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Characterization of a mouse model overexpressing the Beta-site APP-cleaving enzyme 2, BACE2, reveals a new role for the aspartic protease in the trophism of noradrenergic neurons

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Abstract

BACE2 is homologous to *BACE1,* a β-secretase that is involved in the amyloidogenic pathway of APP, and maps to the Down syndrome critical region. Alzheimer disease neuropathology is common in Down syndrome patients at relatively early ages and it has thus been speculated that BACE2 co-overexpression with APP would promote the early neurodegenerative phenotype. However the in vivo function of BACE2 has not yet been elucidated. The aim of the present work has been to analyze the impact of *in vivo* BACE2 overexpression using a transgenic mouse model. Our results reinforce the concept that BACE2 is not involved in the cognitive dysfunction or cholinergic degeneration. However, TgBACE2 animals showed increased anxiety-like behavior along with increased numbers of noradrenergic neurons in locus coeruleus, thus suggesting an unexpected role of BACE2 overexpression.

Introduction

Progressive formation and extracellular aggregation of amyloid-β (Aβ) peptide has been considered one of the causal factors for the pathogenesis of Alzheimer's disease (AD) (Yan *et al.* 2001). This neuropathological hallmark is also common in Down syndrome (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). In the amyloidogenic pathway, a β-secretase cleaves APP to generate APPsβ, a soluble N-terminal fragment, and a C-terminal fragment (C99). A γ-secretase cleaves C99 to form the mature Aβ peptide comprising 39-42 amino acids (Mattson 2004). An increased accumulation of the C99 fragment has been observed in the brain of DS individuals (Busciglio *et al.* 2002; Sun *et al.* 2006), suggesting that abnormal processing at the APP β-site might be involved in the common degenerative phenotype of DS and AD patients.

One of the principal players in the amyloidogenic pathway is the β site APP cleaving enzyme 1 (BACE1), encoded by a gene located on HSA11 (Sambamurti *et al.* 2004). BACE1 is expressed in the hippocampus and the cerebral cortex, colocalizes with APP in the Golgi compartment, and its overexpression results in an increase of Aβ peptide (Hussain *et al.* 1999; Vassar *et al.* 1999). However, there is still no consistent evidence of its role in the early appearance of DS neurodegenerative phenotypes (Cheon *et al.* 2008). *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). It has been thus speculated that BACE2 and APP co-overexpression would promote the early appearance of amyloid plaques in DS patients. BACE2 is a

transmembrane glycoprotein with aspartic protease activity (Bennett *et al.* 2000). Overexpression of BACE2 protein in DS fetal brain samples has been reported in some studies (Barbiero *et al.* 2003), but is controversial (Cheon *et al.* 2007). Of interest to the AD phenotype, immunoreactivity for BACE2 has been observed in neurofibrillary tangle-bearing neurons from elderly DS brains with AD-type neuropathology (Motonaga *et al.* 2002).

There has been an intense debate about BACE2 function. *In vitro* studies showed that BACE2 cleaves APP at the β-secretase site (Farzan *et al.* 2000; Hussain *et al.* 2000), although cleavage at the α-secretase site has been also reported (Basi *et al.* 2003; Farzan *et al.* 2000; Yan *et al.* 2001). However, a recent study demonstrated that BACE2 cleaves APP at a novel site named θ, generating APP C80 fragments, an effect that would reduce Aβ production (Sun *et al.* 2006).

It is thus necessary to elucidate the function of BACE2 in vivo and its possible involvement in DS phenotypes. We here demonstrate for the first time that overexpression of BACE2 in a transgenic mouse model gives rise to significant alterations in the noradrenergic system accompanied by increased anxiety-like behavior, thus suggesting a novel role for this enzyme.

Materials and Methods

General procedures

Animals were housed in standard macrolon cages (4-5 animals per cage, 40 x 25 x 20 cm) with freely available food and water in standard environmental conditions (constant humidity and temperature of 22 ± 1ºC) 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-IMIM and 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).

Construction of Thy-1/*BACE2*HA transgene

The open reading frame corresponding to the longest isoform of human *BACE2* (Solans *et al.* 2000) was C-terminally fused in-frame to the hemaglutinin (HA) tag. A blunted DNA fragment containing these sequences was inserted into the blunted XhoI site of the murine Thy1 cassette (Moechars *et al.* 1996), which was kindly provided by D. Moechars (Janssen Pharmaceutica NV, Belgium). The orientation of the cDNA within the cassette was verified by sequencing with specific primers, and the complete expression cassette was designated Thy1-BACE2.

Generation of transgenic mice and genotyping

Transgenic mice were generated by standard pronucleus microinjection of the 8.7 kb fragment from the Thy1-BACE2 construct, without plasmid vector sequences, both on a

hybrid B6/CBA (retinal degeneration mutation free) and on a B6/SJL genetic background. The presence of the transgene was assessed in DNA from tail biopsies by digestion with BamHI and Southern blot analysis by using a fragment of *BACE2* cDNA as a probe. Two transgenic lines were obtained and maintained by backcrossing to a B6/CBA or a B6/SJL background in heterozygosity. Genotyping was performed routinely by PCR analysis using the primer pairs: BACE2f, 5'-ATCCACAAATGCGCTGGT-3'; and BACE2r, 5'-GCGGCCGTTACTAGTGGA-3'. Hybrid founders were backcrossed extensively in order to attenuate littermate's genetic differences. All experiments were performed in mice from the F15 generations. In all cases, transgenic mice were directly compared with non-transgenic littermates.

RNA expression analysis

For expression analysis of *BACE2* mRNA, total RNA from brain samples was isolated with the TriPure kit (Boehringer Mannheim, Germany). RT-PCR was carried out by reverse-transcribing total RNAs (1 µg) using Superscript reverse transcriptase (Gibco BRL, San Francisco, CA, USA), and followed by PCR amplification with primers BACE2f and BACE2r. Absence of genomic DNA contamination was determined by the amplification of a 125 bp PCR fragment from cDNA samples with primers for GdX transcript (GdXf, 5'-GGCAGCTGATCTCCAAAGTCCTGG-3' and GdXr, 5'-AAC GTTCGATGTCATCCAGTGTTA-3').

Western blot

Animals were sacrificed, brains rapidly removed, and the cerebral cortex, the hippocampus and the brainstem dissected on ice. 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 (1400xg, 20 min at 4ºC), 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 4ºC. The following antibodies were used as primary antibodies: goat anti-Bace2 antibody (1:500; D-20; Santa Cruz, Heidelberg, Germany), rabbit anti-HA antibody (1:1000; Eurogenic, Emeryville, CA, USA), mouse anti-tyrosine hydroxylase (TH) antibody (1:15000, Sigma, St Louis, MO, USA) and anti-actin antibody (1:2000; 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, Rockord, IL, USA) assay allowed detection. Quantification was made by densitometric analysis of non-saturated films (Quantity One image software).

Immunohistochemistry

Four animals for each age and genotype were perfused transcardially with 0.1 M PBS, pH 7.4 and then with 4% paraformaldehyde (Sigma, St Louis, MO). The brains were removed from the skull and left in the same fixative for 24 h, then cryoprotected in 30% sucrose and kept frozen at -80ºC. Coronal sections were obtained using a cryostat (50 µm). Serial tissue sections were processed free-floating using the streptavidin-biotin-peroxidase complex immunohistochemical method (DAKO, LSAB system, Ely, UK).

Briefly, after peroxidase blocking, sections were incubated with 10% fetal bovine serum (FBS) and 0.25% gelatine. Then incubation with the primary antibody was performed overnight at 4ºC at a dilution of 1:1000 for anti-ChAT antibody (choline acetyltransferase, Chemicon, Temeluca, CA, USA), 1:2000 for anti-$p75^{NGFR}$ antibody (Chemicon, Temeluca, CA, USA), 1:8000 for anti-TH antibody (Sigma, St Louis, MO) or 1:1000 for anti-HA antibody (Eurogenic, Emeryville, CA, USA) in PBS containing 0.2% Triton X-100 and 1% FBS. The sections were then incubated with the biotinylated link and the streptavidin-HRP as indicated in the manufacturer's instructions. Peroxidase activity was visualized with 0.05% diaminobenzidine and 0.01% hydrogen peroxide.

Stereological analysis: quantification of ChAT, $p75^{NGFR}$ and TH positive cells

Two basal forebrain (BF) cholinergic nuclei, the medial septal nucleus (MSN) and the vertical diagonal band (VDB), and the locus coeruleus (LC) were analyzed. Stereological estimations of the total number of ChAT and $p75^{NGFR}$ positive neurons in BF and of TH positive neurons in LC were obtained. For the stereological analysis the CAST-GRID software package (Olympus, Denmark) adapted to an OLYMPUS BX51 microscope was used. The studied areas were MSN and VDB (Bregma 1.54 mm to 0.26 mm) and LC (Bregma -5.34 mm to -5.8 mm) according to the stereological coordinates adopted from the Mouse Brain Atlas (Keith B.J 1997). Estimation of the volume of the selected regions was performed using the Cavalieri method and the optical dissector method was used as previously described by (Dierssen *et al.* 2006).

Behavioral analysis

To check behavioral age-associated changes, we studied male mice of two different age groups, adult (6 months; WT, n = 24, TgBACE2, n = 17) and old (22 months; WT n = 14, TgBACE2, n = 12). The behavioral characterization consisted in a neurological test battery, analysis of the locomotor activity, anxiety-like behavior and cognitive profile (see below). The experiments were performed with an increasing gradient of stress to avoid interference in the results.

Neurological assessment (SHIRPA protocol)

SHIRPA primary screen is a comprehensive semiquantitative 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). Assessment of each animal began with observation of undisturbed behavior in a cylindrical clear Perspex viewing jar (15 cm height, 11 cm diameter) for wild running or stereotypes. Mice were then transferred to an arena (56 x 34 cm) for observation of motor behavior and sensorial function. Animals underwent screening exams for visual acuity, vibrissae, corneal and pinna responses to an approaching cotton swab, auditory (Preyer reflex) and vestibular function (contact righting reflex and negative geotaxis), and grip strength and body tone. In the last part of the test battery, changes in excitability, aggression, general fear, vocalization and salivation, and piloerection (for analysis of autonomic function) were recorded.

Locomotor activity

Locomotor activity was measured by using actimetry boxes (45 x 45 cm; Panlab SL, Spain) contained in a soundproof rack mount cabinet. Back and forward 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, during 4 consecutive days. The measured parameters in the present study were total distance travelled by the animals (cm) and mean velocity (cm/sec).

Open field test

The open field was a white melamine box (70 x 70 x 25 cm high) divided into 25 equal squares and under high intensity light levels (300 Lux). Mice tend to avoid brightly illuminated, novel, open spaces, so the open field environment acts as an anxiogenic stimulus and allows for measurement of anxiety-induced locomotor activity and exploratory behaviors. Thus, two zones, centre and periphery, were delineated being the centre more anxiogenic. At the beginning of the test session, mice were left in the periphery of the apparatus and during 5 min we measure and analyze 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, middle area).

Light and dark box

The light and dark box test is based on the innate tendency of mice to seek refuge in a dark box. We used a box consisting of a small (15 × 20 × 25 cm) compartment with black walls and black floor dimly illuminated (25 Lux), 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 Lux). 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 x 5 cm wide) set in cross from a neutral central square (5cm x 5cm). Two opposite arms were delimited by vertical walls (closed arms) and the two other arms had unprotected edges (open arms). The maze was elevated 40 cm above the ground and placed in indirect light (100 Lux). At the beginning of the 5 min observation session, each mouse was placed in the central neutral zone, facing one of the open arms. The total numbers of visits to the closed arms and the open arms, and the cumulative time spent in open and close arms were recorded. An arm visit was recorded when the mouse moved all four paws into the arm.

Zero maze

The zero maze consisted of a circular path (runway width 5.5 cm , 46 cm diameter) with two open and two closed segments (walls 8 cm high) and was elevated 50 cm above ground. Animals were placed into the close segment and their movements were recorded for 5 min. The latency to enter to the open segment, the number of entries and the total time spent into both segments were measured.

Morris Water Maze

The swimming pool, 120 cm diameter and 25 cm depth, was filled with water (24ºC ± 1) made opaque with non-toxic white paint. In the first day (training session) animals were trained using a visible platform (15 cm diameter, 24 cm height) to escape from

water. During the following 5 days animals were tested for place learning acquisition with the escape platform located in the center of northwest quadrant, 1 cm below water surface. In each of 4 daily trials, mice entered the pool randomly using one of the starting positions (north, south, east or west) and were allowed to swim until they located the platform. Mice failing to find the platform within 60 sec were placed on it and left there for 20 sec, as the successful animals. Several fixed room cues were constantly visible from the pool. On the 7th day, the removal test, with the platform removed from the pool was performed and the time spent and distance traveled in the trained and non-trained quadrants were recorded during 60 sec. On the next day, cued learning was tested, and the platform was elevated 1 cm above the water surface and its position was clearly indicated by a visible cue. The last day, mice performed the reversal learning session. The platform's position was changed to the opposite quadrant (southeast) and four trials were performed. All the trials were recorded and traced with an Image tracking system (SMART, Panlab SL, Spain) connected to a video camera placed above the pool.

Passive Avoidance

Short- and long-term memories were analyzed using a step-down passive avoidance test. The apparatus consisted of a transparent Plexiglas circular cage (40 cm in height, 30 cm in diameter) with a grid floor with 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 on the grid with all four paws was measured. Immediately after stepping down on the grid, animals received an electric shock (0.6 mA, 2 sec). Retention test session was carried out 24 h (short-term) and 7 days (long-term) after

training and was procedurally identical to training. Step-down latency was used as a measure of memory retention. A cut-off time of 300 sec was set.

Data analysis

Data are reported as means ± standard error of the mean (S.E.M.). Results from both transgenic lines were combined because no significant differences between them were detected by two-way ANOVA in all behavioural tests. Statistical analysis was performed using one-way or repeated measures ANOVA and Bonferroni post-hoc test. For the analysis of the Western blot and immunohistochemistry results, Student's t analysis was used. Significant levels were set at $P < 0.05$. The statistical analysis was performed using the SPSS 12.0 software.

Results

Generation and general characterization of TgBACE2 mice

To generate transgenic mice overexpressing *BACE2*, we cloned the human *BACE2* open reading frame (518 amino acids isoform) fused at the C-terminus to the HA-tag under the control of the mouse neuron-specific *Thy-1.2* promoter (Fig. 1a). We used the *Thy-1.2* promoter because it has been shown to drive efficient and specific expression of transgenes in the brain (Moechars *et al.* 1996), and we decided to generate the TgBACE2 mice in two different genetic backgrounds (B6/CBA and B6/SJL) to discard influences of the background (Milner & Crabbe 2008). Two transgenic lines, designated as line 2 (B6/CBA background) and 11 (B6/SJL background), carrying 5 and 10 copies of the transgene, respectively, were established (data not shown). Transgene expression at the protein level was confirmed by Western blot with an anti-HA antibody (Fig. 1b) and increased BACE2 protein levels were detected as shown by Western blot analysis with an anti-BACE2 antibody in adult brain from (line 2: 21% ± 4.6; n = 4; line 11: 23% ± 3.7; n = 4; Fig. 1c). Immunohistochemistry studies showed that overexpressed BACE2-HA was present in the cytoplasm and the membrane of the soma in agreement with endogenous BACE2 subcellular localization (Fig. 1d).

Physical characteristics such as body weight and the presence of bald patches and appearance of behavioral anomalies in the home cages were registered systematically with no differences between genotypes. Neurological assessment using modified Primary SHIRPA protocol revealed that spontaneous activity or sensory, motor and autonomic functions were not affected by *BACE2* overexpression (Supplementary Table 1). No genotype- or age-dependent differences were found in

total locomotor activity in any of the four days recorded, and the results showed that there was a habituation process in all mice during the consecutive days (Fig. 2).

BACE2 overexpression does not lead to alterations in learning and memory or to cholinergic degeneration

No genotype-related differences were observed in procedural learning in the Morris water maze, as demonstrated by the similar escape latency and travelled distance registered during training session and no significant motor or motivational problems were detected in the cued session. Both genotypes were equally efficient in learning the location of the platform as indicated by the progressive decrease in latency and distance during acquisition sessions. In addition, cognitive flexibility was not altered in TgBACE2 mice as shown in the reversal session (Fig. 3a). Finally, no differences were observed during the removal session (Fig. 3b), thus suggesting that reference memory was intact in transgenic mice.

The data obtained in the passive avoidance demonstrate that overexpression of BACE2 affects neither short-term nor long-term memories in an aversive context (Fig. 3c).

In a second series of experiments, it was relevant to determine whether BACE2 overexpression had an impact in cholinergic neurodegeneration similar to the observed in Ts65Dn, a DS mouse model (Granholm *et al.* 2000). To this end, unbiased stereological methods were used to compare the number of cholinergic neurons of basal forebrain in MSN and VDB. Cholinergic neurons were stained with specific markers, namely ChAT, the rate-limiting enzyme in the acetylcholine synthesis, and $p75^{NGFR}$, the low affinity nerve growth factor receptor. No genotype-dependent differences were detected in the total volume of the nuclei studied (data not shown) or in ChAT or

p75^{NGFR} cell density at the ages analyzed (Table 1). These results suggest that BACE2 overexpression does not produce early degeneration of the cholinergic system.

Behavioral and molecular characterization revealed increased anxiety-related phenotype in TgBACE2

In the open field there was a significant increase in the latency to cross from centre to periphery in both age groups of transgenic animals (Fig. 4a; $P < 0.05$), along with an increase in the time spent in the periphery (Fig. 4b; $P < 0.05$). Importantly, distance travelled and speed were unaffected (Fig. 4c,d), indicating that the anxiety-like behavior was not because of alterations of locomotor activity. In the light and dark box, young TgBACE2 mice exhibit a significant increase to cross from light to dark compartment (Fig. 5a; $P < 0.05$) and an increase in the time spent in the dark box, an accepted measure of anxiety-like behaviour (Fig. 5b; $P < 0.05$). Both young and old transgenic animals presented reduced percentage of entries to the light compartment during the test (Fig. 5c; $P < 0.05$).

For a further characterization of the anxiety-like behavior, we tested the animals in the elevated plus maze. There were no differences in the time spent in the close arms (Fig. 6a), but young transgenic mice presented reduced entries to the open arms comparing to their control group (Fig. 6b; $P < 0.05$). The latency to cross from close to open arm in the zero maze was higher in TgBACE2 old mice comparing to their control group (Fig. 7a; $P < 0.05$). Transgenic young mice showed a decrease in the time spent in the open arm (Fig. 7b; $P = 0.07$), being significant in old transgenic animals (Fig. 7b; $P < 0.05$). Finally, TgBACE2 young mice showed reduced entries to the open arms (Fig.

7c; $P < 0.05$). Taken together results indicate an increased anxiety-like behavior in TgBACE2 mice.

To explain the increased anxiety phenotype observed in TgBACE2 mice we hypothesized that the noradrenergic system could be affected in these mice. To explore this possibility, we determined the expression levels of TH, the rate-limiting enzyme of the synthesis of catecholamines, in the medulla-pons region by Western blotting analysis. Our results revealed a significant increase in TH levels in young transgenic mice vs. wild type (Fig. 8a; $P < 0.01$) and a tendency to an increase in old TgBACE2 animals (Fig. 8a; $P = 0.088$).

Since these results may indicate an increase in either LC cellularity or noradrenergic neuron activity, we quantified TH-positive cells in the LC. The density of TH-positive of cells was higher in transgenic animals of both ages comparing to their respective controls (Fig. 8b; $P < 0.05$) without differences in the total volume of the LC (data not shown).

Discussion

The present study analyzes the effect of overexpression one HSA21 gene, *BACE2*, focusing on the central nervous system. Our results demonstrate that overexpression of BACE2 did not lead to morphological alterations in TgBACE2, similar to what was described in *Bace2* knockout mice (Dominguez *et al.* 2005). Moreover, TgBACE2 mice did not show alterations in locomotion or general activity during four consecutive days of record.

Due to its high homology to BACE1, during many years β-secretase activity was attributed to BACE2 (Acquati *et al.* 2000; Yan *et al.* 1999); if confirmed, it could contribute to the Alzheimer's like neuropathology observed in DS patients by the concomitant overexpression with *APP* (Acquati *et al.* 2000; Murphy *et al.* 1990). However, *in vitro* studies have shown controversial results. Whereas some authors found that BACE2 can cleave wild type or Swedish mutant APP at β-secretase (Farzan *et al.* 2000; Hussain *et al.* 2000) and at α-secretase sites (Yan *et al.* 2001), more recently, it has been demonstrated that BACE2 generates a C80 fragment, as a result of a novel cleavage site designated as APP θ-secretase cleavage site (Sun *et al.* 2006). Furthermore, it has been shown that the overexpression of BACE2 in APP-expressing cells profoundly reduced Aβ formation (Bennett *et al.* 2000; Farzan *et al.* 2000; Hussain *et al.* 2000; Sun *et al.* 2006; Yan *et al.* 2001), and that selective down-regulation of BACE2 by RNA interference increased Aβ secretion (Basi *et al.* 2003). All these data point to a protective role for BACE2 more than a pro-amyloidogenic factor.

On the other hand, a role of β-amyloid and/or inflammation has been suggested in the degeneration of cholinergic synaptic structures, and premature loss of basal forebrain cholinergic neurons, one of the neuropathological hallmarks of DS and AD

(Casanova *et al.* 1985; Mann 1988; Mann *et al.* 1985). This alteration, also observed in Ts65Dn mice (Cooper *et al.* 2001; Granholm *et al.* 2000), is associated with learning and memory deficits in cognitive tasks (Fodale *et al.* 2006; Granholm *et al.* 2000; Hyde *et al.* 2001). In our experiments, TgBACE2 mice did not show any impairment in visuo-spatial learning and memory, or in recent memory in the passive avoidance test, which has been shown to depend on the integrity of the cholinergic system (Dierssen *et al.* 1992). Along with the lack of cognitive impairment, TgBACE2 mice did not present signs of cholinergic degeneration, thus indicating that single overexpression of BACE2 is not able to develop the cholinergic and cognitive phenotype observed in DS, AD patients and DS mouse models.

Interestingly, behavioral characterization revealed an increased anxiety-like behavior in TgBACE2. This anxiety-like behavior is similar to that described for *Bace1* knock-out mice, but opposite to that shown by BACE1 transgenic mice which exhibited a less anxiety-like behavior (Harrison *et al.* 2003), suggesting antagonistic roles for BACE2 and BACE1 in anxiety pathways. Several mechanisms could be involved in the anxiety-like behavior including an elevated noradrenergic activity or inappropriate activation of the LC, which has a physiological role in emotionality (Priolo *et al.* 1991). Consistent with this, we have observed an increase of TH-positive neurons density in LC of both transgenic group and a significant increase of TH levels in the medulla-pons region of TgBACE2 young mice, and a tendency in old transgenic animal, suggesting that although the density of noradrenergic neurons density is still higher than their control group, maybe the activity of TH is reduced due to the age.

Since there are no studies analyzing noradrenergic phenotype in the other available BACE1 mouse models, we cannot establish any comparison between the effects of the two ß-secretases in this particular aspect. However, DS and AD patients

and the Ts65Dn mice, a DS trisomic model, show alterations in noradrenergic synaptic transmission in the cerebral cortex and the hippocampus (Dierssen *et al.* 1997; Dierssen *et al.* 1996; Lumbreras *et al.* 2006), although no anatomical differences were observed in the cholinergic, serotonergic and catecholaminergic systems in these trisomic mice (Megias *et al.* 1997) and in DS fetuses (Lubec *et al.* 2001). Phenotypically, reduced behavioral inhibition and/or reduced attention to relevant stimuli in the open-field and elevated plus-maze (Coussons-Read & Crnic 1996; Escorihuela *et al.* 1995; Escorihuela *et al.* 1998) and anxiety and panic behavior responses to a predator in the Mouse Defense Test Battery (Martinez-Cue *et al.* 2006) have been reported in Ts65Dn mice, thus suggesting that functional or structural alterations of the noradrenergic system may be at least partially contributed by BACE2 overexpression.

In conclusion, our results indicate that BACE2 overexpression *in vivo* is not involved in the cholinergic-dependent cognitive dysfunction observed in DS patients. However, we demonstrate that BACE2 overexpression induces an anxiety-related behavior and gives raise to structural changes in the noradrenergic system.

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Figure Legends

Figure 1. Construction of Thy-1/*BACE2*HA transgene. Genotyping and expression analysis of TgBACE2 mice. **(a)** Schematic representation of the Thy-1/*BACE2*HA chimeric gene. Dark grey boxes represent exons E1-E4 from mouse *Thy1* gene. **(b)** Western blot analysis of adult brain from control (WT) and TgBACE2 (Tg) with anti–HA antibody. **(c)** Western blot analysis of adult brain from control (WT) and transgenic mice using anti-Bace2 antibody; two animals per genotype are shown. Actin detection was used as a control of equal loading. **(d)** Imnunohistochemical analysis in different brain areas of TgBACE2 using anti-HA antibody. Panels **a,** cerebral cortex; **b,** cerebellar cortex (Purkinje cells); and **c;** medulla-pons region. Arrows show stained cells. *Anti-HA staining was also observed in blood vessels. Similar results were obtained in different transgenic lines. Scale bar: 50 μm.

Figure 2. Overexpression of BACE2 does not change locomotion or habituation. Total distance travelled in the actimetry box during 24h in four consecutive days. No differences were observed comparing WT vs. TgBACE2 mice of each age and each day. Habituation is observed along days in both genotypes and ages. The typical age-associated reduction in activity is similar in both genotypes. Data are expressed as mean ± S.E.M.

Figure 3. Age-associated memory loss is similar in TgBACE2 and WT mice. Morris water maze. **(a)** Escape latencies along the different sessions of the test. No differences between genotypes were observed at either studied ages. (T = training; A = acquisition; REV = reversal). **(b)** 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). Data are expressed as mean ± S.E.M. **(c) Step-down passive avoidance.** No differences were observed between genotypes and ages in the latency to exit the platform. Data are shown as the median and interquartile rates.

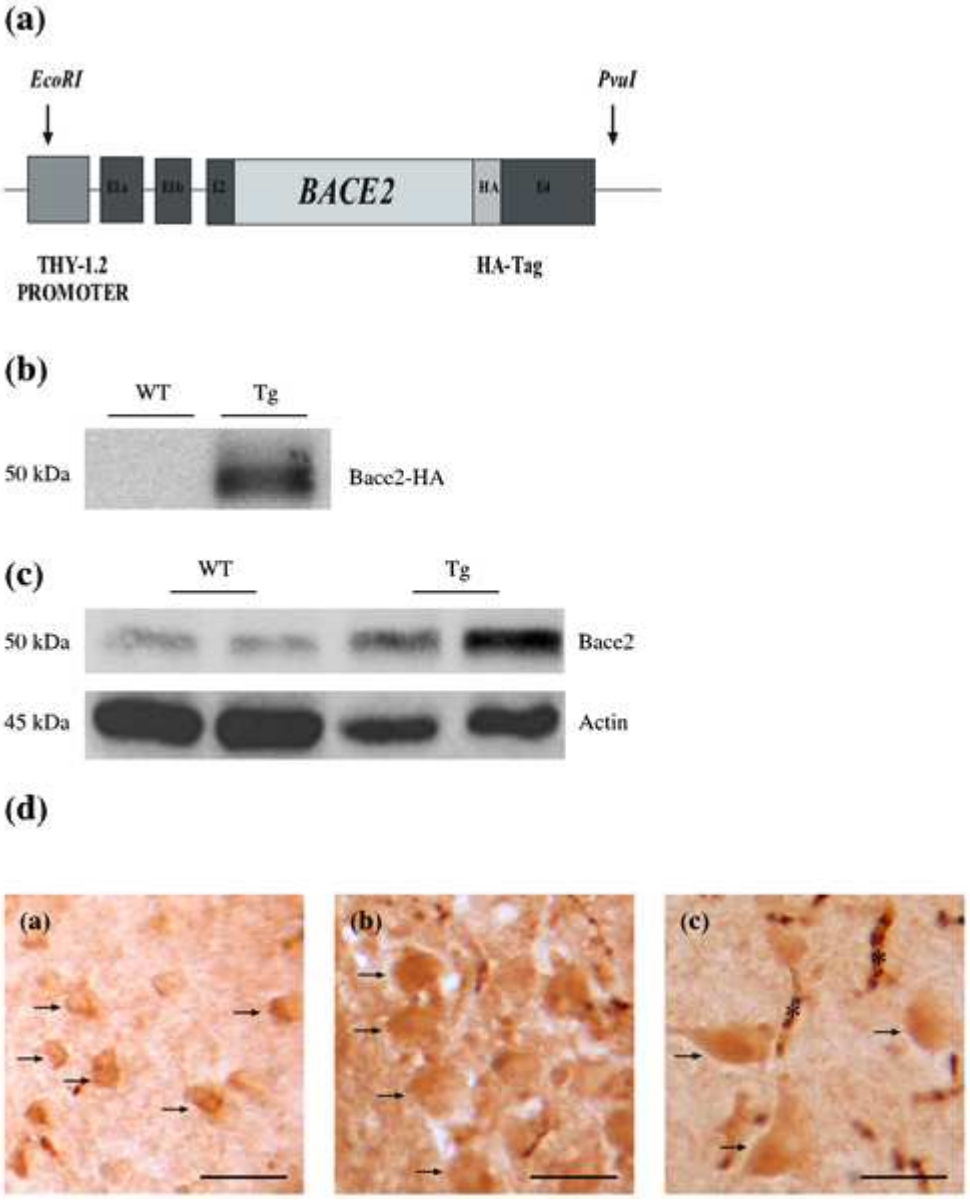
Figure 4. Open field test. (a) Latency to cross from periphery to centre expressed in seconds. Transgenic animals show an increased latency that was more marked in older mice. **(b)** Transgenic animals spent more time in the periphery of the maze. TgBACE2 displayed normal activity levels **(c)** and velocity **(d)**. Data are expressed as mean ± S.E.M.*P < 0.05.

Figure 5. Light and dark box. (a) Latency to cross from light to dark compartment expressed in seconds. Young transgenic animals show a significantly increased latency. **(b)** Percentage of time spent in the dark zone. Transgenic young mice spent more time in this zone as compared to their control group. **(c)** Percentage of entries to the light side. Both transgenic groups entered less often to the lit side than their controls. Data are expressed as mean ± S.E.M.*P < 0.05.

Figure 6. Elevated plus maze. (a) Percentage of time spent in the protected (close) arms, indicated no differences between genotypes, and (b) young transgenic animals visited less the unprotected (open) arms. Data are expressed as mean ± S.E.M.*P < 0.05.

Figure 7. Zero maze. (a) Latency to enter in the unprotected (open) arm in seconds. Transgenic animals showed a higher latency to enter to the open arms. **(b)** Percentage of time spent in the close arms. Transgenic animals spent more time in protected (close) arms. **(c)** Number of entries to the unprotected (open) arms. A reduction was observed in young transgenic animals. Data are expressed as mean ± S.E.M.*P < 0.05.

Figure 8. Molecular characterization of the anxiety phenotype. (a) Western blot analysis to evaluate TH expression levels in the medulla-pons region of TgBACE2 (Tg, n = 4 per age) and control mice (WT; n = 4 per age) at two different ages, as indicated. Actin expression was used for normalization. **(b)** Increased density of TH positive cells in LC from TgBACE2 (n = 4 per age) vs. control (WT, n = 4 per age) mice. Data are expressed as mean ± S.E.M. *P < 0.05; **P < 0.01.



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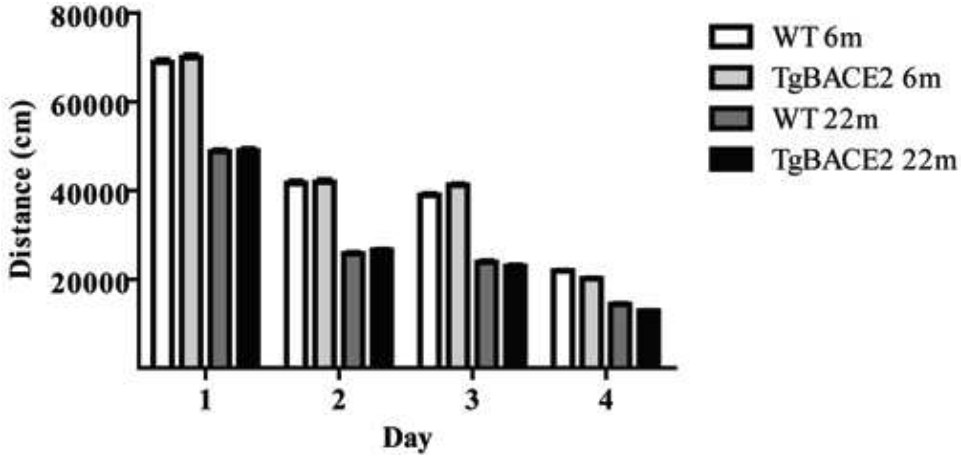

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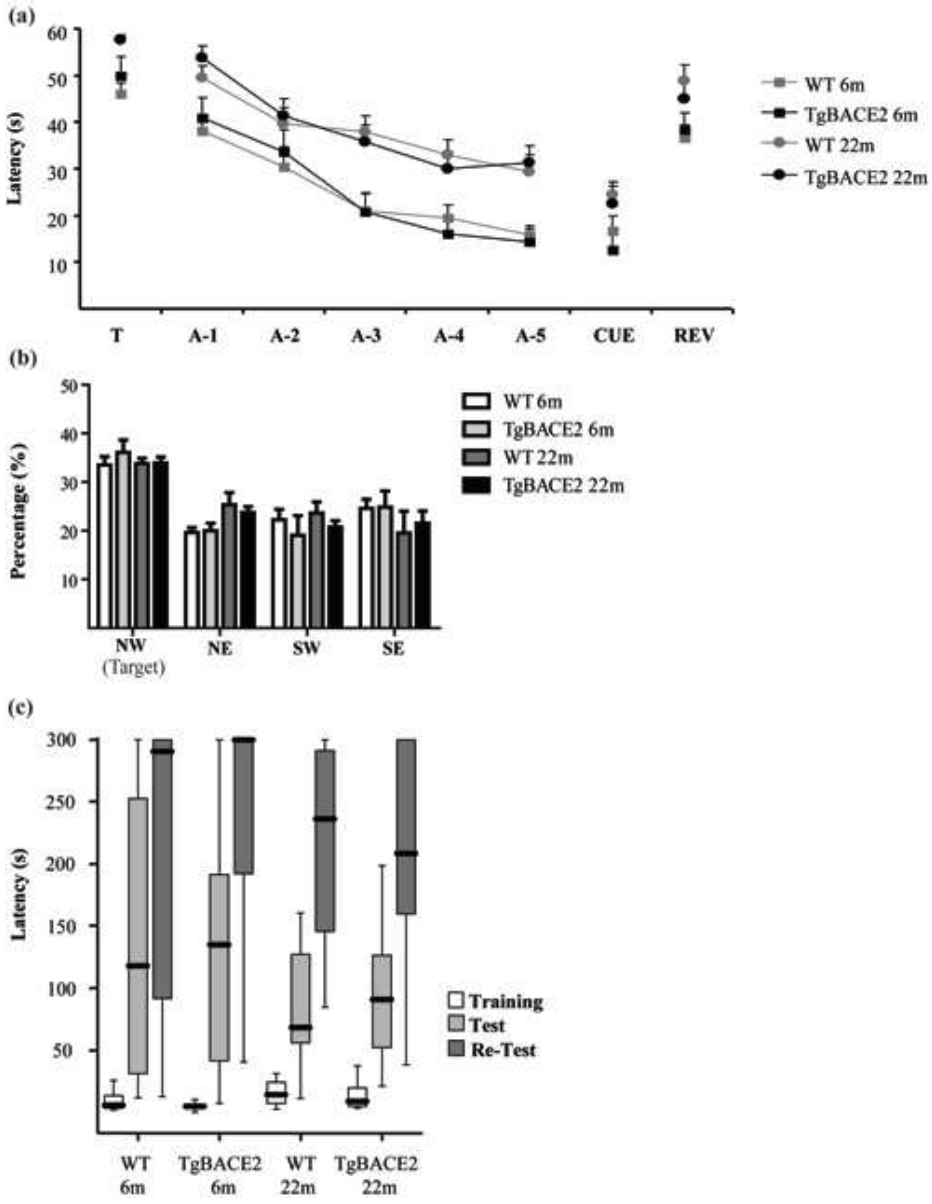

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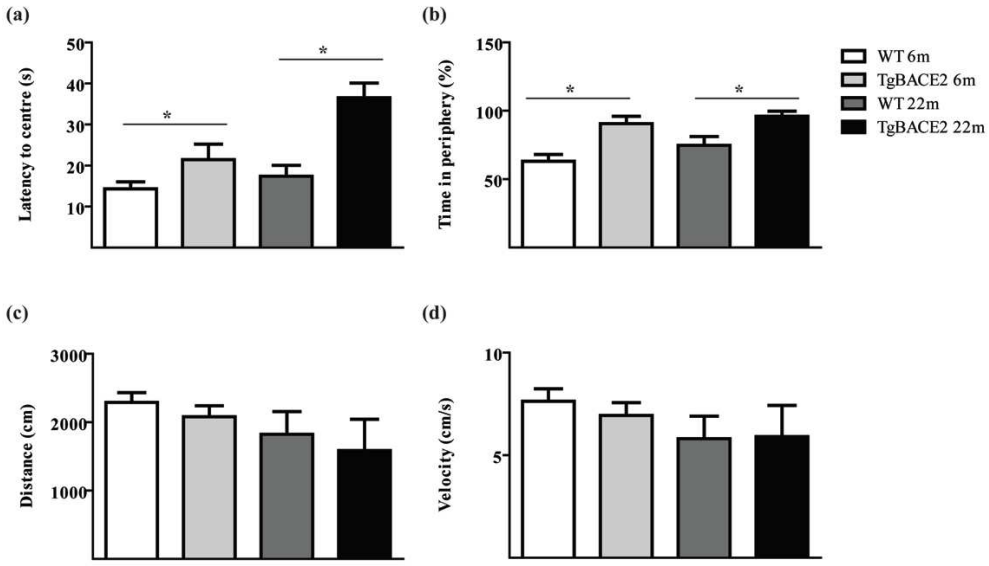

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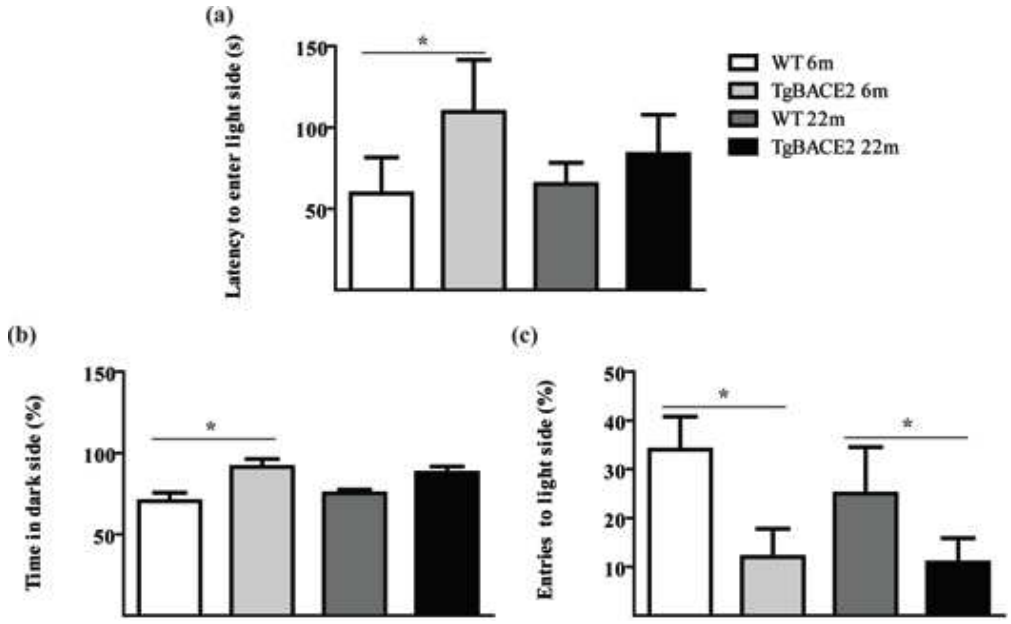

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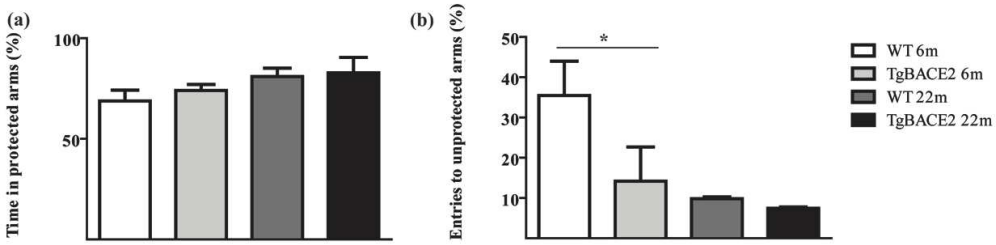

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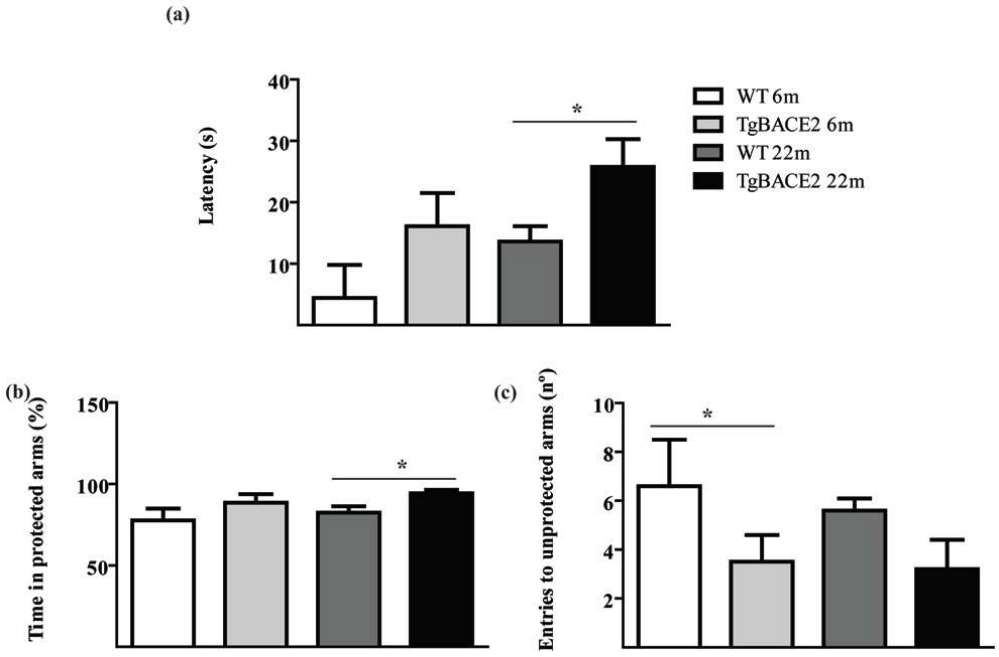

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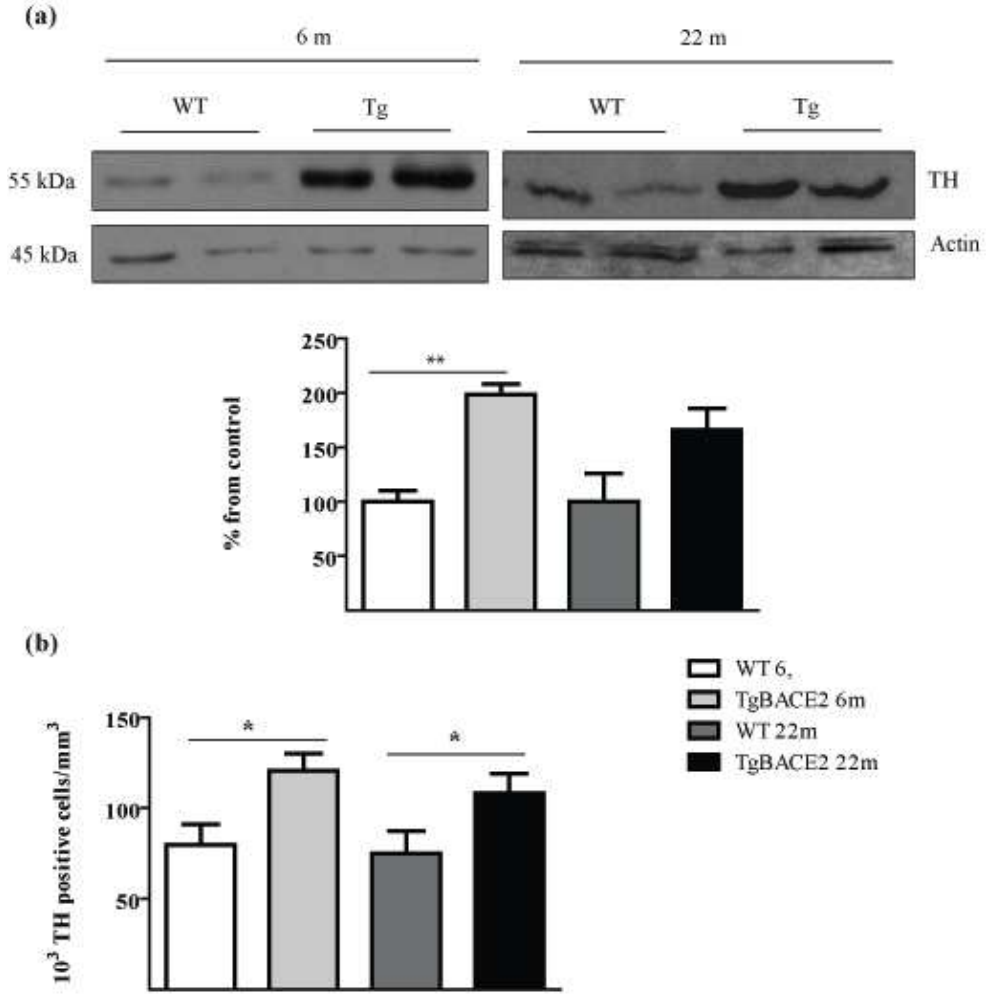

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Table 1. **Stereological analysis of cholinergic neurons in the basal forebrain.**

	ChAT density		$p75^{NGFR}$ density	
	6 months	22 months	6 months	22 months
WT	5282.012 ± 972.68	5603.85 ± 450.02	6622.73 ± 225.56	6250.01 ± 974.78
TgBACE2	4199.37 ± 1185.61	4870.68 ± 247.27	6519.74 ± 877.96	4401.88 ± 317.66

ChAT and $p75^{NGFR}$ cell density in MSN and VDB. No differences were observed between genotypes and ages. Wild type (WT; n = 4 per age) vs. TgBACE2 (L11, n = 4 per age). Data (nº of cells/mm^3) are expressed as means ± S.E.M.