

**Title:**

Lenti-GDNF gene therapy protects against Alzheimer's disease-like neuropathology in 3xTg-AD mice and MC65 cells

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**Abstract:**

**Aims:** Glial cell-derived neurotrophic factor (GDNF) is emerging as a potent neurotrophic factor with therapeutic potential against a range of neurodegenerative conditions including Alzheimer's disease (AD). We assayed the effects of GDNF treatment in AD experimental models through gene therapy procedures. **Methods:** Recombinant lentiviral vectors were used to overexpress GDNF gene in hippocampal astrocytes of 3xTg-AD mice in vivo, and also in the MC65 human neuroblastoma that conditionally overexpresses the 99-residue carboxyl-terminal (C99) fragment of the amyloid precursor protein. **Results:** After 6 months of overexpressing GDNF, 10-month old 3xTg-AD mice showed preserved learning and memory, whilst their counterparts transduced with a green fluorescent protein vector showed cognitive loss. GDNF therapy did not significantly reduce amyloid and tau pathology, but rather, induced a potent upregulation of brain derived neurotrophic factor that may act in concert with GDNF to protect neurons from atrophy and degeneration. MC65 cells overexpressing GDNF showed an abolishment of oxidative stress and cell death that was, at least partially, mediated by a reduced presence of intracellular C99 and derived amyloid  $\beta$  oligomers. **Conclusions:** GDNF induced neuroprotection in the AD experimental models used. Lentiviral vectors engineered to overexpress GDNF showed to be safe and effective, both as a potential gene therapy and as a tool to uncover the mechanisms of GDNF neuroprotection, including crosstalk between astrocytes and neurons in the injured brain.

**Keywords:**

Glial Cell Line-Derived Neurotrophic Factor, Alzheimer Disease, Transgenic Mice, Neuroblastoma, Gene Therapy.

## Introduction

Alterations in the neurotrophic levels, either due to age, genetic background or other factors have been reported to lead to neurodegeneration. Glial cell-derived neurotrophic factor (GDNF) gene expression is reduced in the cerebral cortex of aged mice [1] and in the hippocampus of the murine model of accelerated aging SAMP8 [2]. Analysis of GDNF in human brain has demonstrated a decrease in substantia nigra in PD [3], but its regulation in Alzheimer's disease (AD) brain is poorly documented. However, GDNF decreases in plasma of mild cognitive impairment and AD patients were found to be a precursor to progression into AD [4]. Furthermore, the expression patterns of novel human GDNF isoforms were found deregulated in AD brains [5].

Neurotrophic factors have been assayed in aged rodents and primates in order to search for effective and harmless treatments so as to reverse mild memory loss of normal aging and also mitigate memory loss in pathological aging. As regards to GDNF, we previously showed that lentiviral-induced GDNF overexpression in hippocampal astrocytes improved the cognitive deficits of aged rats [6]. The role of GDNF against AD pathogenesis has not been previously analyzed in any AD transgenic mouse model. However, two previous studies show encouraging protective effects of GDNF against A $\beta$ -induced neuronal cell death in rabbit hippocampus [7] and in cultured septal neurons [8]. There is also suggestive evidence that several pharmacological agents of proposed use against AD [9] may partially work by way of GDNF enhancement in the brain [10]. In the case of the clinical drugs amantadine and memantine, brain astrocytes are proposed as operating as the mediators of amantadine and memantine-elicited neurotrophic effects through an increased production of GDNF by these glial cells [11,12].

GDNF and its receptor complex GFR $\alpha$ -1 are expressed in both neurons and astrocytes [13], although GDNF is mainly produced in neurons in the normal mature brain [14]. The ability of activated astrocytes of up-regulating GDNF production after brain injury is believed to play an active role in neuron survival and plasticity [14]. In fact, activated astrocytes have been observed to up-regulate neurotrophic factors, antioxidants and other key molecules, all of which support neurons and oligodendrocytes survival as well as tissue repair [15]. This cross-talk between neurons and astrocytes after brain injury, provides an avenue for new therapeutic approaches targeting these glial cells in age-related neurodegeneration and AD.

In the present study we investigated the neuroprotective effects of GDNF against AD-related neuropathology by using a gene therapy approach in the 3xTg-AD mouse model [16]. This transgenic mouse model presents progressive AD-like pathology, including A $\beta$  deposits and hyperphosphorylated tau (p-tau), oxidative stress, disorders of brain physiology and behavior, and cognitive loss [16,17]. As viral vectors can be engineered to selectively transduce astrocyte cells, we addressed the feasibility of potentiating the neuroprotective role of these glial

cells in the mouse brain. Furthermore, we analyzed the neuroprotection afforded by lenti-GDNF transduction against cell damage induced by conditioned overexpression of the 99-residue carboxyl-terminal (C99) fragment of the amyloid precursor protein (APP) in the human neuroblastoma MC65 [18]. MC65 is an established AD neuronal model, where cell death is induced by intracellular accumulation of C99 and its derived cleavage fragments such as A $\beta$  peptide; with a prominent cytotoxic role of oxidative stress [18,19].

## Methods

### *Animals and treatment groups*

Male 3xTg-AD mice and non-transgenic (NTg) mice with the same genetic background were used for this study. The 3xTg-AD mouse strain harboring human transgenes for presenilin-1 (PS1) with M146V mutation, APP with Swedish mutation and tau with P301L mutation, was genetically engineered at the University of California Irvine [16]. Animals were maintained in Macrolon cages under standard laboratory conditions of food and water ad libitum, 22 $\pm$ 2°C and 12 h light:dark. Genotypes were confirmed by PCR analysis of DNA obtained from tail biopsies.

At four months of age, 3xTg-AD and NTg mice were assigned to either GDNF treatment by gene overexpression in the hippocampus or control group which expressed green fluorescent protein (GFP) gene. The treatment groups (n = 8-9) were thus: NTg-GFP, NTg-GDNF, Tg-GFP and Tg-GDNF. Animals were individually housed in cages with filters, fulfilling level 2 safety requirements. The study was terminated at ten months of age, following a six month period of gene overexpression.

Animal handling and procedures were approved by the local animal ethics committee (Ref: DAAM 4664, CEEA, UB), in accordance with Spanish legislation and the EU Directive 2010/63/EU for animal experiments.

### *Lentiviral vectors*

Recombinant lentiviral vectors encoding human GDNF were constructed for the specific transduction of mouse astrocytes *in vivo* or MC65 cells *in vitro*. The combination of the lyssavirus Mokola glycoprotein (Mokola-G) pseudotype with the human cytomegalovirus promoter (CMV) allowed efficient transgene expression in astrocytes after intrahippocampal injection, as previously reported [6]. Furthermore, the viral particles used for transduction of neuroblastoma MC65 cells were pseudotyped with the vesicular stomatitis virus G glycoprotein (VSV-G). Vectors encoding GFP instead of GDNF were used as control vectors. We used the plasmids Flap-CMV-GDNF-WPRE and Flap-CMV-GFP-WPRE. For details of obtaining and handling the viral stocks see [6]. The viral titers were obtained using a real-time quantitative PCR (qPCR)-based method previously described [20].

Levels of GDNF in the hippocampus of an additional group of mice injected with lenti-GDNF and in the culture medium of transduced MC65 cells were determined by ELISA with the GDNF Emax ImmunoAssay System kit (Promega, Madison, WI), following the manufacturer's instructions.

#### *Surgical procedures and sacrifice*

Mice were anesthetized with 10 mg/kg xylazine (Rompun 2%, Bayer, Leverkusen, Germany) i.p. and 80 mg/kg ketamine (Ketolar 50 mg/ml, Pfizer, Alcobendas, Madrid, Spain) i.p., and placed in a stereotactic apparatus. Bilateral infusions of lenti-GDNF and lenti-GFP were performed into the CA1 area of the mouse hippocampus by stereotactic procedures. Injections of lentiviral vectors were performed at a rate of 1  $\mu$ l/min and at coordinates relative to Bregma of -2.0 mm A/P,  $\pm$ 1.2 mm M/L, +2 mm V/D. Coordinates were selected in preliminary studies with fast green colorant injection (not shown). One microliter per side of the GDNF vector solution, containing  $8.81 \times 10^9$  vector genomes/ml, was delivered to the application point with a 25-gauge stainless steel cannula (Small Parts Inc., Miami, FL) connected to a Hamilton syringe through a Teflon tube. The syringe was attached to a micro-infusion pump (Bioanalytical systems Inc., West Lafayette, IN). The cannula was left in position for 5 min after delivery to prevent the solution from surging back. The incision was sutured, and the mice were allowed to recover from anesthesia on a thermal pad and placed back into their cages. Control animals transduced with lenti-GFP received 1  $\mu$ l injections of  $6.28 \times 10^9$  vector genomes/ml solution.

At the end of the study, animals were sacrificed by decapitation and the brains were removed and processed as described below.

#### *Behavioral testing*

Testing of cognitive behavior was performed with the Morris water maze test (MWM), as previously described [17]. MWM was used to determine acquisition and retention of a hippocampus-related spatial task.

Additional tests to check for changes in general behavior were performed with the corner test and the open field test, in which the former is used to measure neophobia to a new home cage, and the latter to measure exploration, emotionality and locomotor behavior [17].

#### *MC65 cell line*

The stably transformed MC65 cell line is derived from the human neuroblastoma SK-N-MC and conditionally expresses a partial APP fusion protein including the C99 fragment [18]. Once the promoter is activated by the absence of tetracycline (TC), the C99 and derived fragments accumulate intracellularly leading to A $\beta$ -related cytotoxicity [18,19,21 22].

MC65 cells were routinely cultured in DMEM supplemented with 10% FBS (Gibco-BRL, Carlsbad, CA) (DMEM10) and 2  $\mu$ l g/ml TC (Sigma, St Louis, MO) (TC+). For activation, cells were seeded at a density of  $1.2 - 1.5 \times 10^5$  cells/cm<sup>2</sup> in 24 well plates, without TC (TC-). After 2 - 3 days the media was changed to OptiMEM (Gibco-BRL) TC-. Control cells were maintained in TC+ media. For GDNF transduction, 0.75  $\mu$ l of the vector solution containing  $8.9 \times 10^9$  vector genomes/ml were added to each culture well 24 h before inducing C99 expression. Well cultures transduced with GFP received  $5.55 \times 10^9$  vector genomes/ml of lenti-GFP. Upon infection, culture wells contained 300  $\mu$ l DMEM10. All experiments were terminated after 24 h of MC65 cell activation (i.e. after 48 h of viral infection). Results were obtained from 4-6 independent experiments performed in duplicate wells. Transduction efficiency was assessed by analyzing the number of cells that showed positive for GFP fluorescence after fixation (see below) and by staining all cell nuclei with Hoechst bisbenzimidazole (Sigma). For each experiment, more than 200 cells were counted in each of two microphotographs, using ImageJ software (<http://rsb.info.nih.gov/ij/>).

#### *Cytotoxicity and oxidative stress tests*

Cell death in MC65 cell cultures was measured by the spectrophotometric assay of lactate dehydrogenase (LDH) leakage following standard procedures.

Intracellular reactive oxygen species (ROS) production of MC65 cells was determined using 2',7'-dichlorofluorescein diacetate (DCFH-DA) (Molecular Probes, Leiden, The Netherlands). Cultures were loaded with 10  $\mu$ M of DCFH-DA and maintained in the cell incubator for 24 h. DCFH oxidation yielded the fluorescent molecule 2',7'-dichlorofluorescein (DCF). For a direct measure of intracellular DCF, cells were washed with PBS and lysed in 10 mM Tris-HCl with 0.5% Tween-20. The homogenates were centrifuged at 10,000 x g for 10 min and the supernatants measured for DCF fluorescence in a plate reader (Spectramax Gemini XS, Molecular Devices, Wokingham, UK) at 485 nm excitation/530 nm emission. Results were expressed as the percentage of DCF compared to control cultures wells.

#### *Immunofluorescence*

At termination, the brain of all mice was bisected sagittally at the midline and a hemibrain of each mouse was processed for immunofluorescence. Tissue was fixed by immersion in 4% paraformaldehyde solution for 24 h, followed by an additional 24 h period in fresh fixative solution. It was then cryopreserved in successive 10%, 20% and 30% sucrose solutions and frozen on dry ice. Sagittal slices of 25  $\mu$ m were stained with the following primary antibodies: anti-GFP 1:100 from Abcam, (Cambridge, UK); anti-GFAP, clone GA5, 1:400, from Sigma; anti-GDNF 1:100 from Santa Cruz Biotechnology (Santa Cruz, CA); anti-

A $\beta$ , clone 6E10, 1:100, from Covance (Emerville, CA); anti-p-tau, clone AT8 specific for double phosphorylated tau at Ser202/Thr205 (p-tau), 1:50, from Pierce (Rockford, IL); anti-NeuN (1:100) from Millipore (Bedford, MA); and anti-phospho-Ca<sup>2+</sup> calmodulin-dependent protein kinase II,  $\alpha$  subunit, phosphorylated at Thr286 (p-CaMKII), 1:100, from Abcam. Secondary antibodies used were: Alexa Fluor 488 1:1000 and Alexa Fluor 546 1:1000 (Molecular Probes). The fluorescence intensity of the CA1 pyramidal neurons after staining with anti-p-CaMKII was measured by image analysis using ImageJ software. In brief, fluorescence intensities of histological slide images obtained in a Leica TCS SP2 confocal microscope were transformed to grayscale intensities, the background was subtracted, and the average values of the integrated optic densities of body neurons in each slide were registered.

MC65 cells grown onto glass coverslips were processed for immunofluorescence using anti-APP C-terminal fragment raised against residues 676-695 of APP695 (APP-CTF) 1:100, from Covance. Cell nuclei were counterstained with Hoechst bisbenzimidazole.

#### *Western immunoblotting*

Hippocampi were quickly dissected from the non-fixed hemibrain of each mouse and snap frozen in liquid nitrogen. Frozen tissues were crushed into a fine powder under liquid nitrogen and aliquoted for subsequent Western blotting and qPCR analyses.

Freshly collected MC65 cells and frozen tissue powder were homogenized in ice-cold RIPA buffer supplemented with protease and phosphatase inhibitors and processed for Western blot analysis by standard procedures. The following primary antibodies were used: anti-A $\beta$  1:1000, clone 6E10, and anti-APP-CTF 1:1000 from Covance; anti-p-tau 1:1000, clone AT8, from Pierce; and anti-actin (20-33, pan-actin) 1:10,000 from Sigma. Secondary antibody was a horseradish peroxidase-conjugated antibody (BD Amersham, Arlington Heights, IL). Immunoreactive bands were detected with a chemiluminescence reaction and digitized. Densitometric results were normalized to actin.

#### *Quantitative real-time PCR*

Total RNA was isolated from cells with TRIzol reagent (Life Technologies, Paisley, UK), and from mouse tissue using *mirVana*<sup>TM</sup> RNA Isolation Kit (Applied Biosystems Foster City, CA) following the manufacturer's instructions. RNA samples were analyzed using a NanoDrop spectrophotometer (ND1000, Fisher Scientific, Waltham, MA) and stored at -80 °C until assay. Random-primed cDNA synthesis was performed using the High-Capacity cDNA Archive kit (Applied Biosystems). Gene expression was measured in an ABI Prism 7900HT qPCR system using TaqMan FAM labeled specific probes (Applied Biosystems). Gene expression of GDNF, disintegrin and metalloproteinase domain-containing protein 10 (ADAM10), beclin 1 and neuritin was analyzed in MC65 cells, and that of GDNF, brain derived

neurotrophic factor (BDNF), tyrosine receptor kinase B (TrkB) and NAD-dependent deacetylase sirtuin-1 (SIRT1) was analyzed in mouse hippocampus. Data were normalized to TATA-binding protein (Tbp) gene expression.

### *Statistics*

Results are expressed as mean  $\pm$  SEM. Statistical analyses were performed using GraphPad Prism v4.02 (La Jolla, San Diego, CA). Repeated measures ANOVA was used for the analysis of the acquisition curves in the MWM. Column statistics was used in the probe trial of the MWM. Results of the p-CaMKII immunostaining were analyzed with one-way ANOVA followed by Newman-Keuls multiple comparison test. C99 Western blots immunolabelled with anti-APP-CTF antibody were analyzed with unpaired Student's *t*-test. All other data were analyzed by two-way ANOVA, whilst Bonferroni's post-hoc test was used for comparison of means.

## **Results**

### *Expression of GFP and GDNF in 3xTg-AD mice*

The injection of lenti-GFP and lenti-GDNF particles into the dorsal CA1 hippocampus yielded a selective expression of transduced proteins in astrocytes, as demonstrated by the overlapping of GFAP immunostaining with either GFP or GDNF immunostaining (Fig. 1A and B, respectively). Protein expression was restricted to the injected area (Supplementary Fig. S1). Mean levels of GDNF determined by ELISA of the whole hippocampus of an additional group of 3xTg-AD sacrificed one week after the injection were  $0.7 \pm 0.04$  pg/mg tissue in GFP mice and  $18.5 \pm 2.02$  pg/mg tissue in GDNF mice ( $n=4$ ). Similar levels of GDNF attained in the striatum through engineered macrophage resulted neuroprotective in a mouse model of Parkinson's disease [23].

### *Beneficial effects of GDNF on cognitive behavior of 3xTg-AD mice*

In the MWM, control 3xTg-AD mice (treated with lenti-GFP) showed severe cognitive loss as expected for this mouse strain [17]. Interestingly, the treatment with lenti-GDNF led to an improvement in both: place task acquisition (Fig. 1C) and retention of learning (Fig. 1D). In the acquisition of learning, repeated measures ANOVA showed significant differences in the daily escape latencies between groups ( $p<0.05$ ), and Bonferroni test showed differences between control 3xTg-AD mice and both control NTg mice and 3xTg-AD treated with lenti-GDNF ( $p<0.01$ ). In the probe trial, column statistics by one sample *t* test showed that all NTg mice and 3xTg-AD treated with lenti-GDNF spent more time than expected at random in the platform quadrant ( $p<0.05$ ), but not 3xTg-AD treated with lenti-GFP. No differences in average



swimming speed were detected between groups (not shown). Namely, GDNF overexpression enhanced spatial learning and memory in 3xTg-AD mice to a level similar to that of NTg mice.

As regards to non-cognitive behavior, GDNF overexpression in the hippocampus did not induce significant changes in the 3xTgAD phenotype of behavioral and psychological symptoms of dementia (BPSD)-like [17]. Selected results are shown in Supplementary Fig. S2. As BPSDs are mainly driven by AD pathology in the cortical and basal ganglia areas, these results showed a lack of GDNF effects beyond the hippocampus target area and also refuted the idea that GDNF could generate unwanted effects on general behavior.

#### *Neuroprotective changes of GDNF in the hippocampus tissue*

A significant A $\beta$  load in the hippocampus of 3xTg-AD mice was demonstrated in immunostained pyramidal neurons (Fig. 2A) and in Western blots of homogenized tissue (Fig. 2B). Treatment with GDNF barely decreased A $\beta$  deposits. With respect to tau pathology, presence of p-tau in hippocampus pyramidal neurons (Fig. 2C) and in homogenized tissue (Fig. 2D) was partially reduced after GDNF overexpression. However, ANOVA showed only a significance of mouse genotype ( $p < 0.01$ ) and no significant effect of GDNF treatment in both pathologies.

Analysis of p-CaMKII immunostaining showed reduced levels of this signaling enzyme in the CA1 pyramidal neurons of the 3xTg-AD mice when compared with NTg mice, whereas an upregulation to normal physiological levels was observed in 3xTg-AD mice treated with lenti-GDNF (Fig. 2E and F) (ANOVA,  $p < 0.05$ ).

The RNA quantification showed a significant increase of GDNF gene expression in the lenti-GDNF transduced mouse hippocampi that was paralleled by an increase of BDNF (Fig. 3A and B, respectively). The expression of the BDNF receptor TrkB was decreased in these mice following chronic upregulation of BDNF (Fig. 3C). Gene expression of the histone deacetylase involved in longevity and neuroprotection SIRT1 was tested for a possible enhancement but, on the contrary, GDNF was found to actually induce a decrease of this deacetylase after chronic overexpression (Fig. 3D). ANOVA showed a significance of GDNF treatment ( $p < 0.01$  for GDNF and SIRT1;  $p < 0.001$  for BDNF and TrkB) and no effect of mouse genotype in all analyzed genes.

#### *Cytoprotective effects induced by GDNF in MC65 cells*

The infection of MC65 cultures with lenti-GFP viral particles demonstrated high efficiency of transduction with  $93 \pm 2\%$  ( $n=6$ ) of cells showing GFP fluorescence (Fig. 4A). Incubation of MC65 with lenti-GDNF induced an elevated secretion of GDNF to the culture media measured by ELISA of  $1865 \pm 57$  pg/ml as compared to that of control cultures of  $36 \pm 4$  pg/ml ( $n=6$ ). Gene expression of GDNF in transduced cells is shown in Fig. 4B (ANOVA,

effect of GDNF treatment  $p < 0.001$ ). Immunostaining with anti-APP-CTF antibody showed an increase in C99 expression after MC65 activation and a tendency toward decreased immunoreactivity after GDNF transduction (Fig. 4C).

Western blot analysis of MC65 cells showed activation of the expression of C99 regardless of the culture medium (DMEM10/TC- or OptiMEM/TC-), with the two antibodies used (anti-APP-CTF and anti-A $\beta$  antibodies; see representative membrane images in Fig. 5A and B, respectively). Additional increases of A $\beta$  aggregates, detected with anti-A $\beta$ -specific antibody, were only found in cells activated in OptiMEM/TC- (Fig 5B). A non-specific 37-kDa band was detected with anti-A $\beta$  antibody. Therefore, we analyzed the effect of GDNF transduction on a major band corresponding to C99, two upper bands of A $\beta$  oligomers with approximate molecular weights of 18 kDa and 25 kDa, and a lower band probably corresponding to an aggregate of A $\beta$  dimers and cleaved CTFs (A $\beta$ /CTF) previously described [24,25]. Cells transduced with GDNF showed a significant decrease of C99 immunolabelled with anti-APP-CTF antibody (Fig. 5A, Student's *t*-test), whereas the results with anti-A $\beta$  antibody showed higher variability and absence of statistical significance for C99. Furthermore, GDNF transduction induced a decrease of A $\beta$  oligomers and A $\beta$ /CTF (Fig. 5B). ANOVA indicated an effect of the factor GDNF treatment on the levels of A $\beta$  oligomers ( $p < 0.001$ ) and CTFs ( $p < 0.001$ ), and no effect of the factor APP fragment species (Fig.5B).

The activation of the neuroblastoma MC65 cells to generate the C99 induced an increase of hydroperoxide and cell death, as previously described [19]. GDNF transduction suppressed both oxidative stress and cytotoxicity (Fig. 6A and B, respectively). ANOVA indicated an effect of GDNF treatment factor in both DCF and LDH results ( $p < 0.05$ ), and of cell activation factor ( $p < 0.001$ ) and interaction between both factors ( $p < 0.001$ ) in LDH.

Gene expression of several genes tested to further explore GDNF mechanisms did not give significant changes. Namely, the relevant  $\alpha$ -secretase ADAM10, the autophagy regulator beclin 1, and neuritin which is involved in neurite outgrowth (not shown).

## Discussion

Gene expression of GDNF mediated by lentiviral vectors was able to induce neuroprotective effects in both, *in vivo* and *in vitro* experimental models of AD. *In vivo*, the chronic overexpression of GDNF in astrocytes from the dorsal CA1 hippocampus protected against cognitive loss in 3xTg-AD mice, as demonstrated by their good performance in the MWM. Spatial cognitive abilities require the dorsal hippocampal function to be intact, with the target CA1 area being particularly crucial for spatial learning [26] and memory [27]. The sustained secretion of GDNF by transduced astrocytes exerted neuroprotective effects on the hippocampal neurons. *In vitro*, GDNF over-expression induced a potent cytoprotective action in

MC65 neuroblastoma. There was a recovery from cell death induced by the intracellular generation of C99 in this AD cell model.

The brain neuroprotection driven by lenti-GDNF transduced astrocytes confirmed previous results obtained in cognitively deficient aged rats [6]. It also supports the mediation of GDNF secreted by astrocytes in the neuroprotection afforded by several anti-AD drugs [10,11]. There was a small and non-significant reduction of amyloid and tau pathologies *in vivo*, whereas activation of BDNF gene expression was substantial. BDNF has already been demonstrated to induce a broad neuroprotection in several models of AD, including reversion of synapse loss and restoration of learning and memory in APP-Tg mice and prevention of death induced by A $\beta$  in primary neuron cultures [28]. In APP-Tg mice, BDNF had no effect on amyloid plaque load and its neuroprotection was reported as mediated through amyloid-independent mechanisms [28]. The decreased levels of BDNF reported in the hippocampus and cortex of AD patients are suggested to contribute to the atrophy and cognitive dysfunctions [29]. Similarly, a reduction of p-CaMKII in the hippocampus and cortex of AD patients is believed to contribute to memory impairment [30]. Activation of the  $\alpha$  subunit of CaMKII by autophosphorylation at Thr286 is essential for the induction of long-term potentiation (LTP) and for consolidating learning through synaptic plasticity changes [31]. Furthermore, cognitive deficiencies in APP/PS1-Tg mice [32], in SAMP8 mice [33] and in diabetic rats [34] have been associated with p-CaMKII reduction. Therefore, recovery of p-CaMKII levels after GDNF treatment may contribute to reversing the synaptic plasticity impairment of pyramidal neurons in the hippocampus of 3xTg-AD mice. Upregulation of BDNF by GDNF could be at least partially mediated through the signaling pathway CaMKII – cyclic AMP-response element binding protein cAMP (CREB) – BDNF, in which p-CaMKII phosphorylates CREB which, in turn, binds to the promoter of BDNF. A mediator of GDNF-induced BDNF expression, as recently described in nigrostriatal dopaminergic neurons, is the transcription factor pituitary homeobox 3 (Pitx3) [35]. Both, GDNF and BDNF are increased in the hippocampus of mice by a known plasticity enhancer therapy such as physical exercise [36-38], although the sequence of gene activation has not been analyzed. Furthermore, similar enhancement of both neurotrophic factors have been described in astrocyte cultures treated with the protective polyphenol resveratrol [39]. In addition to the neuroprotection attained through BDNF upregulation, GDNF itself is a potent neurotrophic factor. Therefore, both factors could have worked in concert to improve synaptic plasticity and hippocampal circuitry in the 3xTg-AD injected with lenti-GDNF. Neurotrophic effects of GDNF secreted by astrocytes enhanced neurotransmitter functionality, as previously demonstrated by local increases in the synthesis of the neurotransmitters acetylcholine, dopamine and serotonin in aged rats similarly transduced with lenti-GDNF [6]. An *in vitro* study with septal cholinergic neurons has shown that GDNF increases the expression of choline acetyl transferase, in addition to protecting against the cytotoxicity induced by oligomeric A $\beta$ 42

[8]. GDNF is also a potent inductor of cell growth pathways and progenitor cell differentiation throughout brain embryonic development and adulthood. GDNF-induced neurogenesis has been described as being present in the dentate gyrus of adult rats [40], whereas *in vitro* studies have shown GDNF facilitation of astroglialogenesis from hippocampal neural progenitors [41]. Although hippocampal neural progenitors are based in the gyrus dentate zone, we cannot rule out the contribution of GDNF overexpression in dorsal CA1 to the recovery of the impaired adult neurogenesis, as reported in the 3xTg-AD mice [42]. Therefore, we can speculate that GDNF-mediated hippocampal neurogenesis and/or astroglialogenesis contributed to the rescue of the cognitive impairment in these mice.

GDNF protected from the oxidative stress induced by C99-derived fragment in activated MC65 cells. Similarly, it has been reported that GDNF neuroprotection involves antioxidant mechanisms in both *in vivo* and *in vitro* models of PD [43-46] and of other pathologies [47,48]. GDNF has been demonstrated to induce the activity of the antioxidant enzymes glutathione peroxidase, superoxide dismutase and catalase in brain tissue [49] and neuronal cultures [46,50]. In this sense, the anti-oxidant action of GDNF may be a significant mechanism of neuroprotection also against AD. The presence of oxidative damage is well documented in the AD brain and oxidative stress is considered an early event that plays an important role in the pathogenesis of the disease [51-53]. The basis of this oxidative damage appears to derive from mitochondrial dysfunction, overaccumulation of iron, defective proteolysis and other interacting mechanisms that generate ROS in vulnerable neuronal populations, such as those of the hippocampus CA1 region [54-56]. Previous studies had reported that A $\beta$  induces ROS generation in neurons [57], whereas oxidative stress, in turn, activates the production of pathological levels of A $\beta$  through a positive feedback relationship between  $\gamma$ - and  $\beta$ -secretase activities [58]. Furthermore, brain tissue and cultured neurons from the 3xTg-AD mouse model have showed early ROS damage and disturbances in the antioxidant defense [17,59]. Also, C99-induced cell death in the MC65 cell model of AD has been demonstrated to involve oxidative stress pathways [19]. It is known that CTFs appear in neuritic plaques [60] and accumulate intracellularly in patients with hereditary AD [61]. Furthermore, C99 and derived fragments have proven to be highly neurotoxic in several experimental systems *in vivo* and *in vitro*, through mechanisms that include generation of ROS [62]. Regarding tau pathology, several oxidative stress-induced mechanisms have been demonstrated to exacerbate hyperphosphorylation of tau [63,64]. The neuroprotection conferred to both AD models by overexpressing GDNF in this study is at least partially mediated by antioxidant mechanisms, as demonstrated by the fact that the inhibition of ROS generation in MC65 cultures was paralleled by the recovery of neuronal survival. An enhanced functionality of the proteasome system in the degradation and processing of toxic proteins could also be involved in the GDNF neuroprotection, as suggested by the decrease of overexpressed CTFs in MC65 cells.

Multiple pathways are involved in GDNF signaling and survival pathways [6,7,65-68], in some of which, there is an evident neuroprotective role of astrocytes. For instance, inflammatory mediators have been shown to induce the release of GDNF from astrocytes, leading to neuron survival [69] and recovery from cognitive impairment [70]. Furthermore, it has been reported that GDNF induces an upregulation of the astrocyte glutamate transporter GLAST-1 against cell death mediated through excitotoxic processes [71]. There was also an upregulation of GDNF secretion by astrocytes after ischemic brain injury [48]. Furthermore it has been proposed that injured dopaminergic neurons signal astrocytes trigger GDNF upregulation [72]. Therefore, in addition to GDNF direct survival effects as seen in MC65 cells, this neurotrophic factor appears to be involved in a neuron-astrocyte crosstalk to generate astrocyte-mediated neuroprotection. This work further demonstrates the feasibility of using astrocytes as engineered minipumps that stably over secrete GDNF at neuroprotective doses [6,73,74].

## **Conclusion**

GDNF demonstrated to be neuroprotective against AD through astrocyte transduction with a lentiviral vector in hippocampus of 3xTg-AD mice, with a preservation of spatial learning and memory. The most significant molecular change was an upregulation of BDNF. Overexpression of GDNF by lentivirus transduction of neuroblastoma MC65 cells protected against oxidative stress and cell death mediated by C99. Engineered lenti-GDNF showed high effectiveness and safety, which might contribute to the advancement of gene-therapy-based approaches in AD experimental and clinical research for this promising neurotrophic agent.

## **Acknowledgments**

MC65 cells were kindly given by Bryce Sopher and George M Martin (University of Washington, Seattle, WA). This study was supported by grants: SAF2009-13093, SAF2012-39852 and CSD2010-45 from the Spanish *MINECO*; 2009/SGR/214 from the *Generalitat* and 062931 from the *Fundació La Marató de TV3*, of Catalonia; and the European Regional Development Fund (ERDF).

## **Disclosure**

The authors declare that there are no actual or potential conflicts of interest.

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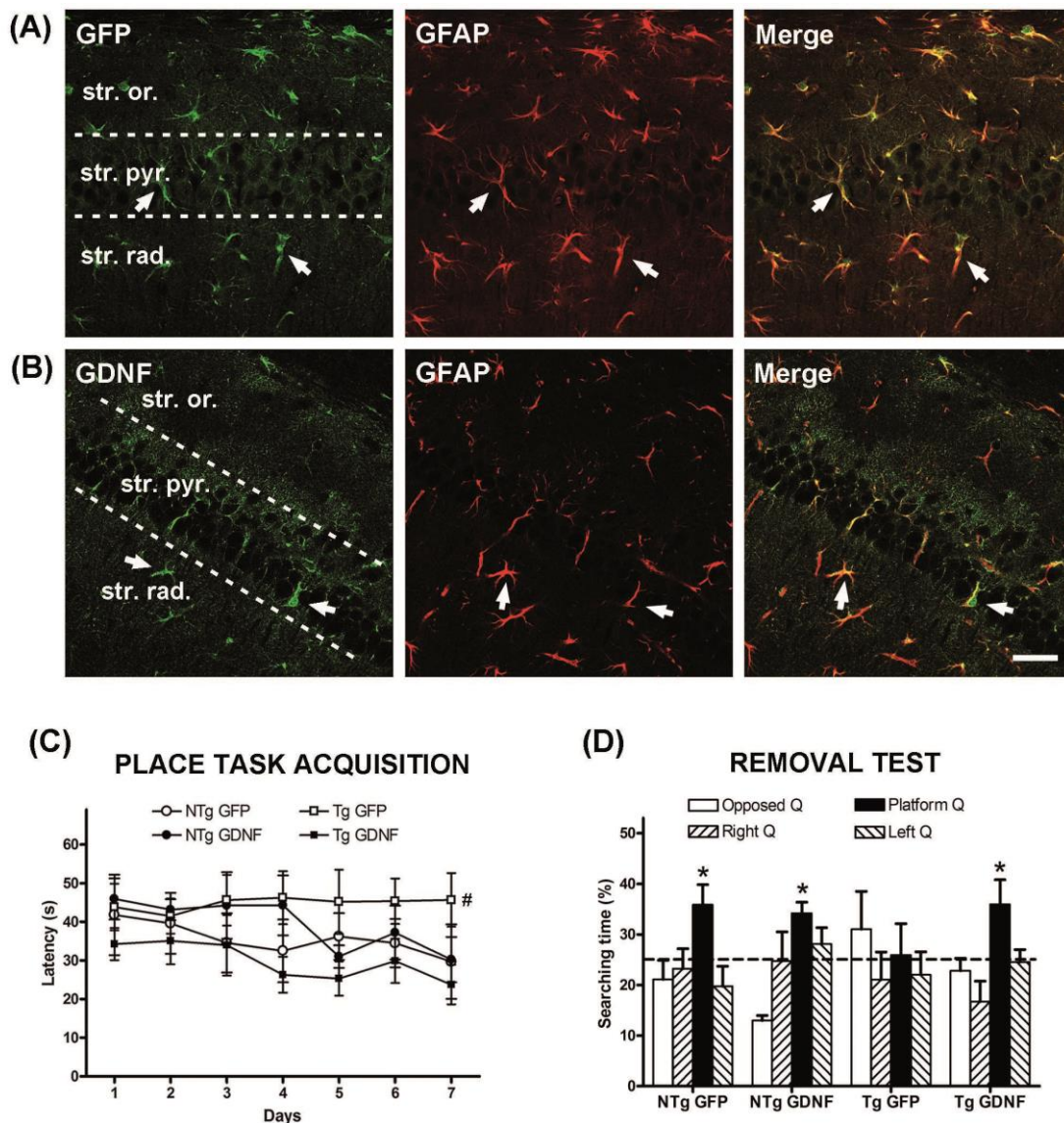
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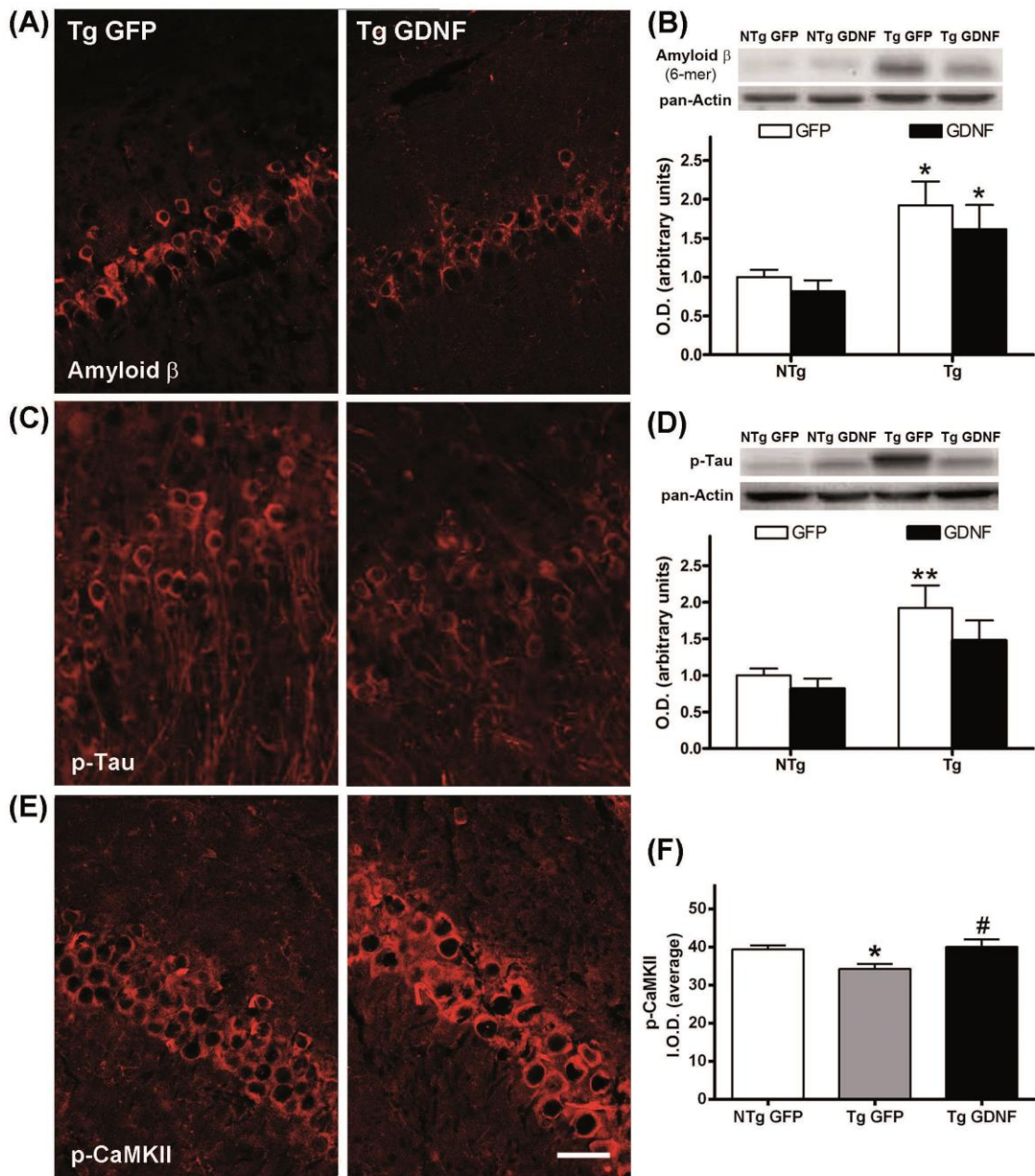
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## Figures



**Figure 1** GDNF gene therapy and its effects on spatial learning and memory in the 3xTgAD mice. (A-B) Transgene expression of GFP and GDNF driven by lentiviral vectors stereotactically injected in the hippocampus. Both, GFP (A) and GDNF expression (B) in transduced astrocytes of the CA1 hippocampal area, was confirmed by co-localization with the astrocyte marker GFAP in the merge images for GFP plus GFAP and GDNF plus GFAP, respectively. Representative confocal images of double immunohistochemistry. Abbreviations: str. or., stratum oriens; str. pyr., stratum pyramidale; str. rad., stratum radiatum. Arrows indicate the same astrocyte cells along each row. Scale bar = 50  $\mu$ m. (C-D) Behavioral testing in the Morris water maze of 3xTgAD mice (Tg) and non-transgenic mice (NTg) treated with GFP or GDNF. (C) Latency to reach the escape platform during the 6 days of training indicating lower

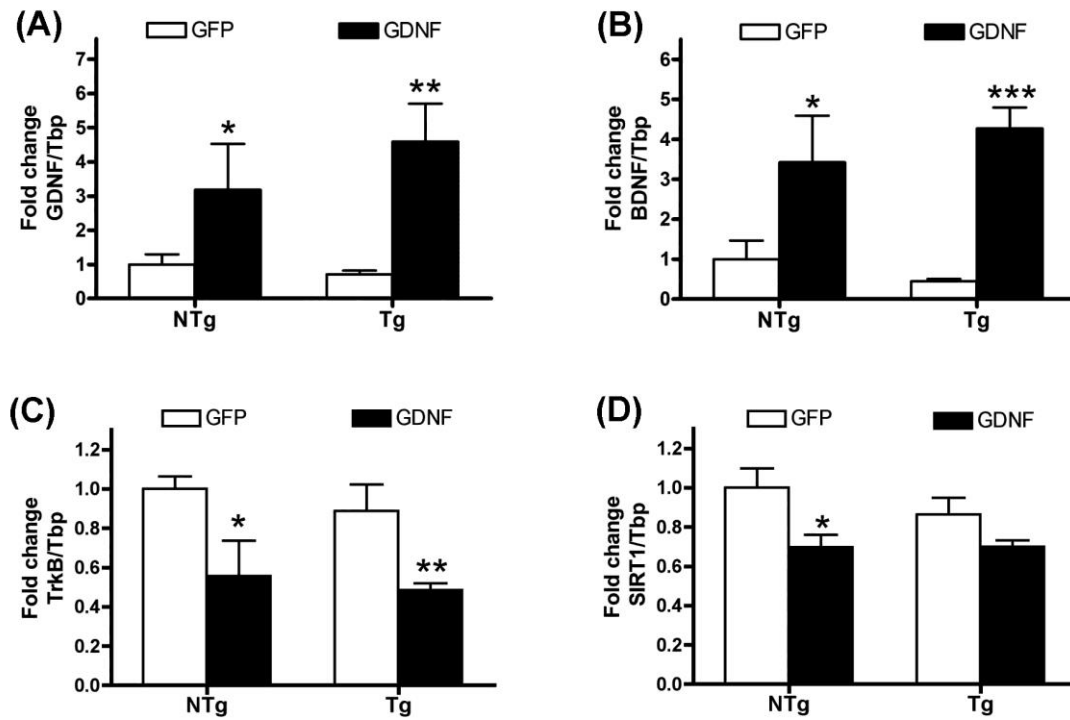
spatial learning in the Tg mice treated with GFP than in the other groups. (D) Time spent swimming in the platform quadrant of the pool (platform Q) when the platform was removed to test the retention of learning was different from chance in NTg mice and Tg mice treated with GDNF, but not in Tg-GFP mice. Dotted line indicates chance performance. Values are the mean  $\pm$  SEM, n = 8-9. Statistics: (C) #p<0.01 compared to the acquisition curve of NTg-GFP and Tg-GDNF; (D) \*p<0.05 compared to chance value; see text for details.



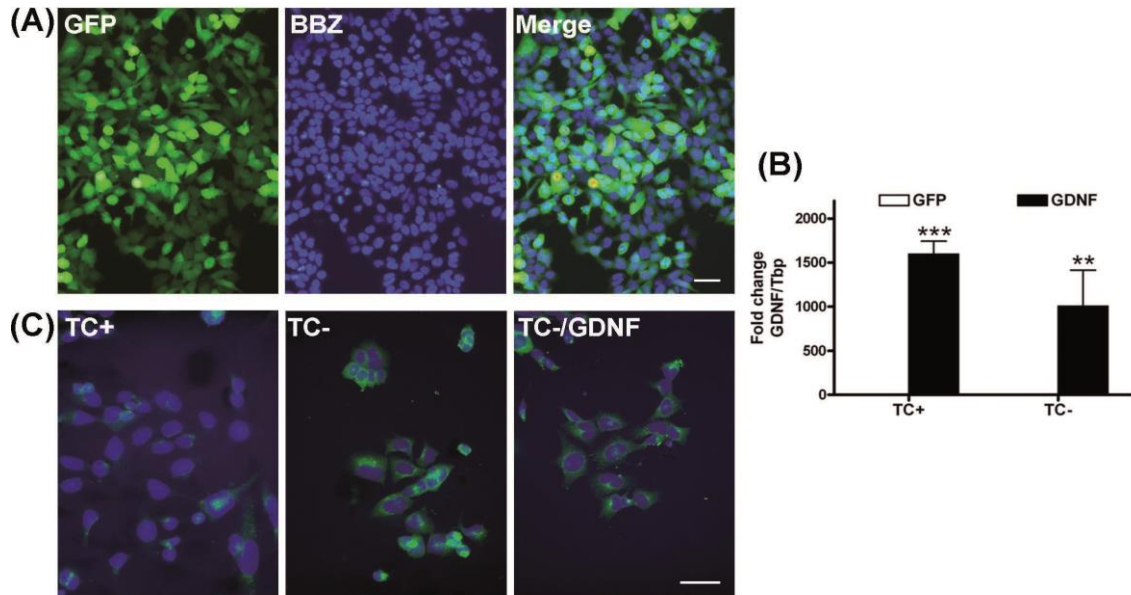
**Figure 2** Amyloid and tau pathology and synaptic plasticity status in the hippocampus of 3xTg-AD mice (Tg) treated with GFP or GDNF. (A) Representative images of immunostaining of intraneuronal amyloid  $\beta$  deposits in the CA1 pyramidal cell layer. (B) Levels of amyloid  $\beta$  oligomers shown by Western blot of the whole hippocampus, in Tg and non-transgenic mice (NTg). (C) Representative images of immunostaining of intraneuronal p-tau deposits in the CA1 pyramidal cell layer. (D) Levels of p-tau shown by Western blot of the whole hippocampus. (E) Representative images of immunostaining of p-CaMKII in the CA1 pyramidal neurons. (F) Intensity of p-CaMKII staining, see text for details. Abbreviations: O.D., optical density of Western blots in (B,D); I.O.D., integrated optical density of neurons in (F). Values are the mean

± SEM, n= 8-9. Statistics: \*p<0.05, \*\*p<0.01 compared to non-transgenic mice (NTg) with the same treatment; #p<0.05 compared to Tg GFP. Scale bar = 50 μm.

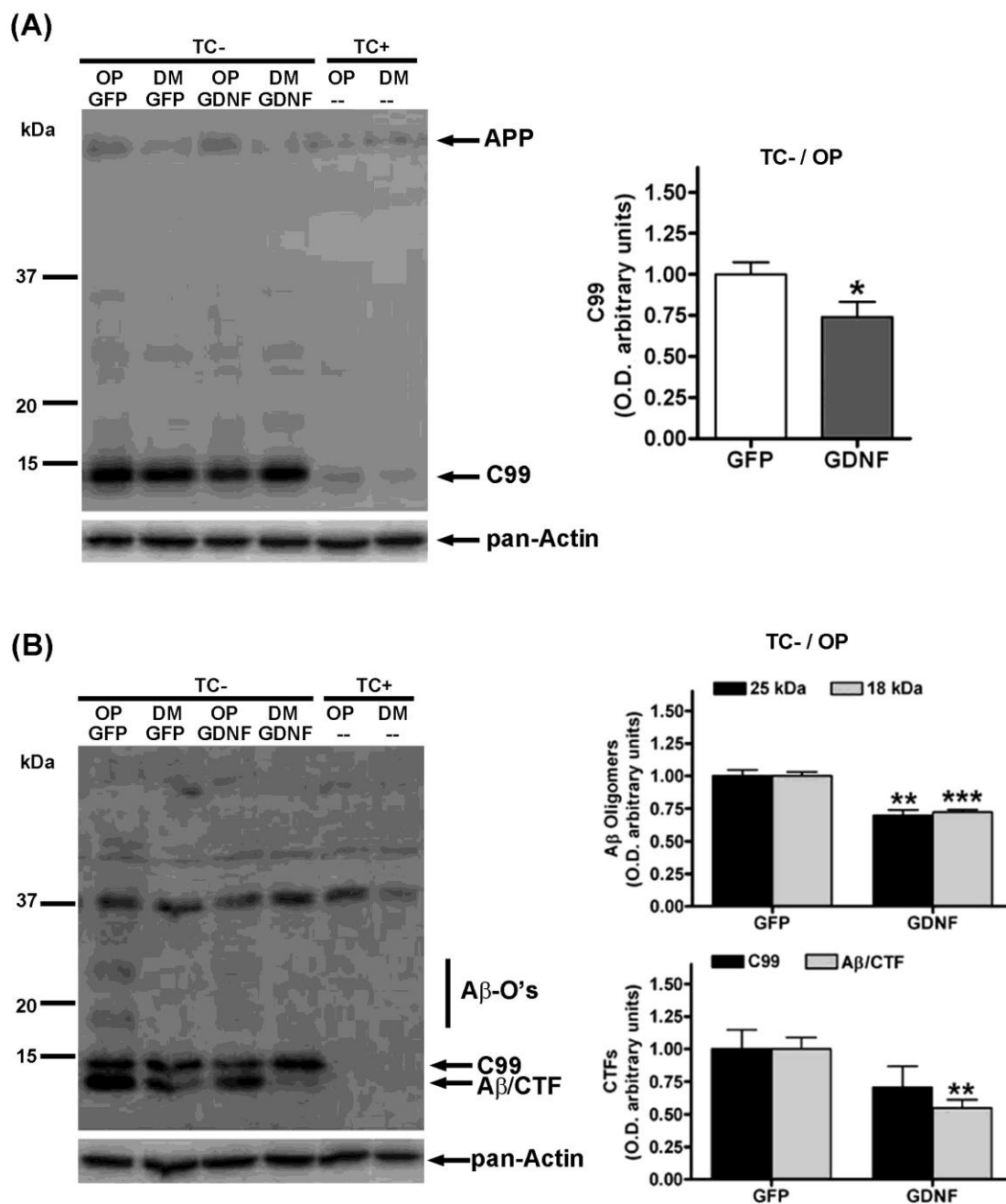




**Figure 3** Gene expression induced by GDNF treatment in the hippocampus of 3xTg-AD mice (Tg) and non-transgenic mice (NTg). RNA levels of GDNF (A), BDNF (B) TrkB (C) and SIRT1 (D) were obtained by quantitative real-time PCR. GDNF overexpression by lenti-GDNF induced a chronic increase of BDNF. Values are the mean  $\pm$  SEM, n=6-8. Statistics: \* $p$ <0.05, \*\* $p$ <0.01, and \*\*\* $p$ <0.001 compared to the same strain with a GFP treatment.

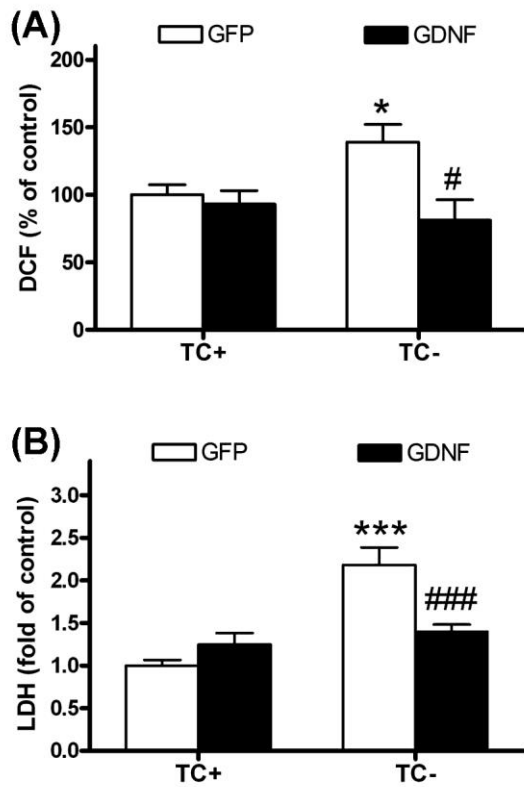


**Figure 4** MC65 neuroblastoma characterization after GFP and GDNF transduction. (A) GFP fluorescence after transduction with lenti-GFP is shown in most cells, as identified by nuclear counterstaining with Hoechst bisbenzimidazole (BBZ). (B) GDNF gene expression after transduction with lenti-GDNF, determined by quantitative PCR analysis. (C) Representative immunostaining images of MC65 cells with anti-APP C-terminal fragment antibody in non-activated cell cultures in presence of tetracycline (TC+), activated for C99 generation in the absence of tetracycline (TC-), and effect of lenti-GDNF reducing C99 accumulation (TC-/GDNF). Values are the mean  $\pm$  SEM,  $n = 4-6$ . Statistics: \*\* $p < 0.01$ , and \*\*\* $p < 0.001$  compared to GFP. Scale bars = A, 50  $\mu\text{m}$ ; B, 20  $\mu\text{m}$ .



**Figure 5** Western blot analyses of C99 and amyloid  $\beta$  levels in MC65 cells after GFP and GDNF transduction. Lysates from cells cultured in OptiMEM (OP), activated for C99 generation in the absence of tetracycline (TC-) and transduced with lenti-GFP or lenti-GDNF were analyzed with an anti-APP-C terminal antibody (A) and an anti-amyloid  $\beta$ -specific antibody (clone 6E10) (B). Lysates from cells cultured in growth medium DMEM10 (DM) in the absence of tetracycline (TC-) and also lysates from non-activated cells grown in the presence of tetracycline (TC+) were added as reference. The levels of amyloid  $\beta$  oligomers (A $\beta$ -O's) and aggregates of amyloid  $\beta$  dimers and C99 shorter fragments (A $\beta$ /CTF) were increased in OP/TC- medium, and decreased by GDNF as compared to GFP. GDNF also decreased C99

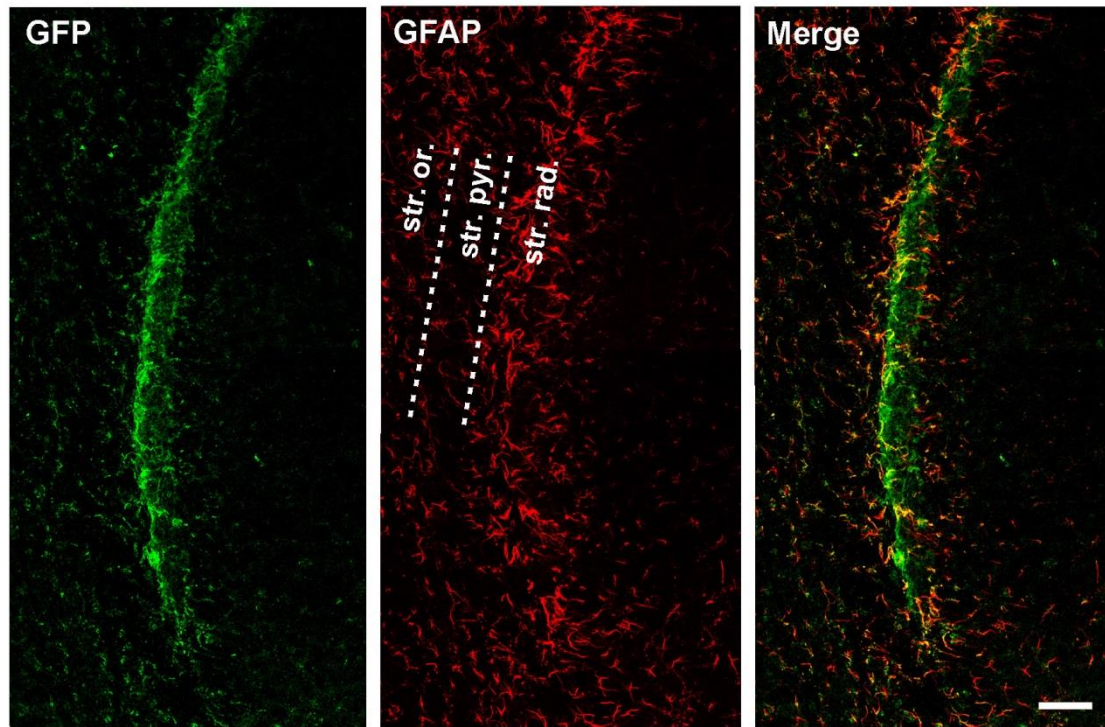
levels. Values are the mean  $\pm$  SEM, n = 4-6. Statistics: \*p<0.05 and \*\*\*p<0.001 compared to the GFP treatment in activated TC- cells.



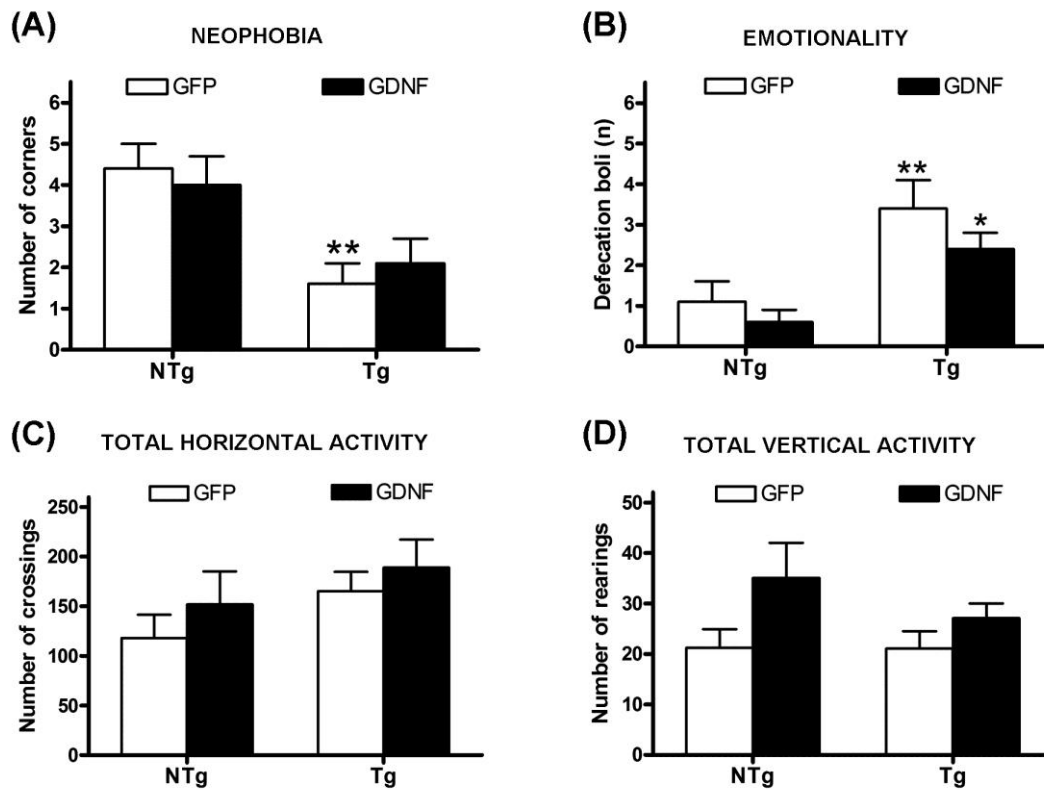
**Figure 6** Cytoprotective effects of GDNF in MC65 cells. (A) GDNF treatment protected from oxidative stress measured by the dichlorofluorescein (DCF) generation method. (B) Cell death measured by LDH leakage was protected by GDNF. Values are the mean  $\pm$  SEM,  $n = 4-6$ . Statistics: \* $p < 0.05$  and \*\*\* $p < 0.001$  compared to the non-activated state with the same treatment (control, TC+; activated for generation of C99, TC-); # $p < 0.05$  and ### $p < 0.001$  compared to the GFP treatment in activated TC- cells.

**Supplementary Material**

Figure S1, Figure S2



**Figure S1** Area of transgene expression of GFP driven by a lentiviral vector in the hippocampus of 3xTgAD mice. GFP in transduced astrocytes of the dorsal CA1 hippocampus was confirmed by co-localization with the astrocyte marker GFAP in the merge images for GFP plus GFAP. Representative confocal image of double immunohistochemistry. Abbreviations: str. or., stratum oriens; str. pyr., stratum pyramidale; str. rad., stratum radiatum. Scale bar = 100  $\mu$ m.



**Figure S2** Behavior of 3xTgAD mice (Tg) and non-transgenic mice (NTg) treated with GFP or GDNF. (A) Neophobia measured by a reduction of the number of corners in the Corner test. (B) Emotionality measured by increased number of defecation boli in the Open field test. (C) Horizontal activity and (D) vertical activity in the Open field test. General non-cognitive behavior was weakly modified by GDNF. Values are the mean  $\pm$  SEM, n=8-9. Statistics: \* $p < 0.05$  and \*\* $p < 0.01$  compared to the corresponding treatment of NTg mice, by Bonferroni test.