Original Paper

Cellular Physiology and Biochemistry

Cell Physiol Biochem 2011;28:793-804

Accepted: October 07, 2011

Inhibition of Leukotriene Receptors Boosts Neural Progenitor Proliferation

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Key Words

Neurogenesis • Neural progenitors • Differentiation • Leukotriene • MAP kinase pathway

Abstract

Neural stem and progenitor cells serve as a reservoir for new neurons in the adult brain throughout lifetime. One of the critical steps determining the net production of new neurons is neural progenitor proliferation, which needs to be tightly controlled. Since inflammation has detrimental effects on neurogenesis and the 5-lipoxygenase/leukotriene pathway is involved in inflammatory processes, we investigated the effects of leukotrienes and montelukast, a small molecule inhibitor of the leukotriene receptors $CysLT_1R$ and GPR17, on neural stem and progenitor cell proliferation. We demonstrate expression of the leukotriene receptor GPR17 by neural progenitors and by neural stem cells. Stimulation with excess amounts of leukotrienes did not affect progenitor proliferation, whereas blockade of GPR17 with montelukast strongly elevated neural stem and progenitor proliferation, while maintaining their differentiation fate and potential. This effect was associated with increased ERK1/2 phosphorylation suggesting an involvement of the EGF signaling cascade. Based on our results, montelukast and the inhibition of the 5-LOX pathway might be potent candidates for future therapies employing neurogenesis to promote structural and functional improvement in neurodegeneration, neuropsychiatric disease and ageing.

Introduction

While it is well-established that neurogenesis, i.e. the generation of new neurons, occurs not only in the developing but also adult mammalian brain [1-3] (for review see [4]), it is unambiguous that neurogenesis is not at a constant level throughout adulthood. For example, neurogenesis is dramatically diminished in the aged brain, where it experiences a more than 100 fold reduction compared to the young adult brain [2]. Also, alterations or defects in neurogenesis are implicated in age-related

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cognitive impairments, in neurodegenerative diseases and in major neuropsychiatric diseases such as depression [5-11]. Vice versa, stimulation of neurogenesis seems to provide an attractive strategy to generate new neurons in the aged or degenerative brain aiming to regain structure and function [4]. This makes the molecular mechanisms that regulate neurogenesis in the adult and diseased brain potential drug targets for regenerative therapies.

Lipid metabolism and lipid mediators of inflammation have recently been moving into the center of anti-ageing research and regenerative medicine. Here, members of the eicosanoids, in particular the leukotrienes, which have been well-studied in the field of allergy and immunity, are involved in mediating brain inflammation associated with age-related dementia and neurodegenerative diseases [12, 13]. Leukotrienes are lipid mediators of inflammation and are metabolized from arachidonic acid through the 5-lipoxygenase (5-LOX) pathway. The end-products leukotriene B4 (LTB_4) and the cysteinyl (Cys) leukotrienes LTC_4 and LTD_4 are involved in asthma, allergic rhinitis, atopic dermatitis, in cardiovascular diseases and cancer. Besides that, a number of studies were able to demonstrate that inhibition of the 5-LOX pathway has neuroprotective effects, reduces brain damage and promotes functional recovery in experimental models of stroke. The 5-LOX inhibitors AA-861 and BW-B70C, for instance, protect the brain against ischemic damage [14, 15]. Similarly, minocycline, a tetracycline antibiotic which blocks the activation of 5-LOX by inhibiting its translocation from the cytoplasm to the nuclear membrane, inhibits post-ischemic brain inflammation, accelerates functional recovery and has been demonstrated to enhance neurogenesis [16, 17].

At least three correlations point towards a critical role of the 5-LOX pathway and leukotrienes in the pathogenesis of age-related neurodegeneration and in the age-associated reduction in neurogenesis. i) Leukotriene levels are typically increased in brains affected by neurodegenerative diseases such as Parkinson and Alzheimer´s disease [12]. Here, the elevated levels of 5-LOX and leukotrienes cause post-inflammatory brain damage by mediating the generation of reactive oxygen species, resulting in a higher expression of inflammatory mediators such as tumor necrosis factor (TNF) alpha and interleukin-1 (IL-1) beta [18]. This might consequentially result in reduced neurogenesis, as TNF alpha and IL-1 beta signaling is known to inhibit neural stem cell proliferation and adult neurogenesis [19, 20]. ii) The expression of 5-LOX and leukotriene levels in the brain increase significantly during ageing with a 2,5 fold higher expression in the aged (24 - 25 months old) compared to the young (2 - 3 months old) rat and mouse brain [21, 22]. Importantly, the age-related elevation of the 5-LOX pathway in the brain is specific for the neurogenic regions, since it was detected in the hippocampus, but not in non-neurogenic regions such as cortex and cerebellum [22]. iii) The finding that glucocorticoids, which are known to inhibit neurogenesis, up-regulate the expression of 5-LOX [23] further points towards a role of the 5-LOX pathway in age-related decline of neurogenesis and possibly in the pathogenesis of age-related dementia.

In this study, we investigated the involvement of leukotrienes and leukotriene signaling in the regulation of neural progenitor cell (NPC) biology, in particular proliferation and differentiation, *in vitro*. We demonstrate expression of leukotriene receptors as well as endogenous leukotriene secretion in NPCs that were derived from the adult rat brain and cultured as neurospheres. Moreover, we modulated leukotriene signaling in those NPCs using montelukast (MTK), a competitive inhibitor of the leukotriene receptors $CysLT_1R$ and GPR17 and analyzed cell proliferation, downstream signaling and cell differentiation. The main findings were confirmed with clonally derived adult neural stem cells (NSCs).

Materials and Methods

Adult rat NPC cultures

Adult female Fischer 344 rats (Charles River Deutschland GmbH, Germany) served as donors for NPC cultures. All experiments were carried out in accordance with the European Communities Council Directive (86/609/EEC) and institutional guidelines. Neurosphere cultures from the adult hippocampus were obtained as described [24]. Briefly, 2-4 month-old female Fischer-344 rats (Charles River Deutschland GmbH, Germany) were decapitated. Hippocampi were aseptically removed and dissociated [24]. Cells were resuspended in Neurobasal (NB) medium (Gibco BRL, Germany) supplemented with B27 (Gibco BRL, Germany), 2 mM L-glutamine (PAN, Germany), 100 U/ml penicillin/0.1 mg/l streptomycin (PAN, Germany), hereafter referred to as NB/B27. For maintenance and expansion of the cultures, the NB/B27 was further supplemented with 2 mg/ml heparin (Sigma, Germany), 20 ng/ml FGF-2 (R&D Systems, Germany) and 20 ng/ml EGF (R&D Systems, Germany). Cultures were maintained at 37°C in a humidified incubator (Heraeus, Germany) with 5% CO_2. Medium was changed 4-5 days after seeding and neurospheres were passaged after 1 week. For dissociation, spheres were incubated with Accutase (Innovative Cell Technologies Inc., distributed by PAA, Germany) for 10 min at 37°C and triturated using a pipette before being resuspended in NB medium. Cell number was determined by Trypan blue exclusion and 5 x 10^4 cells/ml were plated in new T75 culture flasks in NB medium. Neurosphere cultures

from passage number 2 to 6 were used throughout this study and termed NPCs.

Clonally derived adult rat neural stem cell (NSC) cultures

Expanded NPCs cultures grown as neurospheres were dissociated with Accutase as previously described. Cell suspension was limitedly diluted and single cells were seeded in 96-well plates. The presence of single cells-containing wells was confirmed by microscopy. Medium change was performed once a week. After 25 days several primary spheres were individually dissociated and cells were seeded for culture expansion. One of the cultures was used for further analysis.

MSC cultures and MSC-CM preparation

MSCs were prepared as described previously [25]. Briefly, bone marrow plugs were harvested from femurs and tibias of 2-4 month-old female Fisher-344 rats (Charles River Deutschland GmbH, Germany). Plugs were mechanically dissociated in alpha-MEM (Gibco Invitrogen, Germany) and recovered by centrifugation. Cell pellets were resuspended in alpha-MEM-10% FBS and seeded at $1x10^6$ cells/cm^2. After 3 days, medium was changed and non-adherent cells were removed. Adherent cells were incubated in fresh alpha-MEM-10% FBS until a confluent layer of cells was achieved. Cells were trypsinized using 0.25% Trypsin (Gibco Invitrogen, Germany) and seeded in alpha-MEM-10% FBS at 8,000 cells/cm^2. After 3-5 days of culture, the resulting monolayer of cells, hereafter named rat bone marrow-derived mesenchymal stem cells (MSCs), was trypsinized and further cultured for experiments or frozen for later use. As demonstrated in our previous work, this cell culture preparation is highly enriched in multipotent MSCs with virtually no hematopoietic contamination [25]. MSC-CM was prepared as described previously [25]. MSCs were plated at 12,000 cells/cm^2 and incubated in alpha-MEM-10% FBS. After 3 days, the conditioned medium was collected and filtered using a 0.22 µm-pore filter and used as an oligodendrogenic stimulus for NPCs.

Treatment with factors for proliferation

After passaging, cells were seeded in 96-well culture plates at a concentration of $5x10^4$ cells/ml in a volume of 100 µl NB medium containing a reduced EGF and FGF concentration of 5ng/ml. The plates were then maintained at 37°C in a humidified incubator (Heraeus, Germany) with 5% CO_2 for 7 days. Before being added, factors (MTK, Leukotriene C4, Leukotriene D4 (Cayman Chemical, USA), U0126 (Calbiochem, USA) and 95% ethanol (Sigma-Aldrich, Germany)) were prediluted in NB medium (containing 5ng/ml EGF and FGF). 10 µl of this solution was added to the corresponding wells (control conditions received medium only) at days 0, 2, 4 and 6.

Analysis of cell proliferation - MTS assay

At day 7, proliferation was assessed using an MTS assay kit (CellTiter 96 AQueous One Solution Cell Proliferation Assay, Promega, Germany) according to the manufacturer's instructions. After 4h of incubation, optical density was measured at 490nm using a plate reader (Emax Precision Microplate Reader, Molecular Devices, USA). Since leukotrienes were dissolved in 95% ethanol, the latter was used as an internal control and leukotriene results were normalized to ethanol. Additionally, the MTS proliferation assay was performed with clonally derived NSCs.

Analysis of neurosphere size and number

Neurosphere size was analyzed using an Olympus SiS CC-12 camera connected to an Olympus IX70 microscope through a C-mount adaptor (IX-TVAD) and a Computer running analySIS Software (Soft Imaging System, Germany). After completion of the MTS assay, two entire wells per condition on one 96-well culture plate were photographed and the number of spheres and their diameters were assessed. From the spheres' diameters, single sphere volumes were calculated and further used for calculation of mean sphere volumes and the total volume of all spheres in one well. Since leukotrienes were dissolved in 95% ethanol, the latter was used as an internal control and leukotriene results were normalized to ethanol.

Analysis of endogenous leukotriene release by ELISA

For determination of endogenous leukotriene release, cells were passaged and then seeded into 12-well culture plates at a concentration of $5x10^4$ cells/ml in a volume of 1 ml NB medium containing a concentration of 5ng/ml EGF and FGF. Cells were cultivated for 7 days and medium was collected at day 0 (2h after passaging) and day 7 and kept at -80°C until further use. To determine leukotriene levels in the medium, we used a Cysteinyl Leukotriene EIA Kit (Cayman Chemical, USA) according to the manufacturer's instructions. Optical density was measured at 405 nm using a plate reader (Emax Precision Microplate Reader, Molecular Devices, Union City, CA, USA).

RNA isolation and RT-PCR

For isolation of RNA, neurospheres grown for 7 days in culture were collected and washed in PBS. After centrifugation, the cell pellet was homogenized in 1ml of Trizol (TRI® Reagent; Sigma, Germany). For phase separation, 200 µl of 1-bromo-3-chloropropane were added, vortexed and centrifuged (15 min at 12000 g). After transferring the aqueous phase into a new tube and adding 350ml of ethanol, we used QIAGEN RNEasy Mini Kit (Qiagen, Germany) according to the manufacturer's instructions for further washing. To remove possible genomic contamination, DNA digestion was performed as suggested and described in the Kit's manual using DNase I and RDD buffer (Qiagen, Germany). For determination of RNA concentration, the RNA was diluted in H_2O (1:25) and absorbance was measured with a photometer. RNA was stored at -80°C until cDNA synthesis.

For reverse transcriptase-polymerase chain reaction (RT-PCR), first-strand cDNA was synthesized from 1 µg of total RNA using the Reverse Transcription System (Promega, Germany) with random primers (Promega, Germany). cDNA was then amplified using 5 PRIME MasterMix (5 PRIME, Germany) and the following primers: CYS-LT1 Receptor forward 5'-ATG TTC ACA AAG GCA AGT GG-3' and reverse 5'-TGC ATC CTA AGG ACA GAG TCA-3', CYS-LT2 Receptor forward 5'-ACC CCT TCC AGA TGC TCC A-3' and reverse 5'-CGT GCT TTG

AAA TTC TCT CCA-3', GPR17 forward 5'-TTG TTT TGT GCC CTA CCA CA-3' and reverse 5'-CTC GTT GGT TTT CCC TTC AA-3', GAPDH forward 5'-GGT CGG TGT GAA CGG ATT TG -3' and reverse 5'-GTG AGC CCC AGC CTT CTC CAT-3' (all synthesized by Invitrogen, Germany). Annealing temperature for all reactions was 55°C. Reaction was also conducted without previous reverse transcription to detect possible genomic contamination of the RNA samples ("rt-" controls). Amplification products were then resolved by agarose (1.5%) gel electrophoresis.

Western blot analyses

For western-blot analysis, passaged cells were seeded at a concentration of $25x10^4$ cells/ml in NB medium containing 5 ng/ml EGF and FGF and cultured for 2 days. The neurospheres were then transferred into NB medium containing no growth factors and cultured for another 24 hours. The same medium was used to dilute MTK (Cayman Chemical, U.S.A). After stimulation with 10 µM MTK spheres were collected at 5, 10 and 15 minutes and immediately put on ice. Since phospho Erk signaling *per se* is known to show oscillating changes in activity and varies over time [26], we performed equivalent time series experiments under control conditions without MTK stimulation. For this, we incubated the cells at 5, 10 and 15 min with NB Medium containing no growth factors. Medium was further removed upon centrifugation in a pre-cooled centrifuge (at 4°C). Again on ice, the pellet was lysed in buffer (0.7% NP40 (Sigma, Germany), 50 mM Tris-HCl (pH 8.0), 0.1 mM EDTA (pH 8.0), 250 mM NaCl, 10% glycerol, 0.2 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 10 mM dithiothreitol, 2 lg/mL Aprotinin and 1 lg/mL Pepstatin), further homogenized using a syringe and a 30G needle, vortexed for 30min at 4°C and centrifuged for 20min at 17.900 g and 4°C. Lysates were stored at -20°C until further use. Protein concentration in the samples was determined using Bicinchoninic Acid Protein Assay Kit (Sigma, Germany) and a plate reader. Protein extracts (10 µg each) were fractionated on 10% SDS-PAGE and blotted on a nitrocellulose membrane (Whatman, Germany) by the semidry electroblot method (Biometra Fast blot, Biometra Biomedizinische Analytik, Germany). Immunoblots were blocked in 4% BSA in TBST buffer (Tris, pH 7.5,0,1M; NaCl, 0.15M; and Tween 20, 0.1 %) and subsequently probed with antibodies. Primary antibodies and their dilutions were as follows: Anti-Actin (rabbit) #A2066 (Sigma, Germany), 1:10.000, Phospho-p44/42 MAPK (Erk1/2) Rabbit mAb #4370 1:1000, p44/42 MAPK (Erk1/2) Rabbit mAb #4695 1:1000 (all antibodies from Cell Signaling Technology, Germany). Bound antibodies were visualized with a horseradish peroxidase conjugated secondary antibody (donkey anti-rabbit IgG-horseradish peroxidase antibody, 1:10.000, Jackson ImmunoResearch Laboratories, Inc, USA) and enhanced chemiluminescence (Amersham ECLplus Western Blotting Detection System; GE Healthcare, Germany) and exposed to Amersham Hyperfilm ECL (GE Healthcare, Germany). Between hybridizations with different antibodies, membranes were washed with RestoreTM Western Blot Stripping Buffer (Thermo Scientific, USA). All experiments were performed in triplicates. To quantify and determine possible alterations in the phospho Erk activation after MTK stimulation over time, the different blot band intensities were evaluated by measuring their individual mean grey values (software ImageJ, Biophotonics). Phospho Erk values were normalized to Actin values. Further, phosphoErk signal intensities at the different time points after MTK stimulation were standardized to the corresponding phosphoErk values under control conditions.

Analysis of cell identity and differentiation fate

To assess the potential of MTK to change the NPCs phenotype, cells were incubated for 7 days under proliferation conditions (NB medium, 5 ng/ml EGF and FGF) with and without 10 µM MTK (MTK was prediluted in medium and added on days 0, 2, 4 and 6). After this period, cell specific marker expression and growth factor withdrawal response was analyzed. In detail, after MTK pre-incubation neurospheres were dissociated with Accutase (Innovative Cell Technologies Inc., distributed by PAA, Germany) and plated on 100 µg/ml poly-L-ornithine (Sigma, Germany) and 5 µg/ml laminin-coated (Sigma, Germany) glass coverslips at a density of 25,000 cells/cm^2 in DMEM Knockout-20% Serum Replacement supplement (SR) (Gibco Invitrogen, Germany) for 12 hours. In order to analyze the cell specific marker profile, cells were immediately fixed for 30 minutes with 4% paraformaldehyde and processed for immunofluorescence. Alternatively, in order to analyze the cell intrinsic differentiation fate (growth factor withdrawal response), cells were incubated for 1 more week in DMEM Knockout-20% SR (Gibco Invitrogen, Germany) without growth factors and in the absence of serum. Finally, cells were fixed for 30 minutes with 4% paraformaldehyde and processed for immunofluorescence.

To analyze cell differentiation potential, NPCs were plated on poly-ornithine (250 mg/ml) and laminin (5 mg/ml)-coated (Sigma, Germany) glass coverslips at a density of 25,000 cells/cm^2 in alpha-MEM-10% FBS (Gibco Invitrogen, Germany) for 12 to 24 hours. Then, NPCs were incubated for 7 more days in alpha-MEM-10% FBS (Control) or in alpha-MEM-10% FBS supplemented with 10 ng/ml BMP-2 and BMP-4 (R&D Systems GmbH, Germany) (astrogenic stimuli) with and without 10 µM MTK. Alternatively, cells were incubated in MSC-CM (oligodendrogenic stimuli) with and without 10 µM MTK. For treatment, MTK was prediluted in alpha-MEM-10% FBS and added on days 0, 2, 4 and 6. After the incubation period cells were fixed for 30 minutes with 4% paraformaldehyde and processed for immunofluorescence to detect the expression of the different cell specific markers tested.

Sphere-forming assay and analysis of multipotency

Expanded NPCs cultures grown as neurospheres were dissociated with Accutase as previously described. Cell suspension was limitedly diluted and single cells were seeded in 96-well plates. The number of single cells-containing wells was confirmed and determined by microscopy evaluation. Medium change was performed once a week. After 25 days the number of generated primary spheres was determined by microscopy evaluation. Primary spheres were dissociated and total cells were seeded for culture expansion and the number of primary spheres able to generate secondary spheres and successfully expand was determined. The percentage of primary

spheres-forming cells as well as of the secondary spheres-forming cells in relation to the total amount of seeded single cells was determined. Multipotency was determined for dissociated secondary spheres. Cells were incubated under the following differentiation stimuli: NB+B27 media supplemented with 1% FBS + 20mM retinoic acid (neuronal differentiation); alphaMEM-10% FBS supplemented with 10 ng/ml BMP-2 and BMP-4 (R&D Systems GmbH, Germany) (astrogenic stimuli); MSC-CM (oligodendrocyte differentiation). After 7days of incubation, cells were fixed for 30 minutes with 4% paraformaldehyde and processed for immunofluorescence to detect the expression of the different cell specific markers tested.

Immunocytochemistry

Fixed cells were washed in TBS (0.15 M NaCl, 0.1 M Tris-HCl, pH 7.5), then blocked with a solution composed of TBS; 0.1% Triton-X100 (only for intracellular antigens); 1% bovine serum albumin (BSA) and 0.2% Teleostean gelatin (Sigma, Germany) (fish gelatin buffer, FGB). The same solution was used during the incubations with antibodies. Primary antibodies were applied overnight at 4°C. Fluorochrome-conjugated species-specific secondary antibodies were used for immunodetection. The following antibodies and final dilutions were used. Primary antibodies: rabbit anti-GFAP 1:1000 (Dako, Denmark); rabbit anti-GalC 1:200 (Chemicon, UK); rabbit anti-NG2 Chondroitin Sulfate Proteoglycan 1:200 (Chemicon, UK); IgM mouse anti-A2B5 1:200 (Chemicon, UK); IgM mouse anti-O4 1:100 (Chemicon, UK); mouse anti-rat Nestin 1:500 (Pharmingen, U.S.A.); mouse anti-Map 2a+2b 1:250 (Sigma, Germany); mouse anti-CNPase 2', 3'-cyclic nucleotide 3'-phosphodiesterase 1:200 (Chemicon, UK); mouse anti-Myelin Basic Protein (MBP) 1:750 (SMI-94, Sternberger Monoclonals Incorporated, U.S.A.); goat anti-GPR17 1:20 (Santa Cruz, CA). Secondary antibodies: donkey anti-mouse (IgG, IgM) or rabbit conjugated with Alexa Fluor® 488 (Molecular Probes, U.S.A.), rhodamine X (RHOX) 1:500 (Dianova, Germany); goat anti-mouse, rabbit or rat conjugated with Alexa Fluor® 488, RHOX 1:500. In cases of detergent-sensitive antigens (i.e. GalC, A2B5 and NG2), Triton X-100 was omitted from FGB buffer. Nuclear counterstaining was performed with 4', 6'-diamidino-2-phenylindole dihydrochloride hydrate at 0.25 µg/µl (DAPI; Sigma, Germany). Specimens were mounted on microscope slides using a Prolong Antifade kit (Molecular Probes, U.S.A.). Epifluorescence observation and photo-documentation were realized using a Leica microscope (Leica Mikroskopie und Systeme GmbH, Germany) equipped with a Spot™ digital camera (Diagnostic Instrument Inc, U.S.A.). For each culture condition, 10 randomly selected observation fields, containing in total 500-1000 cells, were photographed for cell fate analysis. Expression frequency of selected cell type markers was determined for every condition in three independent experiments.

Statistics

Statistical analyses were performed using one-way and ANOVA and Tukey post-hoc. Averages are expressed with their standard deviations. Experiments were performed at least in triplicate. Statistical analysis was performed using Graphpad PRISM 5.

Results

NPCs express leukotriene receptors

Cysteinyl leukotrienes signal via three different receptors, the $CysLT_1$ and $CysLT_2$ receptors and the recently identified GPR17 [27]. In the CNS, previous expression analyses have shown the presence of all three receptors, with strong expression of GPR17 and CysLT2R [27-29] and rather weak expression of CysLT1R [30, 31]. The latter was found to be mainly restricted to microvascular endothelium and only induced in gray and white matter tissue after traumatic injury or around brain tumors [32]. Moreover, GPR17 is expressed by neural and oligodendroglial progenitors of the adult CNS [33-35]

In this study, we analyzed adult rat hippocampus derived NPCs for CysLT receptor expression by RT-PCR. We detected strong expression of GPR17 that was comparable to the overall expression of this receptor in brain tissue (Fig. 1A). $CysLT_2R$ expression was also present in NPCs, however at a much lower level than in whole brain lysate (Fig. 1C), whereas $CysLT_1R$ expression was absent or under the level of detection (Fig. 1B). This is in line with the previous expression data and suggests that in our NPCs, cysteinyl leukotrienes signal mainly through GPR17 and CysLT2R.

Excess of CysLT receptor ligands fails to modulate NPC proliferation

Acute ischemic lesions in the brain induce progenitor proliferation and neurogenesis [36]. Also, the levels of leukotrienes, in particular of LTC_4 and LTD_4 are elevated during and after stroke [37]. Moreover, GPR17 is expressed on oligodendroglial progenitors [38] and on NPCs (Fig. 1A). This points towards a possible involvement of leukotrienes in the modulation of progenitor proliferation after ischemic insults. Furthermore, leukotrienes presumably play a role in lesion-induced neuronal cell death and brain atrophy, since antagonists of CysLTRs or in vivo knockdown of GPR17 by antisense oligonucleotides reduces the infarct volume after stroke in animal models [27, 38]. These findings evoked our interest in potential effects of leukotrienes on NPC proliferation.

Therefore, we analyzed the effects of the cysteinyl leukotrienes LTC_4 and LTD_4 on NPC proliferation *in vitro*. Stimulation with various concentrations

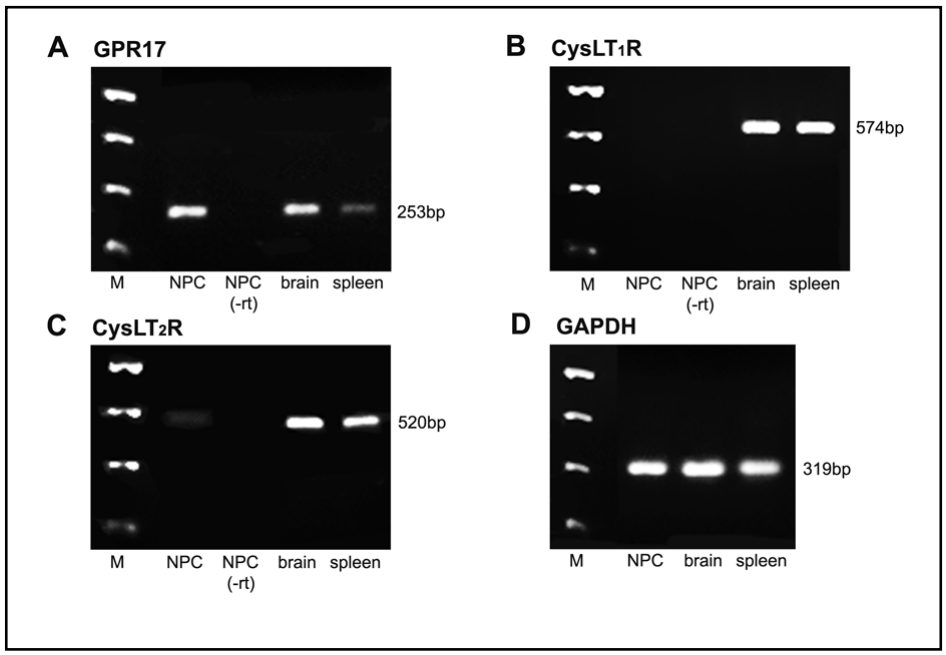


Fig. 1. Qualitative analysis of leukotriene receptor expression in adult rat hippocampal NPCs by RT-PCR. RT-PCR amplification of rat cDNA sequences for GPR17 (A), CysLT1R (B) and CysLT2R (C) in NPCs, brain and spleen. In brain and spleen, which were chosen as positive controls, expression of all three receptors was detected, whereas in NPCs, strong expression was only observed for GPR17 (A), while $CysLT_2R$ was expressed weakly (C), and expression of $CysLT_1R$ was absent (B). No amplification was found in the absence of retrotranscription ("rt-" controls) in NPCs, ruling out possible genomic contamination of the RNA samples. Parallel expression of the housekeeping gene GAPDH is shown (D).

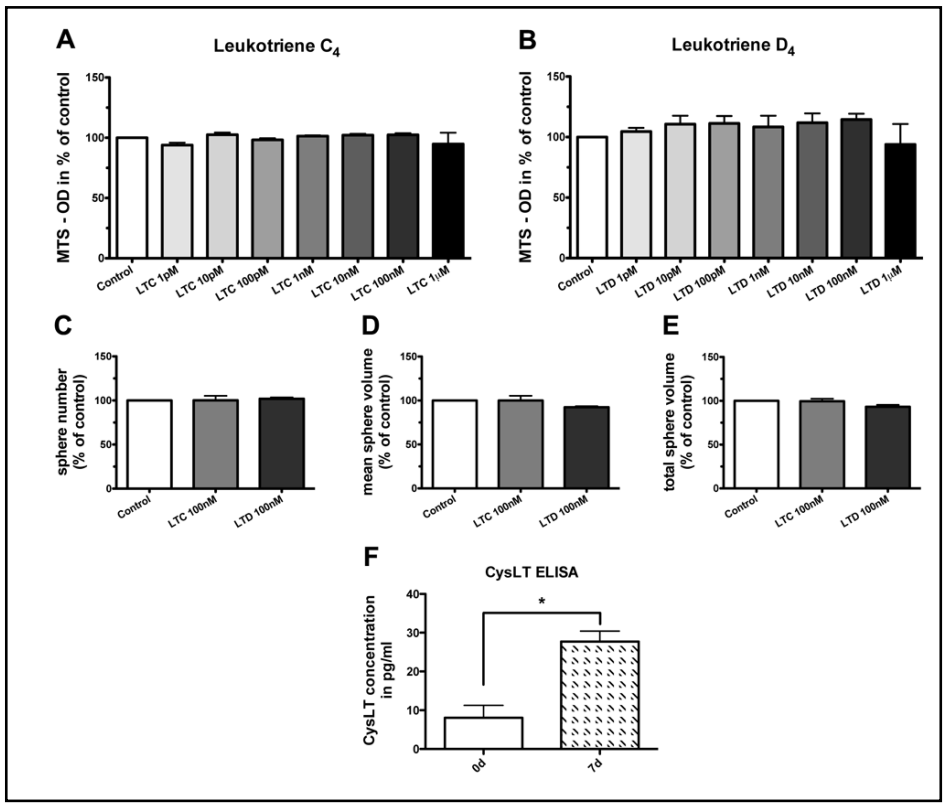


Fig. 2. Effect of Leukotrienes on NPC proliferation. NPC proliferation was measured by an MTS assay (A, B). Stimulation of adult rat hippocampal NPCs with Leukotriene C_4 (LTC) and Leukotriene D_4 (LTD) in various concentrations had no effect on NPC proliferation. Neurospheres were counted and their sizes measured using phase contrast microscopy (C, D, E). Neither neurosphere number (C) nor neurosphere volume (D, E) were affected by these compounds. Data are shown as means ± SD and normalized to control condition and 95% ethanol. For statistical analysis one-way ANOVA was performed. CysLT-Levels in medium 2 hours and 7 days after passaging were measured using a commercially available ELISA kit (F). NPCs released CysLTs into the medium and concentration increased over time. Data are shown as means ± SD. For statistical analysis, paired t-test was used (*= $p<0.05$).

of LTC_4 and LTD_4 ranging from 1 pM to 1 µM over a one-week period failed to affect NPC proliferation in an MTS assay (Fig. 2A, B), as well as number or volumes of neurospheres (Fig. 2C, D, E). These findings prompted the idea that NPCs might secrete and therefore be exposed to leukotrienes in culture and that an autocrine/paracrine leukotriene-mediated mechanism might control NPC proliferation *in vitro*. A leukotriene ELISA confirmed the presence of cysteinyl leukotrienes in medium conditioned by NPCs and showed a three-fold increase in concentration during a cultivation period of 7 days (Fig. 2F).

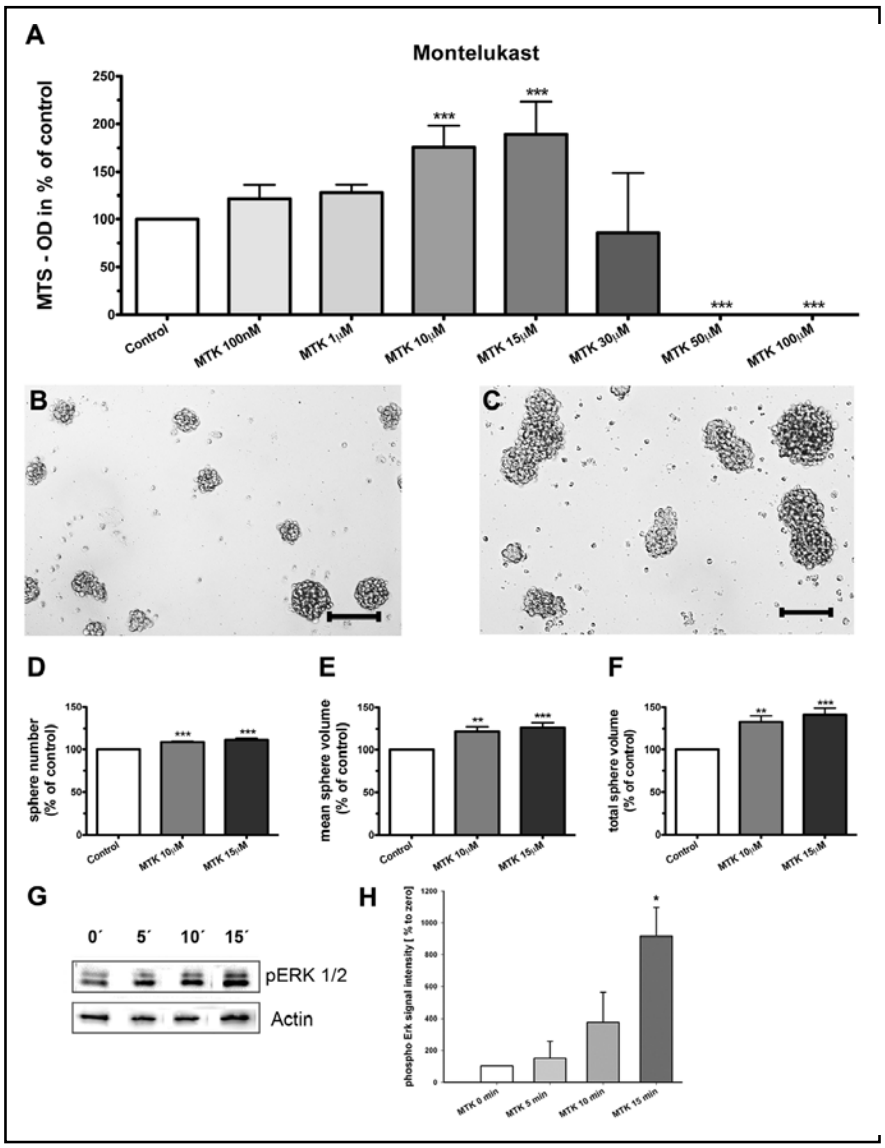


Fig. 3. *In vitro* stimulation of NPCs with MTK. NPC proliferation was measured by an MTS assay (A). MTK dose-dependently increased NPC proliferation, while conentrations beyond 15 µM caused cell death. Phase contrast images of neurospheres grown in absence (B, control) or presence of MTK (C). Scale bars 100 µm. Neurosphere number (D) and volume (E, F) were determined for all conditions using phase contrast microscopy. Neurospheres treated with MTK were more numerous and displayed bigger volumes compared to control condition. Western blot analysis revealed an increase of phospho ERK1/2 signaling after MTK treatment over time (G, H). Quantification of the phospho ERK signal showed a significant increase in signal intensity at 15 min compared to 0 min MTK treatment (H). Phospho Erk values were normalized to Actin and are shown in percent of control (0 min MTK). All experiments were done in triplicates. Data are shown as means ± SD (except western blot quantification, which is illustrated as means ± SEM). For statistical analysis, one-way ANOVA and Tukey post-hoc was performed (*= $p<0,05$; **= $0,01<p<0,05$; ***= $p< 0,001$).

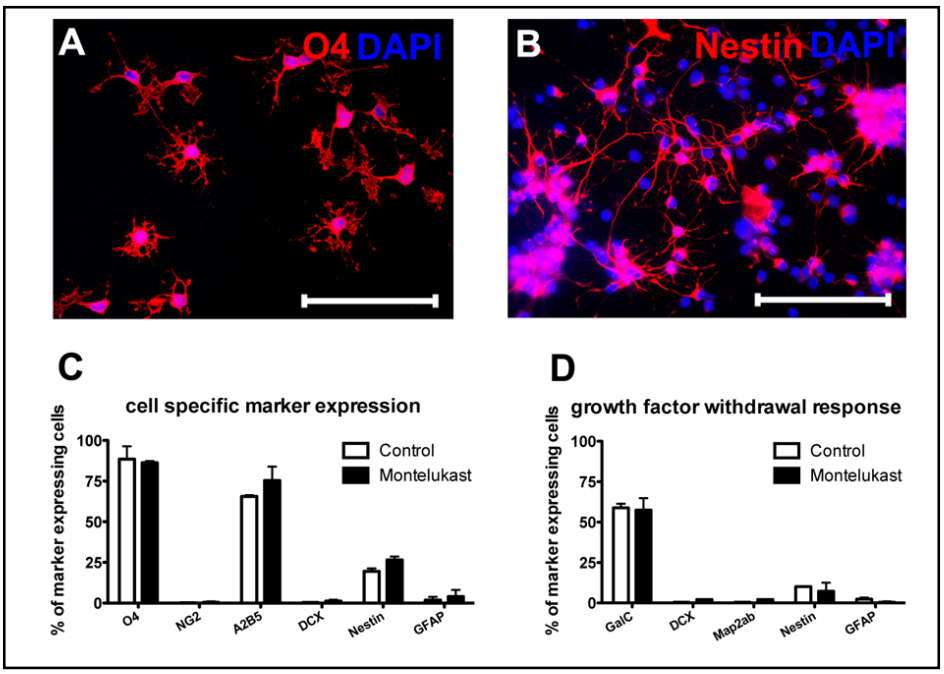
Montelukast stimulates NPC proliferation without affecting the identity and differentiation potential of the cells

Next, we raised the question whether a blockade of leukotriene activity could modulate adult hippocampal NPC proliferation *in vitro*. Therefore, NPCs were exposed to various concentrations of MTK, a competitive inhibitor of the leukotriene receptors $CysLT_1R$ and GPR17, and NPC proliferation was analyzed. A one-week stimulation of NPCs with MTK resulted in a dose dependant and significant increase in the amount of NPCs as measured by an MTS assay (Fig. 3A). At concentrations beyond 15 µM, MTK apparently had a toxic effect on the progenitors (Fig. 3A). MTK treated spheres grew significantly bigger and were more numerous compared to control treated cultures (Fig. 3B-F). Moreover, consistent with a higher proliferation rate, the ERK1/2 signal transduction cascade was transiently induced after MTK stimulation (Fig. 3G, H). Compared to 0 min MTK stimulation, the phosphoErk signal was significantly elevated after 15 min (Fig. 3H).

Apart from its effects on proliferation, we speculated that MTK might change the cell identity and differentiation fate of NPCs. Therefore, we immunocharacterized MTK treated NPCs under proliferation and differentiation conditions (growth factor withdrawal) using markers for neural stem cells (nestin), progenitors (A2B5, O4, Ng2), neuronal precursors (DCX), neurons (Map2ab), oligodendrocytes (GalC) and astrocytes (GFAP) (Fig. 4A, B). MTK and control treated cultures showed a very similar marker expression profile (Fig. 4C, D), suggesting that MTK neither changed cell identity nor the differentiation fate of the NPCs.

Finally, we forced the progenitors to differentiate into astroglial cells using serum or BMPs or into oligodendrocytes using mesenchymal stem cell

Fig. 4. Analysis of cell identity and differentiation fate of NPCs after treatment with MTK. Immunostaining of MTK treated proliferating NPCs with O4 (A) and Nestin (B). Scale bars 100 µm. Cell specific marker expression under proliferation conditions (C) or after growth factor withdrawal (intrinsic differentiation fate, D). In both cases, no differences were observed in the distribution of cell specific marker expression between the MTK and the control group. Data are shown as means ± SD and for statistical analysis one-way ANOVA was performed.



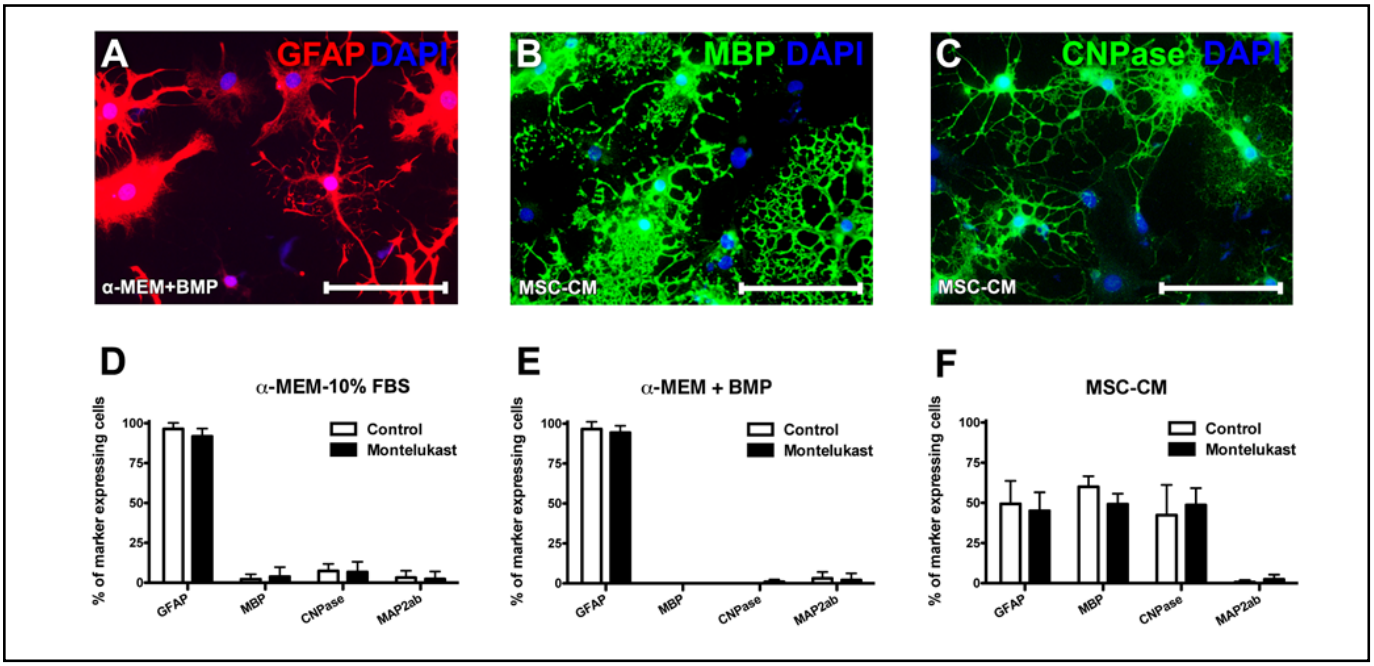


Fig. 5. MTK treatment does not alter NPC differentiation potential. Immunostaining of NPCs with GFAP (A), MBP (B) and CNPase (C). Cells were incubated in alpha-MEM-10% FBS (A) and in MSC-CM (B, C), respectively. Scale bars 100 µm. Expression frequency of GFAP, MBP, CNPase and MAP2ab as determined by fluorescence microscopy (D, E, F). MTK treatment did not affect the differentiation of NPCs incubated in either alpha-MEM-10% FBS alone (D), in alpha-MEM supplemented with BMP (astrogenic stimulus) (E) or in mesenchymal stem cell conditioned medium (oligodendrogenic stimulus) (MSC-CM; F). Data are shown as means ± SD and for statistical analysis one-way ANOVA was performed.

conditioned medium (MSC-CM) [25] and analyzed expression patterns using markers for astrocytes (GFAP), oligodendrocytes (MBP, CNPase) or neurons (Map2ab) (Fig. 5A, B, C). While BMPs and MSC-CM exerted their expected astroglial, respectively oligodendroglial effects, MTK did not affect the differentiation potential of the NPCs in any of the conditions (Fig. 5D, E, F).

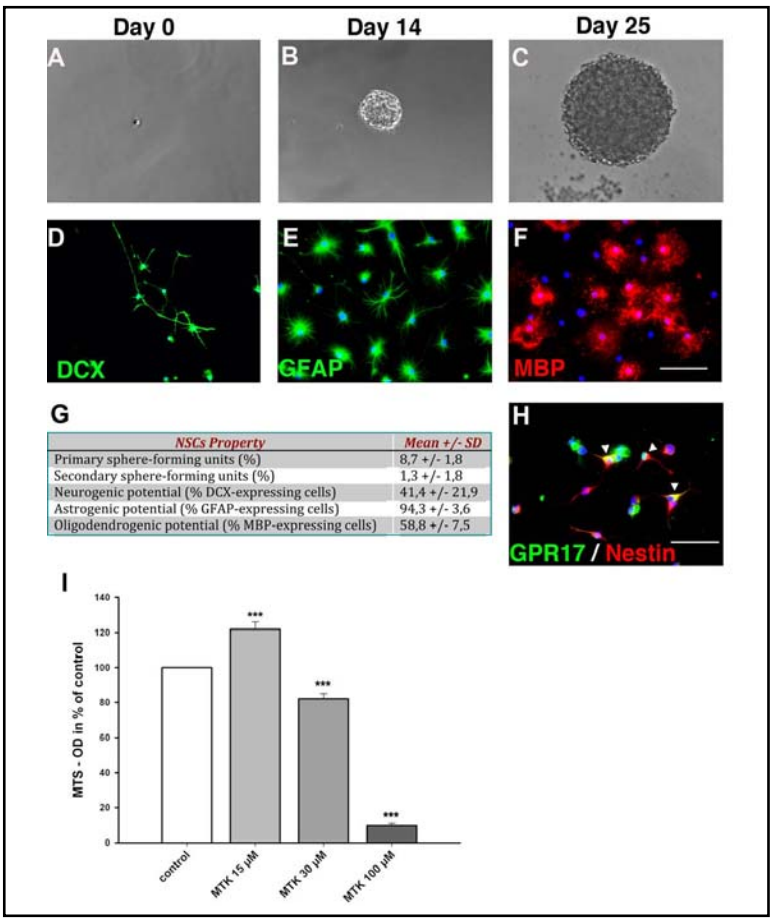


Fig. 6. Montelukast increases adult multipotent NSC proliferation. (A-C) Phase contrast images of clonally derived neural progenitors: adult NPCs were seeded at clonal density (single wells) and incubated up to 25 days in proliferation media where few single cells give rise to growing spheres. (A) Single cell at the moment of seeding; (B) small sphere 14 days after seeding; (C) big sphere 25 days after seeding. (D-F) Fluorescence images show NSC multipotency: spheres were dissociated and tested for differentiation under specific stimuli. (D) neurogenic condition, DCX (green) DAPI (blue). (E) astrogenic condition, GFAP (green) DAPI (blue). (F) oligodendrogenic condition, MBP (red) DAPI (blue). Scale bar 100 µm. (G) Summary table shows data as means ± SD of: the percentage of primary and secondary forming units; percentage of DCX-expressing cells (neurogenic response); percentage of GFAP-expressing cells (astrogenic response); and percentage of MBP-expressing cells (oligodendrogenic response). (H) Fluorescent image shows the expression of GPR17 in adult NSCs: Nestin (red) GPR17 (green) DAPI (blue). Scale bar 50 µm. (I) MTS analysis of the effect of different concentrations of MTK on proliferating NSCs. Note that 15 µM MTK enhances NSC proliferation. Experiments were done in triplicate. One-way ANOVA Tukey post-hoc was used for statistical analysis. *= $p<0.05$; **= $p<0.01$; ***= $p<0.001$.

Montelukast enhances proliferation of clonally derived multipotent neural stem cells

We recently demonstrated that adult rat neurospheres, although being tripotent, consist mainly of oligodendroglial progenitor like cells [39]. Also in the present study, a relatively high proportion of NPCs expressed glial and oligodendroglial progenitor cell (OPC) markers and showed an oligodendrocyte intrinsic fate regardless of MTK treatment (Fig. 4). In order to test if MTK also has a mitogenic effect on neural stem cells, we generated clonally derived NPCs that fulfill the criteria for neural stem cells, i.e. self-renewal and multipotency. While neural progenitors give rise to spheres that have limited self-renewal potential and cannot be expanded into secondary spheres and subsequent cultures, NSCs are characterized by their potential to self-renew for a longer period of time [40]. Therefore, we seeded adult NPCs at clonal density and incubated them for 25 days. During this incubation period 8,7% of single cells proliferated and gave rise to growing primary spheres (Fig. 6A-C, G). In a subsequent clonal analysis, cells were allowed to generate secondary spheres, an indication for self-renewal and thus a requisite for stem cell identity. As a result, 1,3% of all original single cells gave rise to primary spheres that in turn generated secondary and subsequent spheres (Fig. 6G).

Next, we tested for multipotency. Thus, a clone that generated secondary spheres was dissociated, further propagated and tested for neurogenic, astrogenic and oligodendrogenic potential. Interestingly, 41,4% of cells were found to express DCX when incubated under neurogenic stimuli and 94,3% of cells expressed GFAP in the presence of astrogenic stimuli. In addition, 58,8% of cells were observed to express MBP when cultured in oligodendrogenic conditions (Fig. 6D-G). In summary, the NSC clone used for the present study does self-renew and is multipotent.

Next, we tested whether adult NSCs express GPR17 and respond to MTK. We found several Nestin-positive cells that co-express GPR17 (Fig. 6H). Finally, cells from the adult multipotent NSC clone were incubated in the presence of MTK and proliferation was evaluated by MTS. In consistence with data on NPCs, 15µM MTK enhanced the number of NSCs, while higher concentrations affected cell survival (Fig. 6I). In summary, these data demonstrate that MTK increases NPC as well as NSC proliferation.

Discussion

Here, we demonstrate that MTK, a CysLT1R and GPR17 antagonist significantly and specifically elevates proliferation of NPCs and NSCs *in vitro*. However, it neither affected the cells' fate nor their differentiation potential. The proliferatíve effect exerted by MTK was associated with enhanced ERK1/2 signaling and rather caused by an inhibition of GPR17 than of the $CysLT_1$ receptor, since expression of the latter one was not detectable. GPR17 is expressed by neurons and by OPCs [34, 35, 38]. Moreover, Maisel and coworkers have identified GPR17 in the transcriptome of neural stem cells [33]. It functions as a receptor for uracil nucleotides and for CysLTs and apparently has a dual function in neurodegenerative as well as -regenerative processes. In the ischemic brain, increased expression of GPR17 in neurons correlates with augmented cell death [38]. Moreover, data strongly suggest that GPR17 signaling is actively involved in triggering cell death in ischemic rodent brains, since treatment with the $CysLT_1R$/GPR17 antagonist MTK as well as treatment with the $P2Y_{12,13}$/GPR17 antagonist cangrelor showed neuroprotective effects *in vivo* [27, 41]. Additionally and more specifically, the *in vivo* knockdown of GPR17 by antisense oligonucleotides markedly prevented an increase in tissue damage in ischemic mouse brains [27, 38]. Besides its role in neuronal cell death, GPR17 is also involved in brain remodeling and repair. Activated microglia/macrophages induce expression of GPR17 and GPR17 positive oligodendrocyte progenitors proliferate in response to injury [27]. In the oligodendroglia lineage, GPR17 determines the correct timing of myelination [34]. Furthermore, GPR17 agonists promote cell differentiation and neurite outgrowth in PC12 cells [42]. The molecular mechanisms that mediate the neuro-regenerative effects of GPR17 might involve the ERK1/2 signaling pathway and the generation of outward K^+-channels, which reduces neuronal hyperexcitability and neuronal injury in the brain [42, 43]. This, together with the present data, indicates that GPR17 signaling might have at least four different functions in brain damage and repair. It induces neuronal cell death, it might block neural progenitor proliferation, but it can also be neuroprotective and might stimulate oligodendroglial progenitor proliferation. The differential responses might derive from cell type specific differences and/or context dependency. For example, even though the MAPK pathway is activated in PC12 cells by various growth factor and cytokines, the cellular responses can be very different and depend on the growth factor context [44].

The work presents leukotrienes, leukotriene synthesis and leukotriene signaling as targets for potential therapies modulating neurogenesis to promote structural and functional regeneration in the diseased CNS. This might be attractive particularly during ageing, where the expression of 5-LOX and leukotriene levels are significantly increased in the neurogenic niche [21, 22]. Most likely, the age-related reduction in neurogenesis is caused by an inhibition or a de-activation of neural stem cells rather than by a progressive loss of stem cells. While the number of neural stem cells in the hippocampus during ageing remains stable, the mitotic activity of these cells is progressively reduced [45]. Thus, the proliferation of progenitors appears to be a main target to stimulate neurogenesis in the aged brain with the aim to reconstitute the age-related functional impairment of learning and memory. Indeed, mechanisms related to allergy seem to be critically involved in brain ageing. For example, we have recently demonstrated that eotaxin, a molecule involved in allergic responses, is crucially involved in inhibition of neurogenesis during ageing [46].

Besides its role in ageing, the 5-LOX pathway might be involved in the pathogenesis of neuropsychiatric disorders. Consistent with this hypothesis, the metabolism of arachidonic acid and the 5-LOX pathway are altered in depressive patients [47, 48]. Likewise, the expression level of cytosolic 5-LOX is elevated and 5-LOX is phosphorylated and thus activated in the prefrontal cortex of suicide victims [49]. Conversely, 5-LOX inhibitors have antidepressive activities. Caffeic acid, an inhibitor of 5-LOX, produces anti-depressant like effects in mice and similarly, injections of MK-886, an inhibitor of the 5-LOX activating protein FLAP, induce an antidepressant behavior in a forced swim test [49, 50]. The underlying mechanisms have not been elucidated so far, but our present data suggest an involvement of progenitor proliferation and neurogenesis. This is consistent with the widely accepted view that neurogenesis is impaired in depression [11, 51] and, vice versa, that deficiencies in neurogenesis might contribute to the pathogenesis of depression [52]. Along the same line, serotonin reuptake inhibitors such as fluoxetine stimulate neurogenesis [11, 53, 54]. Indirect evidence of a potential role of the 5-LOX pathway in neuropsychiatric disbalances is provided by notions from allergy research. Interestingly, asthma/allergy patients have an elevated risk to commit suicide [55]. It is of course highly intriguing but attractive to speculate that a 5-LOX-related diminished level of neurogenesis might contribute to a suicidal behavior. Obviously, this requires further investigation and substantial evidence. Nevertheless, in

summary, the 5-LOX pathway appears to be an attractive target for therapy of neurodegenerative and neuropsychiatric diseases.

Acknowledgements

The authors would like to thank the following funding agencies for their support: Bayerische Forschungsstiftung, Munich, Germany (F.R.), the Alexander von Humboldt Foundation - Georg Forster Program (F.R.), the Bavarian State Ministry of Sciences, Research and the Arts (ForNeuroCell grant) (S. CD., L.A.), the state of Salzburg, the Germany Federal Ministry of Education and Research (BMBF grants #0312134, #01GG0706 and #01GN0505) and the PMU research fund PMU-FFF. Moreover, we thank Maria Pia Abbracchio for helpful discussions.

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