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ORIGINAL PAPER

Effects of Cerebrolysin™ on neurogenesis in an APP transgenic model of Alzheimer's disease

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Abstract Cerebrolysin (CBL) is a peptide mixture with neurotrophic effects that might reduce the neurodegenerative alterations in Alzheimer's disease (AD). We have previously shown that in the amyloid precursor protein (APP) transgenic (tg) mouse model of AD, CBL improves synaptic plasticity and behavioral performance. However, the mechanisms are not completely clear. The neuroprotective effects of CBL might be related to its ability to promote neurogenesis in the hippocampal subgranular zone (SGZ) of the dentate gyrus (DG). To study this possibility, tg mice expressing mutant APP under the Thy-1 promoter were injected with BrdU and treated with CBL for 1 and 3 months. Compared to non-tg controls, vehicle-treated APP tg mice showed decreased numbers of BrdU-positive (+) and doublecortin+ (DCX) neural progenitor cells (NPC) in the SGZ. In contrast, APP tg mice treated with CBL showed a significant increase in BrdU+ cells, DCX+ neuroblasts and a decrease in TUNEL+ and activated caspase-3 immunoreactive NPC. CBL did not change the number of proliferating cell nuclear antigen+ (PCNA) NPC or the ratio of BrdU+ cells converting to neurons and astroglia in the SGZ cells in the APP tg mice. Taken together, these studies suggest that CBL might rescue the alterations in neurogenesis in APP tg mice by protecting NPC and decreasing the rate of apoptosis. The improved neurogenesis in the hippocampus of CBL-treated APP tg mice might play an important role in enhancing synaptic formation and memory acquisition.

Keywords Alzheimer's · Amyloid precursor protein · Subgranular zone · Hippocampus · Apoptosis

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Introduction

Considerable progress has been made in recent years towards better understanding the pathogenesis of Alzheimer's disease (AD), a common dementing disorder of the aging population that affects over 2.5 million individuals in the US and Europe alone. This neurological condition is characterized by widespread neurodegeneration throughout the association cortex and limbic system, deposition of amyloid-β protein (Aβ) in the neuropil and around the blood vessels, and formation of neurofibrillary tangles (NFT) [53]. The neurodegenerative process in the initial stages of AD targets the synaptic terminals [30] and then propagates to axons and dendrites, leading to neuronal dysfunction and eventually to neuronal death in later stages of disease [22]. Although the precise mechanisms leading to neurodegeneration in AD are not completely understood, recent studies suggest that alterations in the processing of amyloid precursor protein (APP), resulting in the accumulation of Aβ and APP C-terminal products, might play a key role in the pathogenesis of AD

[24, 50]. Several products are derived from APP through alternative proteolytic cleavage pathways, and enormous progress has recently been made in identifying the enzymes involved [6, 27, 49, 51, 58].

Various lines of evidence suggest that the direct abnormal accumulation of Aβ oligomers in the nerve terminals might lead to the synaptic damage and ultimately to neurodegeneration in AD [49]. More recent studies have uncovered evidence suggesting that another component to the neurodegenerative process in AD might include the possibility of interference with the process of adult neurogenesis in the hippocampus [52]. In transgenic (tg) animal models of AD, previous studies have shown significant alterations in the process of adult neurogenesis in the hippocampus [10, 13, 14, 23, 61]. Moreover, previous studies have shown that neurogenesis in the mature brain is important in the process of synaptic plasticity and memory formation in the hippocampus [57]. Therefore, compounds capable of protecting synapses and promoting neurogenesis might hold a serious promise in the development of new treatments for AD.

The nootrophic agent Cerebrolysin™ (CBL, a mixture of peptides and amino acids obtained from porcine brain tissue) has been shown to improve memory in patients with mild to moderate cognitive impairment [44, 46]. In support of these observations, it has also been shown to display neurotrophic activity in vitro [29] and in animal models of neurodegeneration [17, 31, 59]. Moreover, CBL has been shown to ameliorate the neurodegenerative alterations and amyloid burden in an APP model of AD-like pathology [39]. We have recently shown that CBL might reduce the amyloid pathology by decreasing APP production and proteolysis [42]. Although the regulatory effects of CBL on APP might contribute to the neuroprotective effects of this compound, it is possible that other mechanisms might also be involved. Among them, considerable interest has developed in the potential role of neurogenesis on the effects of neuroprotective compounds [9, 52]. Thus, CBL might ameliorate the neurodegenerative process in AD by stimulating neurogenesis in the adult hippocampus. In this context, the main objective of this study was to investigate the effects of CBL on neurogenesis in the hippocampus of APP tg mice and to better understand the mechanisms involved.

Materials and methods

Generation of APP tg mice, BrdU regimen and CBL treatment

The tg mice generated express mutated human (h)APP751 under the control of the mThy-1 promoter (mThy1-hAPP751) and, for this study, the highest expresser (line 41) tg mice were used [40]. These tg mice are unique in that, compared to other tg models, amyloid plaques are found in the brain at a much earlier age (beginning at 3 months) [40]. The first area in the brain to show amyloid deposition is the frontal cortex at 3 months, followed by the hippocampus. In this latter brain region, amyloid deposits occur at 4–6 months of age in the molecular and pyramidal layers but not in the subgranular zone (SGZ) of the dentate gyrus (DG). Genomic DNA was extracted from tail biopsies and analyzed by PCR amplification, as described previously [41]. Transgenic lines were maintained by crossing heterozygous tg mice with non-transgenic (non-tg) C57BL/6 × DBA/2 F1 breeders. All mice were heterozygous with respect to the transgene. A total of 24 (3-month-old) tg mice and 12 non-tg littermates were utilized for the present study. Each mouse received five injections (one per day) with 5-bromo-2-deoxyuridine (BrdU, Sigma-Aldrich, St. Louis, MO) at 50 mg/kg followed by treatment with vehicle or CBL. For all experiments, saline was utilized as the vehicle control. The 24 tg mice were divided into two groups; for the first, mice were treated for one month with daily intraperitoneal (IP) injections of either vehicle ($n = 6$) or CBL (5 ml/kg) ($n = 6$); for the second, mice were treated for 3 months with either vehicle ($n = 6$) or CBL (5 ml/kg) ($n = 6$). The non-tg mice were treated for one month with either vehicle ($n = 6$) or CBL (5 ml/kg) ($n = 6$). At the end of this period mice were sacrificed for analysis of neurogenesis and neuropathological assessment. All experiments described were approved by the animal subjects committee at the University of California at San Diego (UCSD) and were performed according to NIH recommendations for animal use.

Tissue processing

In accordance with NIH guidelines for the humane treatment of animals, mice were anesthetized with chloral hydrate and flush-perfused transcardially with 0.9% saline. Brains were removed and divided sagitally. One hemibrain was post-fixed in phosphate-buffered 4% paraformaldehyde (pH 7.4) at 4°C for 48 h and sectioned at 40 μm with a Vibratome 2000 (Leica, Germany), while the other hemibrain was snap frozen and stored at −70°C for protein analysis.

Analysis of neurodegeneration and Aβ deposits

To evaluate neurodegeneration and response to CBL, blind-coded 40 μm thick vibratome sections were

immunolabeled with the mouse monoclonal antibodies against synaptophysin (SYN, presynaptic terminal marker, 1:40, Chemicon, Temecula, CA), NeuN (general neuronal marker, 1:1000, Chemicon), and glial fibrillary acidic protein (GFAP, marker of astrogliosis, 1:1000, Chemicon), as previously described [38, 42]. After overnight incubation with the primary antibodies, sections were incubated with FITC-conjugated horse anti-mouse IgG secondary antibody (1:75, Vector Laboratories, Burlingame, CA), transferred to SuperFrost slides (Fisher Scientific, Tustin, CA) and mounted under glass coverslips with anti-fading media (Vector). All sections were processed under the same standardized conditions. The immunolabeled blind-coded sections were imaged with the laser-scanning confocal microscope (LSCM, MRC1024, BioRad, Hercules, CA) and analyzed with the Image 1.43 program (NIH), as previously described [34, 55] to determine the percent area of the neuropil covered by SYN-immunoreactive terminals and the levels of GFAP immunoreactivity (pixel intensity) in the DG. For counting of NeuN cells in the DG, digitized confocal images collected according to the optical disector method were analyzed with the Image-Pro Plus 4.0 (Media Cybernetics, Silver Spring, MD) software as previously described [7, 8].

For detection of Aβ deposits, briefly as previously described, vibratome sections were incubated overnight at 4°C with the mouse monoclonal antibody 4G8 (1:600, Senetek, Napa, CA), followed by incubation with a fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (Vector Laboratories). Sections were imaged with the LSCM (MRC1024, BioRad) as described previously [35] and digital images were analyzed with the NIH Image 1.43 program to determine the percent area occupied by Aβ deposits. Three immunolabeled sections were analyzed per mouse and the average of individual measurements was used to calculate group means.

Immunocytochemical analysis of markers of neurogenesis and cell death

For detection of markers of neurogenesis, briefly vibratome sections oriented in the sagittal plane were pretreated with 50% formamide/ 2 × SSC (2 × SSC: 0.3 M NaCl, 0.03 M sodium citrate) at 65°C, rinsed for 5 min in 2 × SSC, then incubated for 30 min in 2 M HCl at 37°C, followed by a 10-min rinse in 0.1 M boric acid, pH 8.5. Then sections were incubated with antibodies against BrdU (marker of dividing cells; rat monoclonal, 1:100, Oxford Biotechnology, Oxford, UK), proliferating cell nuclear antigen (PCNA, marker of proliferation; mouse monoclonal, 1:250, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or doublecortin (DCX, marker of migrating neuroblasts; goat polyclonal, 1:500, Santa Cruz) overnight at 4°C. Sections were then incubated with biotinylated secondary antibodies directed against rat, mouse, or goat. Following intermittent rinses in tris-buffered saline (TBS), avidin–biotin–peroxidase complex was applied (ABC Elite kit, Vector) followed by peroxidase detection with diaminobenzidine (DAB) in 0.01% H_2O_2, 0.04% NiCl in TBS.

For analysis of the proportion of BrdU-positive (+) cells converting into neurons or astroglial cells, double immmunofluorescence labeling was performed with antibodies against BrdU and NeuN, and BrdU and GFAP. For this purpose, vibratome sections were treated to denaturate the DNA as described above. Afterwards a combination of the two antibodies was applied in TBS-donkey serum for 48 h at 4°C, followed by incubation with secondary fluorochrome antibodies as previously described, transferred to SuperFrost slides (Fisher) and mounted under glass coverslips with anti-fading media (Vector). To investigate the relationship between neurogenesis and markers of AD-like pathology, vibratome sections were double-labeled with monoclonal antibodies against Aβ (4G8, 1:500, Senetek) or human APP (8E5, 1:5000, courtesy of Elan Pharmaceuticals) and the polyclonal antibody against DCX (1:500, Santa Cruz). All sections were processed under the same standardized conditions. The immunolabeled blind-coded sections were imaged with the LSCM (MRC1024, BioRad).

For detection of apoptosis of neural progenitor cells (NPC), the terminal deoxynucleotidyl transferase dUTP Nick End Labeling (TUNEL) detection method using the ApopTag In Situ Apoptosis Detection Kit (Chemicon) was used with modifications for free floating sections as described previously [4, 5, 12]. Detection was performed with Avidin-FITC and sections were mounted under glass coverslips with anti-fading media (Vector) for confocal microscopy analysis. To verify that NPC undergo apoptosis, sections were double-labeled with a monoclonal antibody against activated caspase-3 (1:200, Stressgen Bioreagents, Ann Arbor, MI) and the polyclonal antibody against DCX (1:500, Santa Cruz), followed by incubation with fluorochrome-labeled secondary antibodies and imaging on the LSCM.

Quantitative analysis of neurogenesis in the hippocampus

For this purpose, a systematic, random counting procedure, similar to the optical disector [18], was used as

described previously [62]. For the purpose of the present study, the morphometric analysis was focused on the SGZ of the DG. This area corresponds to the layer of NPCs located directly under the first layer of mature granular cells in the DG, which in addition to the SGZ, includes the granular cell layer (GCL) and the molecular layer (ML). The analysis was centered on the SGZ because a previous study has shown that this is the area most consistently affected in APP tg mice [14]. To determine the number of BrdU+, DCX+, PCNA+ or TUNEL+ cells in the SGZ of the hippocampus, every sixth section (200-μm interval) of the left hemisphere was selected from each animal and processed for immunohistochemistry. The reference volume was determined by tracing the areas using a semi-automatic stereology system (Stereoinvestigator, MicroBrightField, Colchester, VT). Positive cells were counted within a 60 μm × 60 μm counting frame, which was spaced in a 300 μm × 300 μm counting grid. Positive profiles that intersected the uppermost focal plane (exclusion plane) or the lateral exclusion boundaries of the counting frame were not counted. The total counts of positive profiles were multiplied by the ratio of reference volume to sampling volume in order to obtain the estimated number of positive cells for each structure.

To determine the frequency of neuronal and astroglial differentiation of newborn cells, as previously described [10, 64], a series of every sixth section (200-μm interval) was examined using the LSCM equipped with a 63 × PL APO oil objective (1.25 numeric aperture) and a pinhole setting that corresponded to a focal plane of 2 μm or less. On average, 50 BrdU+ cells were analyzed in the SGZ for neuronal differentiation. BrdU+ cells were randomly selected and analyzed by moving through the *z*-axis of each cell, in order to exclude false double labeling due to an overlay of signals from different cells.

Statistical analysis

Analyses were carried out with the StatView 5.0 program (SAS Institute Inc., Cary, NC). Differences among means were assessed by one-way ANOVA with post hoc Dunnett's (when comparing to the non-tg control group) or Tukey–Kramer (when comparing between treatment groups). Comparisons between two groups were done with the two-tailed unpaired Student's *t* test. Correlation studies were carried out by simple regression analysis and the null hypothesis was rejected at the 0.05 level. All values are expressed as mean ± SEM.

Results

Cerebrolysin's neuroprotective effects are associated with increased numbers of BrdU+ and DCX+ cells in the SGZ of APP tg mice

To investigate the effects of CBL in hippocampal neurogenesis, 3-month-old APP tg mice received a series of five BrdU injections and then were treated for 1 and 3 months with CBL, and levels of markers of neurogenesis were analyzed in the SGZ. Compared to vehicle-treated non-tg mice (Figs. 1a, b; 2a, b), vehicle-treated APP tg mice displayed a significant decrease in the numbers of BrdU+ (Fig. 1a, d) [$F(2,11) = 6.24$, $P < 0.02$] and DCX+ (Fig. 2a, d) [$F(2,11) = 4.94$, $P < 0.05$] cells. In contrast, APP tg mice treated with CBL displayed comparable numbers of BrdU+ (Fig. 1a, e) and DCX+ (Fig. 2a, e) cells compared to CBL-treated non-tg controls (Figs. 1a, c; 2a, c). Similar effects were observed after 1 and 3 months of treatment. Moreover, the numbers of BrdU+ cells [$F(3,18) = 21.05$, $P < 0.001$] and DCX+ cells [$F(3,18) = 6.51$, $P < 0.01$] were different between vehicle- and CBL-treated APP tg mice. In some cases, CBL-treated APP tg mice showed numbers of BrdU+ cells greater than the numbers in vehicle-treated non-tg mice (Fig. 1a). Similarly, CBL-treated non-tg mice had a slight increase in the numbers of BrdU+ cells when compared to the non-tg vehicle-treated group (Fig. 1a).

To investigate the relationship between APP expression and neurogenesis in the tg mice, double-labeled sections were analyzed with the confocal microscope. The APP tg mice express the human APP in the GCL, moreover approximately 18% of the DCX+ NPC in the SGZ expressed human APP (Fig. 2f–h). Amyloid deposits are observed at 4–6 months of age in the ML of the DG but no deposits are detected in the SGZ in the vicinity of the NPC that are DCX+ (not shown).

Consistent with the effects in neurogenesis, analysis of the nerve terminals by confocal microscopy showed that in the CBL-treated APP tg mice, levels of SYN-immunoreactive (IR) terminals (Fig. 3a) and NeuN+ cells (Fig. 3b) in the DG were comparable to non-tg control mice. In contrast, in the DG of the vehicle-treated APP tg mice there were decreased levels of SYN-IR terminals (Fig. 3a) [$F(3,18) = 5.75$, $P < 0.01$] and NeuN+ cells (Fig. 3b) [$F(3,18) = 16.9$, $P < 0.001$] compared to CBL-treated APP tg mice. Neuropathological analysis of the APP tg mice showed that compared to the vehicle-treated group, after 1 and 3 months of CBL treatment, there was a significant reduction in the levels of astrogliosis in the DG

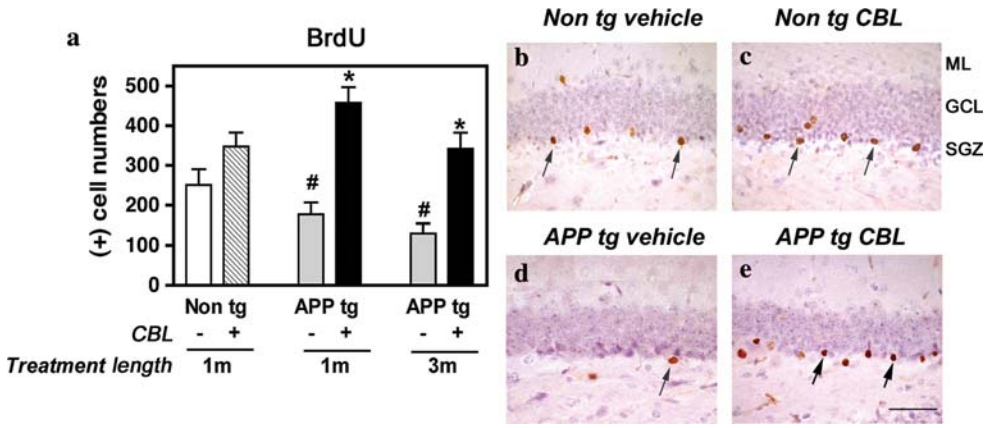

Fig. 1 Immunocytochemical analysis of 5-bromo-2-deoxyuridine (*BrdU*)-positive (+) cells in non-transgenic (*non-tg*) and amyloid precursor protein (*APP*) tg mice treated with Cerebrolysin (*CBL*). **a** Quantitative analysis using the disector method in the subgranular zone (*SGZ*) shows decreased numbers of BrdU+ neurons in vehicle-treated APP tg mice compared to non-tg animals, while CBL-treated APP tg mice at 1 and 3 months (m) show increased levels of BrdU+ neurons compared to vehicle-treated APP tg animals. $^{\#}P < 0.05$ compared to vehicle-treated non-tg control by one-way ANOVA with post hoc Dunnett's ($n = 6$); $^{*}P < 0.05$ compared to vehicle-treated APP tg mice by one-way ANOVA with post hoc Tukey–Kramer ($n = 6$). **b**–**e** All panels are representative images displaying the molecular layer (*ML*), *GCL* (granular cell layer), and SGZ at 400× magnification of mice treated for 1 month. BrdU+ neurons in the SGZ of vehicle-treated non-tg mice (**b**), CBL-treated non-tg mice (**c**), vehicle-treated APP tg mice (**d**), and CBL-treated APP tg mice (**e**). *Scale bar* 60 μm

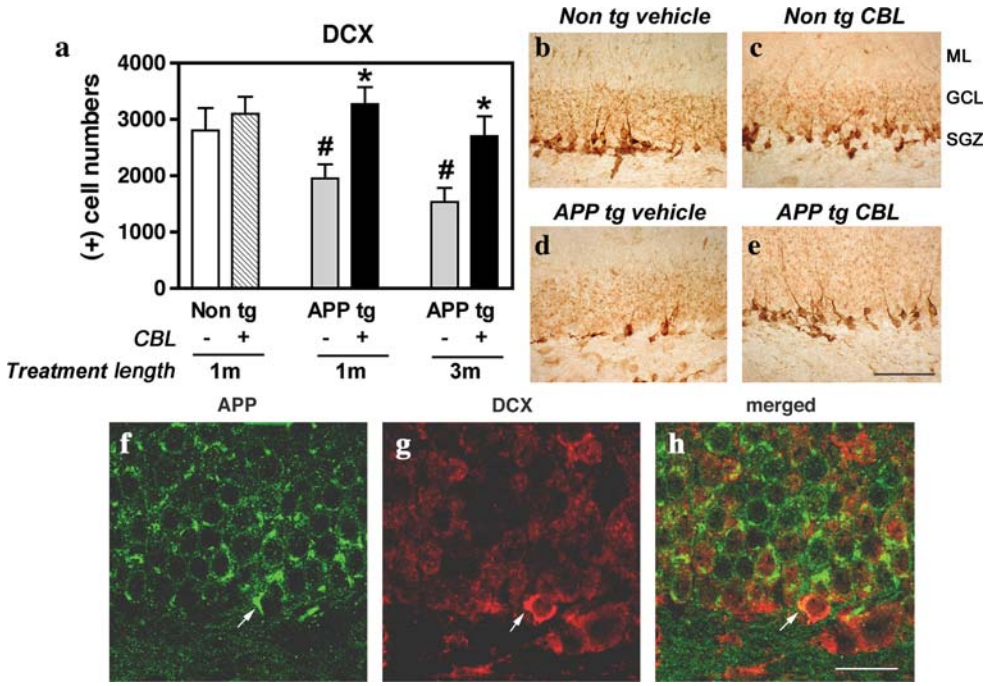

Fig. 2 Immunocytochemical analysis of doublecortin (*DCX*)-positive (+) cells in non-tg and APP tg mice treated with CBL. **a** Quantitative analysis using the disector method in the SGZ shows decreased numbers of DCX+ neurons in vehicle-treated APP tg mice compared to non-tg animals, while CBL-treated APP tg mice at 1 and 3 months (m) show increased levels of DCX+ neurons compared to vehicle-treated APP tg animals. $^{\#}P < 0.05$ compared to vehicle-treated non-tg control by one-way ANOVA with post-hoc Dunnett's ($n = 6$); $^{*}P < 0.05$ compared to vehicle-treated APP tg mice by one-way ANOVA with post-hoc Tukey–Kramer ($n = 6$). **b**–**e** All panels are representative images (400×) displaying the ML, GCL, and SGZ of mice treated for 1 month. DCX+ neurons in the SGZ of vehicle-treated non-tg mice (**b**), CBL-treated non-tg mice (**c**), vehicle-treated APP tg mice (**d**), and CBL-treated APP tg mice (**e**). *Scale bar* 60 μm. **f**–**e** All panels are representative images displaying the CGL and SGZ of a vehicle-treated APP tg mouse. Double-labeling analysis with antibodies against human APP (8E5, **f**) and DCX (**g**) shows colocalization (**h**) of the two markers in cells in the SGZ (*arrows*). *Scale bar* 30 μm

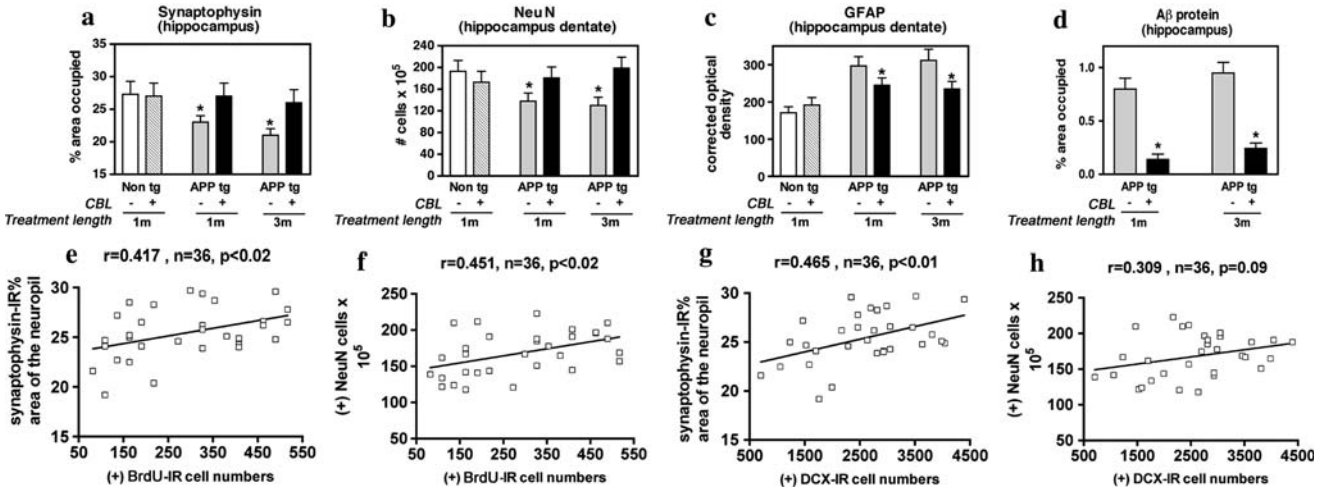

Fig. 3 Analysis of neurodegeneration and amyloid deposition and correlation with markers of neurogenesis in non-tg and APP tg mice treated with CBL. **a** Percent area of the ML of the hippocampal dentate gyrus covered by synaptophysin-immunoreactive terminals. **b** Estimates of the numbers of NeuN+ neurons in the hippocampal dentate gyrus using the disector method. **c** Levels of astrogliosis [glial fibrillary acidic protein (*GFAP*) immunoreactivity] in the hippocampal dentate gyrus. **d** Percent area of the hippocampus covered by amyloid-β (Aβ)-immunoreactive deposits. $^*P < 0.05$ compared to vehicle-treated APP tg mice by one-way ANOVA with post-hoc Tukey–Kramer ($n = 6$). **e**–**h** Linear regression analysis between markers of neurogenesis [BrdU- and DCX-immunoreactive (IR) cells] and markers of neuronal preservation (synaptophysin and NeuN)

(Fig. 3c) and in the percent area of the neuropil covered by Aβ immunoreactivity (Fig. 3d). Linear regression analysis showed that the levels of SYN-IR terminals and NeuN+ cells in the DG were correlated with the numbers of BrdU+ (Fig. 3e, f) and DCX+ (Fig. 3g, h) cells in the SGZ.

The effects of CBL on neurogenesis are associated with increased survival of hippocampal NPC

To determine if the effects of CBL were related to a correction in the baseline alterations in the proportion of NPC in the SGZ converting into neurons or astroglia in the APP tg mice, analysis of sections double-labeled for BrdU and NeuN or GFAP was performed. As expected, in non-tg mice approximately 65% of the BrdU+ cells expressed NeuN (Fig. 4a) and 25% expressed GFAP (Fig. 4b). Although in the vehicle-treated APP tg mice there were fewer BrdU+ (and total NeuN+ in the DG) nuclei, the proportion of cells expressing NeuN (Fig. 4a, c–e) and GFAP (Fig. 4b) was similar to the non-tg controls. Moreover, CBL treatment did not alter this proportion (Fig. 4a, b, f–h), suggesting that the effects of CBL on neurogenesis were not related to an enhanced differentiation of precursors into neurons but rather to increased proliferation or survival of NPC in the SGZ of the hippocampus. To investigate these possibilities, sections immunolabeled with an antibody against PCNA or stained with the Apoptag kit were analyzed. This study showed that vehicle- and CBL-treated mice showed comparable numbers of PCNA-IR cells in the SGZ (Fig. 5a). Similarly, no significant differences were observed between vehicle-treated non-tg and APP tg mice (Fig. 5b–e). In contrast, vehicle-treated APP tg mice displayed increased numbers of TUNEL+ cells in the SGZ compared to the vehicle- and CBL-treated non-tg control group (Fig. 6a–d) [$F(3,18) = 4.26$, $P < 0.05$]. This increased mortality of NPC in APP tg mice (Fig. 6d) was significantly reduced by CBL (Fig. 6e) after 1 and 3 months of treatment (Fig. 6a) [$F(3,18) = 7.69$, $P < 0.01$]. To verify that NPC in the SGZ of the APP tg mice activate pathways involved in apoptosis, sections were double-labeled and analyzed by confocal microscopy. This study showed that in the non-tg mice, approximately 3% of the DCX+ cells in the SGZ are IR for activated caspase-3, and in the APP tg mice, about 5% of the DCX+ cells co-expressed activated caspase-3 (Fig. 7). In contrast, in the APP tg mice treated with CBL, less than 2% of the DCX+ cells displayed caspase-3 activation (Fig. 7).

Together, these findings support the possibility that CBL rescues the defects in neurogenesis in APP tg mice by enhancing the survival (and reducing the rate of apoptosis) of the NPC in the hippocampus.

Discussion

The present study showed that CBL ameliorates the alterations in hippocampal neurogenesis in an APP tg

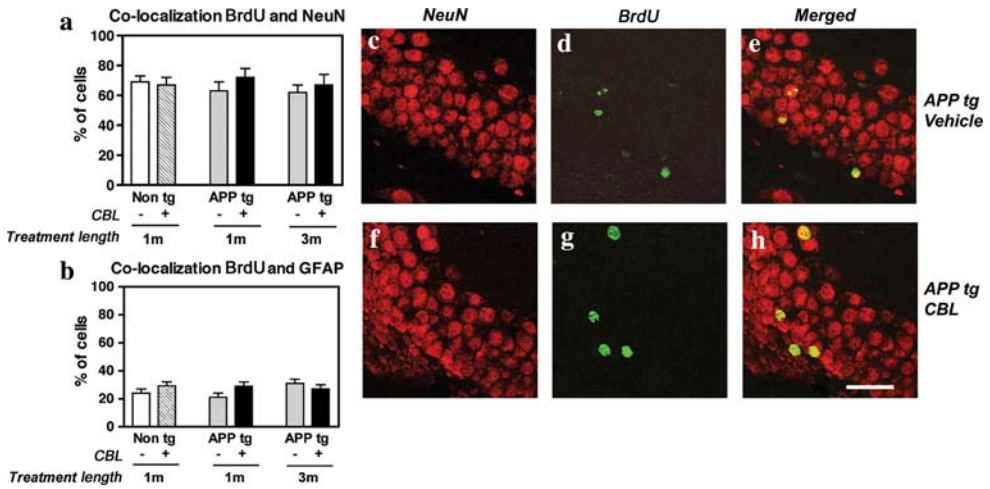

Fig. 4 Double-immunocytochemical analysis to determine the proportion of BrdU-positive (+) cells that are NeuN and GFAP-positive in non-tg and APP tg mice treated with CBL. **a** Percent of BrdU+ cells that are NeuN+. **b** Percent of BrdU+ cells that are GFAP+. **c–h** Representative images obtained from confocal microscopy of sections double-labeled with antibodies against NeuN (*red*) and BrdU (*green*) in the hippocampal dentate gyrus of APP tg mice treated for 1 month (m) with vehicle (**c–e**) or CBL (**f–h**). *Scale bar* 50 μm

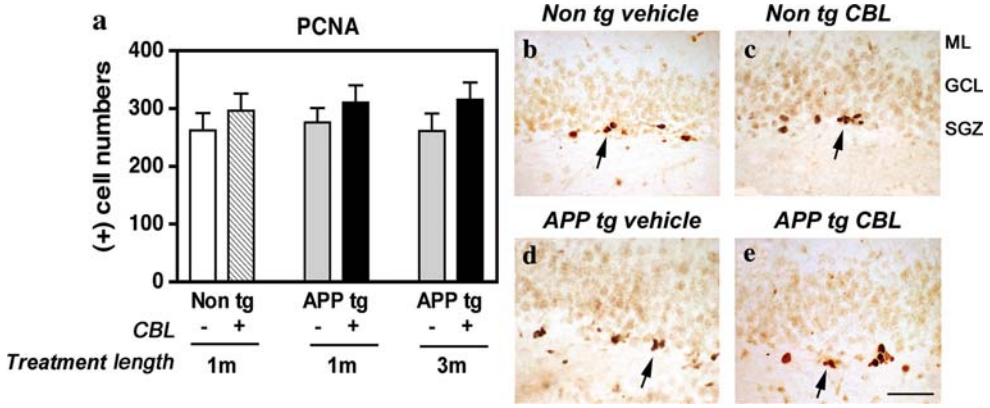

Fig. 5 Immunocytochemical analysis of proliferating cell nuclear antigen (PCNA)-positive (+) cells in non-tg and APP tg mice treated with CBL. **a** Quantitative analysis using the disector method in the SGZ showing numbers of PCNA+ NPC. **b–e** All panels are representative images displaying the ML, GCL, and SGZ at 400× magnification of mice treated for 1 month (m). PCNA+ NPC in the SGZ of vehicle-treated non-tg mice (**b**), CBL-treated non-tg mice (**c**), vehicle-treated APP tg mice (**d**), and CBL-treated APP tg mice (**e**). *Scale bar* 60 μm

model of AD neuropathology. The deficient neurogenesis in the SGZ of the DG found in our APP tg mice is consistent with studies in other lines of APP tg mice that deposit amyloid [13, 21] and other models that do not deposit amyloid but express mutant variants of the presenilin gene associated with familial AD [10, 16, 60]. These models have shown decreased markers of neurogenesis, such as BrdU+ and DCX+ cells, with an increase in the expression of markers of apoptosis [13, 16, 21, 60]. Although a different study reported increased neurogenesis in the PDAPP model [23], a more recent and comprehensive analysis showed that while in the ML of the DG there is an increased number of NPC, in the SGZ markers of neurogenesis are decreased, indicating that in PDAPP animals there is altered migration and increased apoptosis of NPC that contributes to the deficits in neurogenesis [14]. A similar situation might be at play in AD patients since a previous study showed increased expression of markers of neurogenesis in the hippocampus [23].

The increased neurogenesis in the SGZ in the CBL-treated mice was correlated with preservation of the neuronal and synaptic markers in the APP tg mice and with reduced deposition of amyloid aggregates. Moreover, CBL enhanced neurogenesis in the non-tg mice. This is consistent with studies in aged rodents and in animals exposed to an enriched environment that have shown that enhanced neurogenesis promotes synapto-

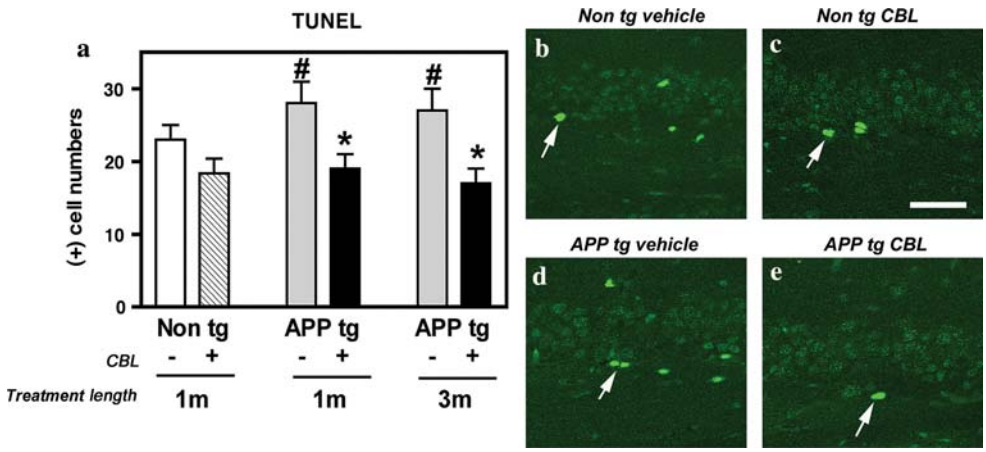

Fig. 6 Analysis of DNA fragmentation by terminal deoxynucleotidyl transferase dUTP Nick End Labeling (*TUNEL*) histochemistry in non-tg and APP tg mice treated with CBL. **a** Quantitative analysis using the disector method in the SGZ to estimate the numbers of apoptotic nuclei show increased numbers of TUNEL+ neurons in vehicle-treated APP tg mice compared to non-tg animals, while CBL-treated APP tg mice at 1 and 3 months (m) show decreased levels of TUNEL+ neurons compared to vehicle-treated APP tg animals. $^{\#}P < 0.05$ compared to vehicle-treated non-tg control by one-way ANOVA with post hoc Dunnett's ($n = 6$); $^{*}P < 0.05$ compared to vehicle-treated APP tg mice by one-way ANOVA with post hoc Tukey–Kramer ($n = 6$). **b–e** All panels are representative images displaying the ML, GCL, and SGZ at 400× magnification of mice treated for 1 month. TUNEL+ neurons in the SGZ of vehicle-treated non-tg mice (**b**), CBL-treated non-tg mice (**c**), vehicle-treated APP tg mice (**d**), and CBL-treated APP tg mice (**e**). *Scale bar* 60 μm

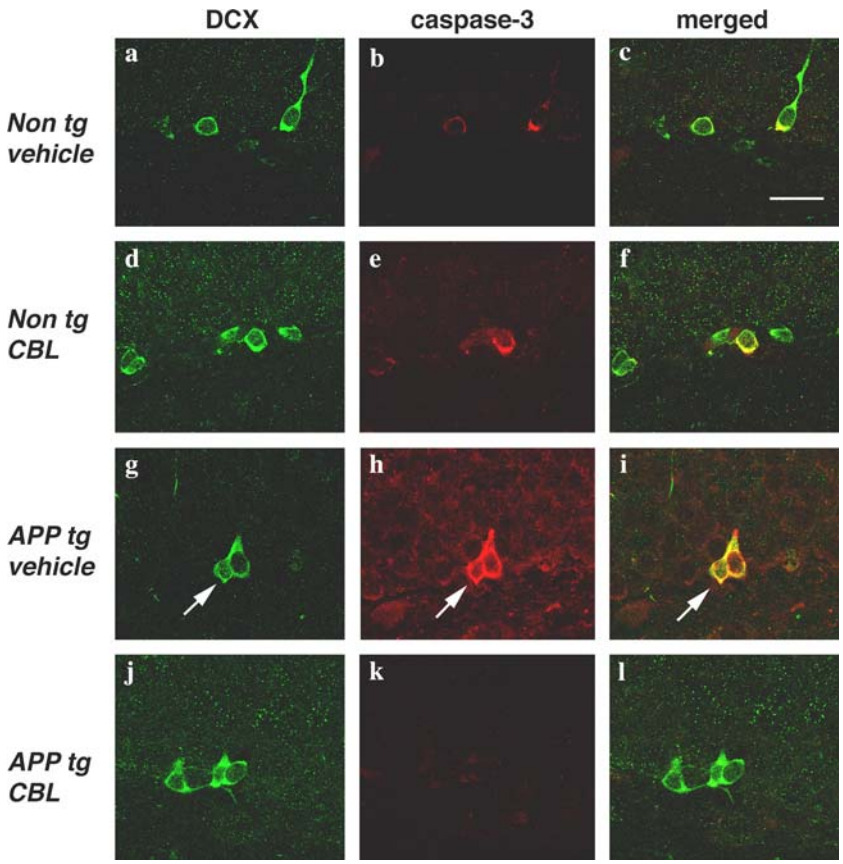

Fig. 7 Double-immunocytochemical analysis of activated caspase-3 in NPCs in APP tg mice treated with CBL. All panels are representative images of the SGZ in mice treated for 1 month. Sections were imaged with the confocal microscope. The panels in *red* correspond to activated caspase-3 and the panels in *green* correspond to DCX. DCX and activated caspase-3 double-immunolabeling in vehicle-treated non-tg mice (**a–c**), CBL-treated non-tg mice (**d–f**), vehicle-treated APP tg mice (**g–i**), and CBL-treated APP tg mice (**j–l**). *Scale bar* 30 μm

genesis and behavioral performance [25, 56]. Although the animals used in the present study display a significant amyloid burden in the hippocampus (at 6 months of age amyloid occupies an average of 1% of the hippocampal area), the changes in hippocampal volume are mild and most likely related to the loss of neuropil.

Therefore, changes in neurogenesis are unlikely to be a result of alterations in hippocampal volume.

The beneficial effects of CBL on neurogenesis in APP tg mice was linked with reduced apoptosis. Interestingly, BrdU uptake was increased in CBL-treated animals, however NPC proliferation as measured by PCNA immunolabeling was unchanged. This difference is likely a direct result of the decreased apoptosis, because while the BrdU labeling identifies dividing cells over the 5 days of injections and there is a 4-week time period between BrdU injection and euthanization, PCNA immunostaining only indicates NPCs in the cell cycle at the time of tissue fixation. Therefore, numbers of NPCs that take up BrdU appear increased because their rate of apoptosis during the 4-week treatment period is reduced, while instantaneous proliferation rates are unchanged. Since markers of NPC proliferation (e.g., PCNA) and differentiation (e.g., ratio of BrdU+/NeuN+ cells) were not altered in the APP tg mice and were not modified by CBL, we concluded that CBL rescues the defects in neurogenesis in APP tg mice by enhancing the survival (and reducing the rate of apoptosis) of NPC in the hippocampus. However, the use of PCNA as an indicator of cell proliferation in the APP tg mice needs to be interpreted with caution because previous studies have shown the Aβ might affect PCNA expression [65]. The effects of CBL on NPC in APP tg mice is consistent with in vitro studies of neuronal cultures [19, 20] and in vivo studies in rodent NPC [9, 52] that have shown that distinct from other neurotrophic factors, CBL influences neurogenesis by improving neuronal survival and decreasing apoptosis. The study by Tatebayashi and colleagues showed that CBL treatment resulted in an increase in the number of BrdU+ cells 12 days after treatment, and these effects were associated with an increase in the numbers of newborn NeuN+ neurons and improved performance in the Morris water maze [52]. The effects of CBL in the in vivo models is also in agreement with studies in neuronal cultures and the mature CNS that have shown that CBL attenuates apoptosis [38] resulting from a variety of neurotoxic challenges. The mechanisms through which CBL might regulate apoptosis and survival of NPC are unclear. Recent studies have shown that in the adult hippocampus the sonic hedgehog (Shh) gene [1, 26, 28], and in the fetal brain the Notch signaling pathway [2] play important roles in neurogenesis. In such a paradigm, it has been proposed that Notch activation in NPC activates Akt, mammalian target of rapamycin (mTor), and STAT3, followed by induction of Hes3 and Shh in a temporally controlled fashion [2]. Interestingly, we have recently shown that CBL reduces amyloid production and neurodegeneration in APP tg mice by modulating glycogen synthase kinase-3β (GSK3β) and cyclin-dependent kinase-5 (CDK5) dependent phosphorylation (and maturation) of APP [42]. Both GSK3β and CDK5 interact with presenilin-1, which participates in the proteolysis of Notch and APP by activating this component of the γ-secretase complex [47, 54]. The GSK3β signaling pathway is regulated by Akt and has been shown to modulate the activity and expression of molecules involved in apoptosis and neuronal survival [11] such as the glycoprotein neuronal pentraxin 1 [15], and the transcription factor NFAT3 [3]. Moreover, GSK3β cross-talks with the wnt-1 [33] and Notch [32] signaling pathways. Taken together, these studies suggest that CBL might play a role in neurogenesis by regulating GSK3β and CDK5 activity. Alternatively, CBL might regulate other aspects of the apoptosis cascades including TNFα-receptor and caspase activation, cytochrome C release and formation of the Apaf-1 complex, among others [66]. Future studies will be necessary to investigate these possibilities and to elucidate if the mechanisms through which CBL regulates apoptosis differ between mature neurons and NPC.

Previous studies have suggested that CBL can exert a neurotrophic-like activity by promoting synaptic formation in 6-week-old [63] and aged [36] rats, protecting against excitotoxicity [59], and ameliorating cognitive deficits and synaptic pathology in animal models of AD-related neurodegeneration [31, 37–39, 42]. Furthermore, in a manner similar to nerve growth factor (NGF) [43], CBL promotes neurite outgrowth and cholinergic fiber regeneration [17, 48] and reduces the synaptic pathology and behavioral deficits in APP tg mice [42]. In support of these results, studies in patients with mild to moderate AD have shown that CBL improves cognitive performance and that these effects are maintained even 6 months after termination of therapy [45]. In conclusion, these studies suggest that the combined effects of CBL on neurogenesis and amyloid production might play a role in alleviating the synaptic and cognitive deficits in patients with AD.

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