wild-type ($n = 14$), TgBACE2 ($n = 10$), TgAPP ($n = 10$) and TgBACE2-APP ($n = 10$) and old (18 months) wild-type ($n = 10$), TgBACE2 ($n = 9$), TgAPP ($n = 8$) and TgBACE2-APP ($n = 8$) male mice. The behavioural characterization consisted of a neurological test battery, analysis of the locomotor activity, anxiety-like behaviour and cognitive profile (see below). The experiments were performed with an increasing gradient of stress to avoid interference in the results.

Neurological assessment

SHIRPA primary screen is a comprehensive semi-quantitative routine testing protocol to identify and characterize phenotype impairments during which 40 separate measurements are recorded for each animal, including somatometry (Rogers et al. 1997).

Locomotor activity

Locomotor activity was measured by using actimetry boxes (45 × 45 cm; Panlab SL, Spain) contained in a soundproof rack mount cabinet. Horizontal movements were monitored by means of an infrared beams grid and used as an index of locomotor activity (counts). Counts were integrated every hour and added to obtain total locomotor activity for a 24-h period maintaining the 12:12 h light–dark schedule. The measured parameters in the present study were total distance travelled by the animals (cm) and mean velocity (cm/s).

Open field test

The open field was a white melamine box (70 × 70 × 50 cm high) divided in two zones, centre and periphery, being the centre more anxiogenic, and under high-intensity light levels (300 lx). At the beginning of the test session, mice were left in the periphery of the apparatus and during 5 min we measured and analysed the latency to cross from the periphery to the centre, total distance travelled, average speed, and time spent in various parts of the field (e.g. the border areas vs. the open, central area).

Light and dark box

We used a box consisting of a small (15 × 20 × 25 cm) compartment with black walls and black floor dimly illuminated (25 lx), connected by a 4-cm-long tunnel to a large compartment (30 × 20 × 25 cm) with white walls and a white floor, intensely lit (500 lx). Mice were individually placed in the dark compartment facing the tunnel at the beginning of the 5 min observation session. Number entries to light and dark zones, and in the tunnel connecting both zones, and time spent in each were recorded, as well as the latency to the first visit to the light zone.

Elevated plus maze

The elevated plus maze consisted of a black Plexiglas apparatus with four arms (29 cm long × 5 cm wide) set in cross from a central square (5 × 5 cm). Two opposite arms were delimited by vertical walls (closed arms), and the other two had unprotected edges (open arms). The maze was elevated 40 cm above the ground under dim light (100 lx). At the beginning of the 5-min observation session, each mouse was placed in the central zone, facing one of the open arms. The total numbers of visits to the closed and open arms, and the time spent in open and closed arms were recorded. An arm visit was recorded when the mouse moved all four paws into the arm.

Morris water maze

The swimming pool, 120 cm diameter and 0.5 m height, was filled with water (24 ± 1°C) made opaque with non-toxic white paint and several fixed room cues were constantly visible from the pool. In the first day (training session), the escape platform (15 cm diameter, 24 cm height) was visible and placed in the centre of the pool to train the animal to escape from water. Four training trials were performed, entering the mice for four starting positions (north, south, east or west). During the following 5 days (days 2–6) animals were tested for place learning acquisition with the escape platform located in the middle of the northeast quadrant, 1 cm below water surface. Four trials per day were performed (30 min inter-trial interval), mice entering randomly from each one of the starting positions and allowed to swim until they located the platform. Mice failing to find the platform within 60 s were

placed on it and left there for 20 s, as the successful animals. On 7th day, we removed the platform from the pool, and four probe trials (60 s) were performed, in which the time spent and distance travelled in the trained and non-trained quadrants were recorded. Finally, a session with a cued visible platform, situated in the centre of the pool and 1 cm above the water surface, was carried out. All the trials were recorded and traced with an image tracking system (SMART, Panlab SL, Barcelona, Spain; J-Tracks, Arqué et al. 2008, 2009) connected to a video camera placed above the pool.

Passive avoidance

We used a step-down passive avoidance test as previously described (Dierssen et al. 1992), which consisted of a transparent Plexiglas circular cage (40 cm in height, 30 cm in diameter) with a grid floor and a circular platform (4 cm diameter) in the center. During the training session, animals were placed on the platform and their latency to step down with all four paws was measured. Immediately after stepping down on the grid, animals received an electric shock (0.6 mA, 2 s). Retention test sessions were carried out 24 h (short term) and 7 days after training (long term). Step-down latency was used as a measure of memory retention. A cut-off time of 300 s was set.

Data analysis

Statistical analysis was performed using the software SPSS 12.0. Data were summarized as mean ± standard error of mean (SEM) when normality might be assumed. For the analysis of the Western blot and ELISA results, Student's *t* analysis was used. Between-group comparisons were analysed using one-way analysis of variance (ANOVA) or a two-way ANOVA with genotype and age as factors and with acquisitions trials as a repeated measure and significant effects were analysed post hoc using Bonferroni test. The passive avoidance test was analysed using Mann–Whitney *U* non-parametric test. In all tests, a difference was considered to be significant if the obtained probability value was $P < 0.05$.

Results

Generation and preweaning behaviour of TgBACE2-APP mice

TgBACE2-APP mice were generated by crossing TgBACE2 females with TgAPP males, and transgenic pups were born at the expected frequency (wild type = 23.2%, TgBACE2 = 28.6%, TgAPP = 19.6% and TgBACE2-APP = 28.6%). Transgene expression at the protein level was confirmed by Western blot. As expected, an increase in App protein levels was observed in the brains of both TgAPP (43.1 ± 14.6%), and TgBACE2-APP mice (78.1 ± 32.2%) compared to wild-type littermates (Fig. 1a). Likewise, TgBACE2 mice showed an increase in Bace2 protein levels in comparison with wild-type littermates (30.7 ± 8.1%, $P = 0.07$). However, the increase of Bace2 protein was significantly higher in TgBACE2-APP double transgenic mice than in single transgenic BACE2

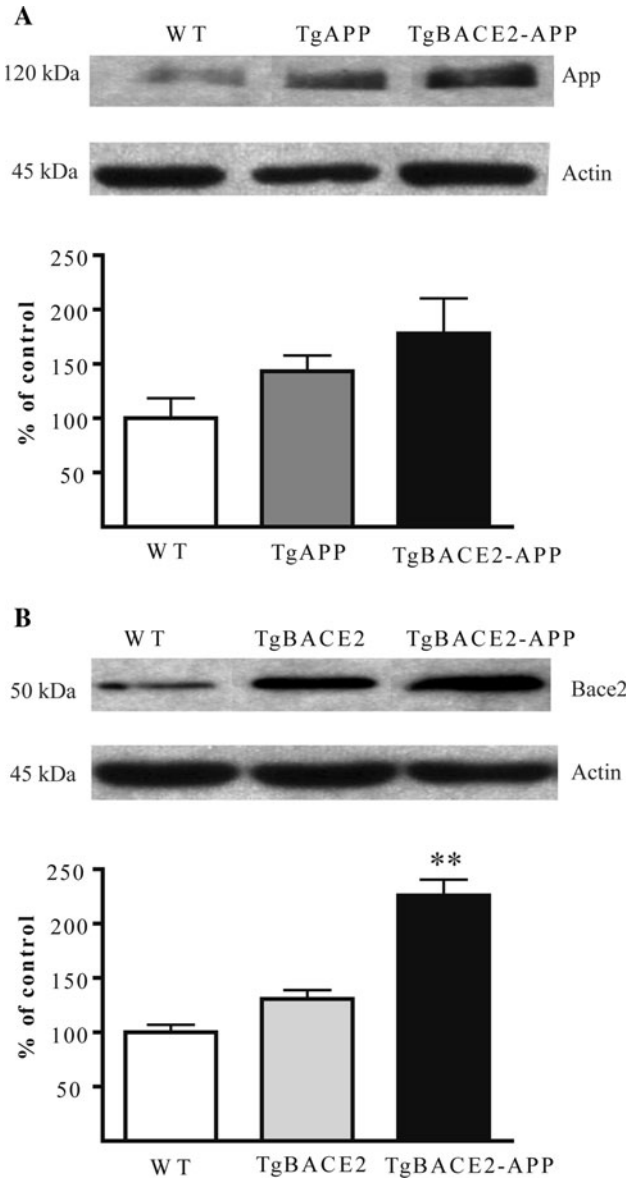

Fig. 1 BACE2 and APP expression. **a** App expression in *APP* overexpressing animals and **b** Bace2 expression in *BACE2* overexpressing animals was higher than wild-type (WT) animals. *Upper panels* representative blots for each genotype. *Lower panels* densitometric quantification expressed as means of the percentage values from control ± SEM. $^{**}P < 0.01$

(95.9 ± 14.4%, $P = 0.012$; Fig. 1b) or wild type (126 ± 14.4%, $P = 0.001$). TgAPP mice did not show Bace2 overexpression and TgBACE2 did not show App overexpression (data not shown).

When analysing the preweaning behaviour, we could not find significant differences among genotypes in the somatometric development, developmental landmarks or in the neurobehavioral development (Supplementary Fig. 1). Nevertheless, in the homing test TgAPP pups spent longer in reaching the home litter sawdust [$F_{(3,50)} = 4.75, P = 0.013$; Fig. 2a] than the rest of the genotypes, thus suggesting a general psychomotor developmental delay, with no differences in neuromotor development (Fig. 2b, c).

General characterization of adult and old TgBACE2-APP mice

Physical characteristics such as body weight and the presence of bald patches and appearance of behavioural anomalies in the home cages were registered systematically with no differences between genotypes. Neurological assessment using modified SHIRPA protocol (see Martínez de Lagrán et al. 2004) revealed that spontaneous activity or sensory, motor and autonomic functions were not affected by *BACE2* and *APP* co-overexpression in adult (Supplementary Table 1) or old (Supplementary Table 2) transgenic mice.

The study of the locomotor activity revealed a significant effect of *APP* overexpression. An increase in total distance travelled and mean speed was observed in adult TgAPP [$F_{(1,23)} = 4.29$, $P = 0.031$; and $F_{(1,23)} = 5.82$, $P = 0.025$, respectively] as compared with wild types, and TgBACE2-APP showed a significant increase in distance travelled as compared with wild type [$F_{(1,23)} = 7.59$, $P = 0.012$], or TgBACE2 adult mice [$F_{(1,19)} = 4.4$, $P = 0.042$], the differences in mean velocity being only significant when comparing wild-type and TgBACE2-APP animals [$F_{(1,23)} = 5.37$, $P = 0.031$]. On the other hand, decreases in distance travelled and mean velocity was observed due to age in all studied genotypes, but no genotype-dependent differences were found among genotype groups (Fig. 3a, b).

In the open field test, increased vertical activity was observed in TgAPP [adult, $F_{(1,23} = 6.13$, $P = 0.022$ and old, $F_{(1,17)} = 4.57$, $P = 0.044$] and TgBACE2-APP [adult, $F_{(1,23)} = 5.11$, $P = 0.034$ and old, $F_{(1,17)} = 4.67$, $P = 0.046$] mice as compared to their respective wild-type controls (Fig. 4a) but both horizontal distance travelled and mean speed were unaffected (data not shown). As was previously described (Azkona et al. 2010), old TgBACE2 animals showed an increased latency to cross from periphery to centre compared to wild type in the open field [$F_{(1,23)} = 4.51$, $P = 0.024$; Fig. 4b]. This anxiety-like phenotype was also observed in the light and dark box, where the latency to cross from dark to light compartment was significantly higher in TgBACE2 adult mice compared to their controls [$F_{(1,23)} = 6.14$, $P = 0.03$; Fig. 4c]. However, all genotype groups spent similar time in the dark box

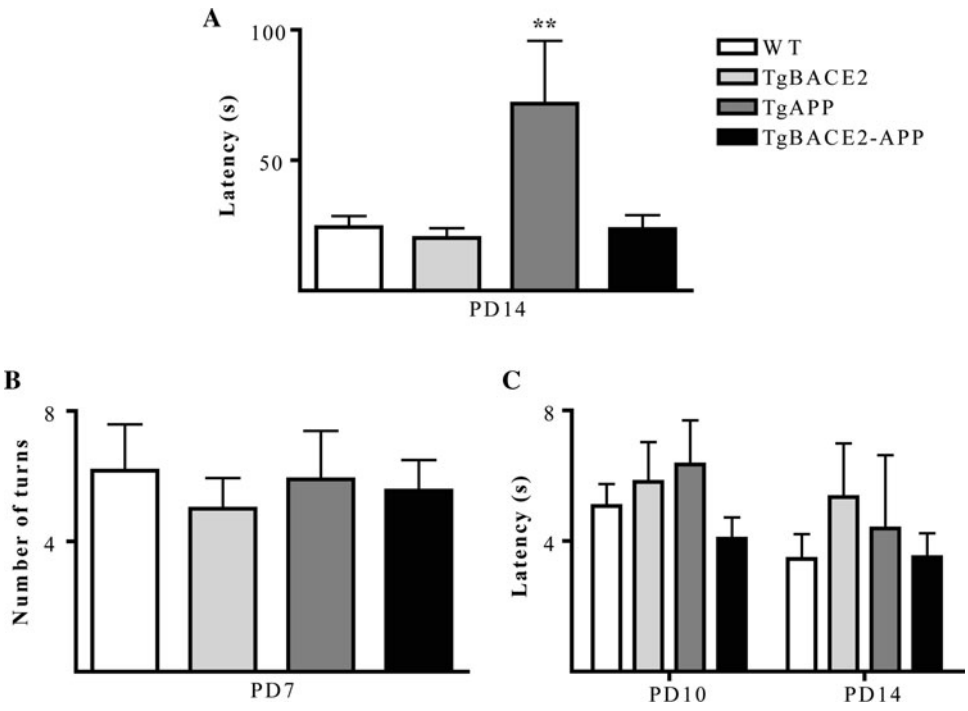

Fig. 2 Preweaning behavioral analysis. TgAPP pups spent longer in reaching the home litter sawdust in the homing test (**a**), while no differences were observed in the pivoting (**b**) or walking (**c**) activity. Data are expressed as mean ± SEM, $^{**}P < 0.01$

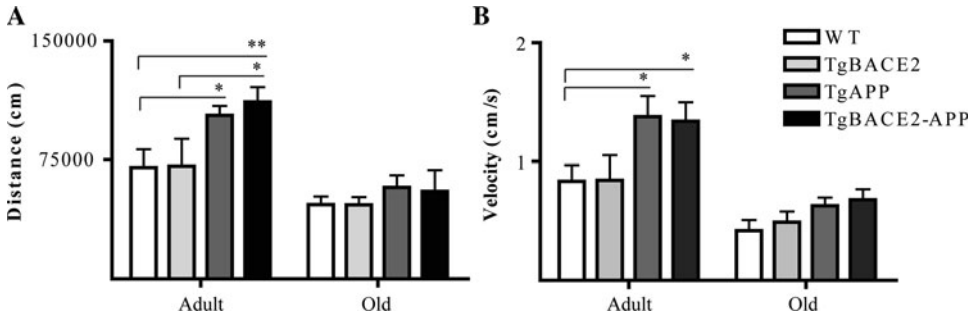

Fig. 3 Locomotor activity. APP overexpressing adult animals showed a significant increase in total distance travelled (**a**) and speed (**b**) in the actimetry box (24 h) as compared to wild type (*WT*). No genotype-dependent differences were found among old groups. Data are expressed as mean ± SEM, $^{**}P < 0.001$

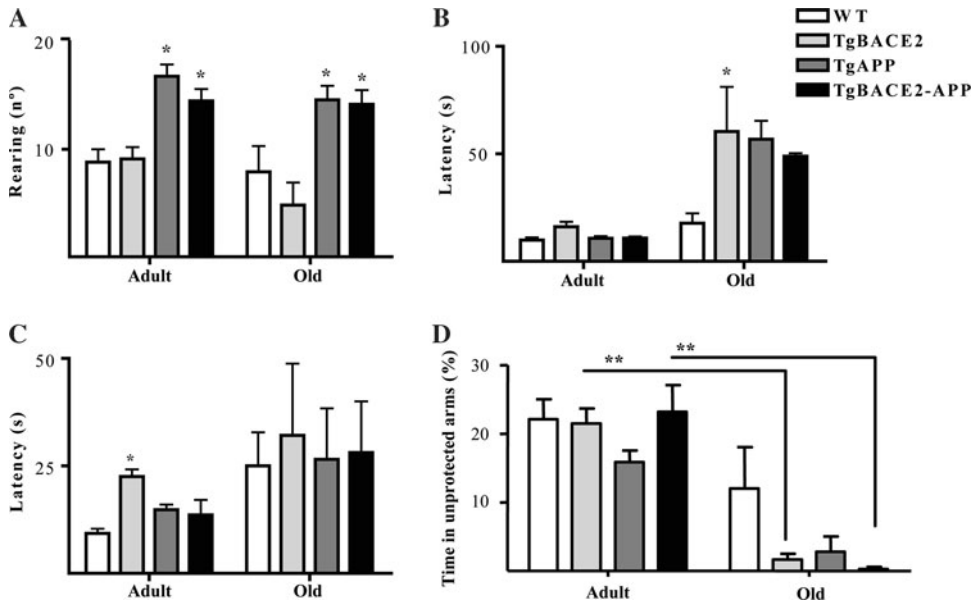

Fig. 4 Open field test (**a**, **b**). **a** APP overexpressing animals showed increased number of rearings compared to their respective wild-type (*WT*) controls. **b** TgBACE2 old animals showed an increase in the latency to cross from periphery to centre. **c** Light and dark box. TgBACE2 adult animals showed an increase in the latency to cross from dark to light compartment. **d** Elevated plus maze. No differences were observed among genotypes in the time spent in the unprotected arms, but the age-related decrease in this parameter was significantly more important in *BACE2* overexpressing mice. Data are expressed as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$

(data not shown). In the elevated plus maze, differences among genotypes in the time spent in the protected arm did not reach statistical significance, but the age-related increase in anxiety-like behaviour was significantly more important in TgBACE2 [$F_{(1,18)} = 43.64$, $P = 0.0001$] and TgBACE2-APP [$F_{(1,17)} = 19.68$, $P = 0.001$; Fig. 4d], suggesting a more important role of BACE2 in anxiety, as previously reported (Azkona et al. 2010).

Cognitive characterization in adult and old TgBACE2-APP

Spatial learning was examined using the Morris water maze. In the training session, no genotype-related differences were observed in either adult or old mice. Along the five consecutive acquisition sessions, all animals learned the task (two-way ANOVA repeated measure; acquisition effect: adult, $F_{(4,43)} = 44.3$, $P = 0.0001$; old, $F_{(4,34)} = 42.8$, $P = 0.0001$), without genotype-related differences (Fig. 5a). In the removal session (Fig. 5b) a significant increase in the percentage of time spent in the trained quadrant (northeast) comparing to non-trained quadrants was observed (two-way ANOVA, $F_{(1,78)} = 68.4$, $P = 0.001$) but no genotype- or age-associated significant differences were observed. Finally, in the cued session, no significant motor or motivational problems were detected (Fig. 5a).

In the passive avoidance, similar step-down latencies were observed in all four genotypes at studied ages, indicating that the co-overexpression of *BACE2* and *APP* do not affect neither the short-term nor long-term memories (Fig. 6a, b).

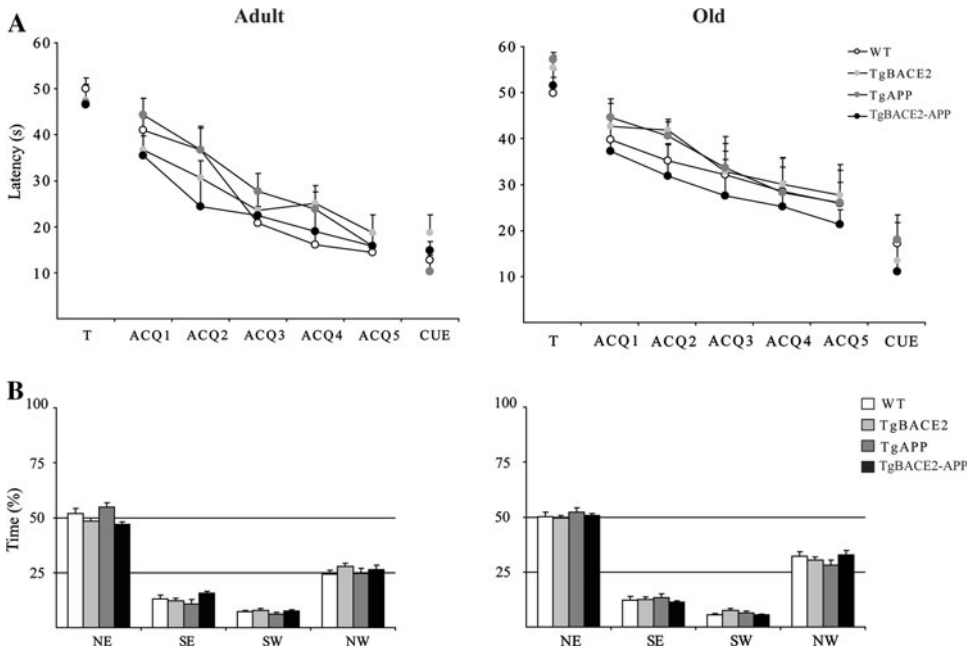

Fig. 5 Morris water maze. Escape latencies along the different sessions of the test. No differences between genotypes were observed at either studied ages and the age-dependent memory loss was similar in both genotypes (*T* training, *A* acquisition) (**a**). In the removal session no differences were observed between genotypes in the preference for the trained quadrant (*NW* northwest, trained quadrant; *NE* northeast; *SW* southwest; *SE* southeast) (**b**). Data are expressed as mean ± SEM

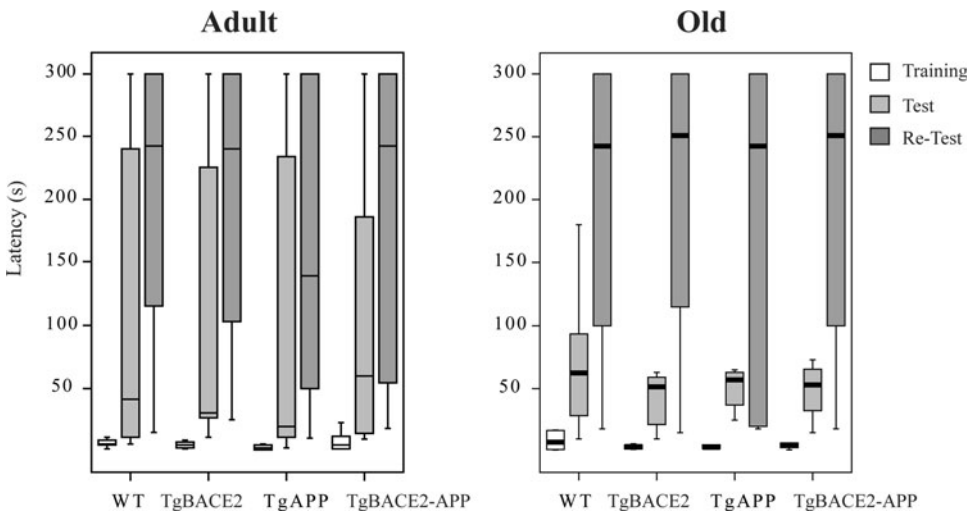

Fig. 6 Step-down passive avoidance. No differences were observed among genotypes in the latency to exit the platform in the ages studied. Data are expressed as the median and interquartile rates

Aβ peptides concentration in adult and old TgBACE2-APP

To determine whether the co-overexpression of *BACE2* and *APP* led to an increase of Aβ peptides, we measured the concentration of $A\beta_{1-40}$ and $A\beta_{1-42}$ by ELISA in wild-type and TgBACE2-APP adult and old animals. The results showed no differences in the amount of any of the peptides in the double transgenic mice of either age when compared to control littermates in the whole brain (data not shown), and the cerebral cortex and the hippocampus, the more affected brain areas in DS and AD (Table 1).

Discussion

The phenotype of DS is thought to result from the triplication of a gene or genes located on the HSA21. The present study analyses the co-overexpression of two HSA21 genes, *BACE2* and *APP*, that could be involved in the AD-like neuropathology and age-related cognitive decline observed in elderly people with DS, but in a background free from dosage effects of other HSA21 genes. We have focused our work in the behavioural and cognitive profiles of double transgenic *APP/BACE2* mice to gain insight into the involvement of the co-overexpression of both genes in DS phenotypes.

Table 1 ELISA quantification of $A\beta_{1-40}$ and $A\beta_{1-42}$ levels in cerebral cortex and hippocampus of wild-type and TgBACE2-APP animals

	Cerebral cortex				Hippocampus			
	$A\beta_{1-40}$		$A\beta_{1-42}$		$A\beta_{1-40}$		$A\beta_{1-42}$	
	Adult	Old	Adult	Old	Adult	Old	Adult	Old
Wild type	0.9 ± 0.2	1.0 ± 0.7	1.0 ± 0.4	1.4 ± 0.5	1.1 ± 0.4	1.3 ± 0.3	0.7 ± 0.8	1.02 ± 0.9
TgBACE2-APP	1.0 ± 0.3	0.9 ± 0.5	1.2 ± 0.7	1.3 ± 0.3	1.1 ± 0.6	1.3 ± 0.5	0.7 ± 0.6	1.07 ± 0.8

No differences were observed between genotypes and ages. $N = 4$ per genotype. Data (pg/ml) are expressed as means ± SEM

Interestingly, in TgBACE2-APP mice the increase of Bace2 protein expression was significantly higher than in single transgenic BACE2 transgenic ($P = 0.012$; Fig. 1b). However, the possibility of a co-regulation seem to be discarded since TgAPP mice did not show expression changes Bace2 protein levels, nor TgBACE2 did show App expression variations.

Since DS individuals would have co-overexpression of APP and BACE2 along the whole life span, it was important to determine if other DS-related phenotypes could be affected in the double transgenic mice. In the preweaning characterization TgAPP mice presented a general psychomotor delay, as tested in the homing test, similar to what has been described in the trisomic mouse model Ts65Dn mice, which possibly resemble the sensorimotor delay observed in DS patients (Holtzman et al. 1996; Escorihuela et al. 1995, 1998). However, this delay was not observed in either BACE2 or double TgBace2/App transgenic mice, thus suggesting that this developmental effect does not depend on *BACE2* overexpression. Moreover, double transgenics did not show the delay, thus suggesting that overexpression of BACE2 may mitigate the developmental effects of *APP* overexpression. None of the adult or old transgenic mice exhibited any sensory or motor deficits, which could interfere with performance in the cognitive tasks, although both TgAPP and TgBACE2-APP showed an increased motor activity. Interestingly, altered activity has been previously described for some TgAPP models (Harris-Cerruti et al. 2004) and DS mouse models (Sago et al. 1998). Since this phenotype was not observed in BACE2 mice the results suggest that App overexpression is sufficient to cause hyperactivity.

Previous results of our laboratory showed that overexpression of BACE2 leads to an increased anxiety-like behaviour (Azkona et al. 2010). In this work we could reproduce these findings, so that increased anxiety was also observed in TgBACE2, but not in the double transgenic mice. In the present work we did not find anxiety-related behaviors in TgAPP mice, although in previous work using different tests (Harris-Cerruti et al. 2004), it was clearly detected.

The most interesting data refer to the age-related cognitive decline. In our experiments, adult TgBACE2-APP mice did not show any impairment in visual–spatial learning and memory, or in recent memory in the passive avoidance test, indicating that co-overexpression of *BACE2* and *APP* does not contribute to the cognitive phenotype observed in DS, AD patients and DS mouse models. In fact, the results of ELISA demonstrate that neuronal co-overexpression of *BACE2* and *APP* does not affect $A\beta_{1-40}$ or $A\beta_{1-42}$ production in the cerebral cortex or the hippocampus. So, our results not only confirmed in vitro and in vivo studies reporting that BACE2 is not involved in the APP amyloidogenic pathway (Sun et al. 2006; Azkona et al. 2010) but also suggested that BACE2 overexpression could have a protective effect on some APP phenotypes. In fact other genes, such as DYRK1A (dual-specificity tyrosine-regulated kinase 1A), or other protein deregulation (Cheon et al. 2001) may also contribute to AD-like phenotypes. In the case of Dyrk1A, it phosphorylates the human microtubule-associated protein tau at Thr212 in vitro, a residue that is phosphorylated in foetal tau and hyper-phosphorylated in AD and is found in sarkosyl-insoluble fractions which are enriched in phosphorylated tau in AD brains, thus suggesting a possible association of Dyrk1A with neurofibrillary tangle pathology (Ferrer et al. 2005).

In conclusion, the present study demonstrates that in vivo co-overexpression of *App* and *Bace2* is not involved in the DS age-dependent cognitive impairment or increased $A\beta$ production as demonstrated in aged TgBACE2-APP mice. Moreover our results implicate App overexpression in DS developmental psychomotor delay, and suggest that co-overexpression of BACE2 may have some protective effects in specific behavioural and cognitive domains.

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