Structural and functional rejuvenation of the aged brain by an approved anti-asthmatic drug

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As human life expectancy has improved rapidly in industrialized societies, age-related cognitive impairment presents an increasing challenge. Targeting histopathological processes that correlate with age-related cognitive declines, such as neuroinflammation, low levels of neurogenesis, disrupted blood-brain barrier and altered neuronal activity, might lead to structural and functional rejuvenation of the aged brain. Here we show that a 6-week treatment of young (4 months) and old (20 months) rats with montelukast, a marketed anti-asthmatic drug antagonizing leukotriene receptors, reduces neuroinflammation, elevates hippocampal neurogenesis and improves learning and memory in old animals. By using gene knockdown and knockout approaches, we demonstrate that the effect is mediated through inhibition of the GPR17 receptor. This work illustrates that inhibition of leukotriene receptor signalling might represent a safe and druggable target to restore cognitive functions in old individuals and paves the way for future clinical translation of leukotriene receptor inhibition for the treatment of dementias.
Promoting successful ageing is highly relevant, since human life expectancy has increased rapidly over the past decades. With increasing age, brain homeostasis changes, cognitive skills decline and the risk to develop dementia or neurodegenerative diseases increases dramatically. Thus, a major goal of basic and applied research is to prevent or revert age-related brain changes responsible for cognitive deficits. Ultimately, this endeavour aims to maintain or restore cognitive abilities in the elderly in order to facilitate high quality of life, even in advanced age.

Histopathological hallmarks that correlate with age-related declines in cognitive function are neuroinflammation, in particular microglia dysfunction, reduced synaptic densities, blood–brain barrier (BBB) disruption and low levels of neurogenesis. Concerning neuroinflammation, microglia in the aged brain produce increased levels of pro-inflammatory cytokines such as interleukin (IL)-6, IL-1beta and tumour necrosis factor (TNF) but also of anti-inflammatory cytokines such as IL-10 and transforming growth factor (TGF) beta1 (refs 2,3). Morphologically, aged microglia have shorter and less motile processes and larger soma sizes compared with microglia in young animals. Also importantly, phagocytosis, one of the principal microglia functions, seems to be altered in the aged brain, and aged microglia exhibit increased expression of the phagosomal/lysosomal associated marker CD68 (refs 6,7).

The aged brain is further characterized by a declining density of presynaptic terminals, as indicated by synaptophysin immunoreactivity, for example. Ample evidence of a partially leaky BBB in the aged brain is demonstrated by greater central nervous system (CNS) transfer of intravenously injected Evan’s blue dye in aged compared with young adult rats, and in dynamic contrast-enhanced magnetic resonance imaging studies of aged humans. Moreover, the rate of adult neurogenesis, that is, the lifelong generation of new neurons from neural stem and progenitor cells in the dentate gyrus (DG) in the hippocampus and in the subventricular zone, decreases dramatically during ageing in rodents, and is also reduced in elderly humans. Countering some, or ideally all, of such age-related changes might rejuvenate the brain and lead to preservation or even improvement of cognitive function in the elderly. The feasibility of such an approach was recently demonstrated by experiments exposing the aged brain to a young systemic environment, that is, young blood, through heterochronic parabiosis. The aged brain responded to young blood by reduced microglia activation, enhanced neurogenesis, and importantly, by improved cognition. Vice versa, old blood caused premature ageing of the young brain and led to impaired cognition. A proteomic approach identified eotaxin, a chemokine involved in asthma pathology, as one of the molecules that is elevated in ageing and that contributes to neuroinflammation, reduced neurogenesis and to impaired cognition. This triggered us to hypothesize that, aside from eotaxin, additional mechanisms that are originally related to peripheral inflammatory conditions such as asthma might act or even be present in the CNS, where they potentially modulate degenerative and regenerative events.

Leukotriene signalling is well studied in the field of asthma. Leukotrienes mediate inflammatory reactions associated with increased vascular permeability, and leukotriene receptor antagonists such as the drug montelukast have been successfully developed to treat asthmatic patients. The role of leukotrienes in the brain, in particular their contribution to degeneration and regeneration, is less clear and sometimes even controversial. Nevertheless, elevated levels of leukotrienes were reported in acute as well as chronic CNS lesions, and also in the aged brain, where they might mediate neuroinflammatory responses including microglia activation. Indeed, microglia express the cysteinyl leukotriene receptor CysLTR1, which mediates pro-inflammatory effects of leukotrienes, and microglia induce the expression of CysLTR2 and of the leukotriene related GPR17 receptor, for example, after ischaemia. Antagonizing CysLTR1 and GPR17 with the specific inhibitor montelukast reduces the levels of inflammatory cytokine expression in particular GPR17 are also expressed on endothelial cells, where they mediate BBB leakage, and montelukast restores BBB integrity. Furthermore, we previously demonstrated leukotriene receptor expression, in particular GPR17, in adult neurospheres, and detected a montelukast-induced dose-dependent increase in progenitor proliferation. Therefore, montelukast is positioned to target at least three of the age-related cellular changes in the brain, that is, microglia activation, BBB integrity and neurogenesis.

Here, we demonstrate that montelukast reduces neuroinflammation, restores BBB integrity and increases neurogenesis specifically in the brain of old rats, the latter being mediated through inhibition of the GPR17 receptor. Most importantly, montelukast treatment restores cognitive function in the old animals, paving the way for future clinical translation for the treatment of dementias.

Results

Montelukast improves learning and memory in old rats. We analysed the effects of a 6-week oral montelukast (10 mg kg$^{-1}$ body weight) treatment of young (4-months old) and old (20-months old) rats on learning and memory. Analysis of the latency times to find a hidden platform over 5 consecutive training days in a Morris water maze test, during which spatial navigation task learning is typically achieved in young animals, confirmed that old individuals have learning deficits (Fig. 1a). Montelukast treatment significantly improved task learning in old rats to a level comparable to young ones, so that on day 5 the drug-treated old animals found the platform as fast as their young counterparts. Learning in young animals was not affected by the drug treatment. Age and treatment groups only differed in the distances moved, but not in swimming speeds, excluding the
latter as a possible cause of different latency times (Fig. 1b,c). Memory was assessed by removing the platform 1 day after the last learning session and by analysing the time spent in the former platform quadrant. For more precise information, we evaluated the time spent in the former platform location, and assessed the frequency entering this area. Surprisingly, old rats spent, irrespective of the treatment, a similar amount of time in the former platform quadrant compared with their young counterparts (Fig. 1d). However, the old individuals had significant deficits in the ability to remember the exact platform location (Fig. 1e,f).
Montelukast treatment completely reverted this age-related phenotype, but did not affect microglia in the young rats (Fig. 2a,d). Next, we speculated whether the increased microglia soma size in old rats, as well as its reduction by montelukast, are linked to changes in phagocytosis. We therefore analysed the expression pattern of CD68, a protein associated to the phagosomal/lysosomal pathway of microglia\(^3^9\). Old animals had a significant higher number of Iba1\(^+\) microglia co-expressing CD68 (Fig. 2f) and bigger CD68\(^+\) particles (Fig. 2e,g) compared with the young animals, suggesting altered phagosomal/lysosomal processing of microglia in old rats. Although montelukast did not influence the number of Iba1\(^+\) CD68\(^+\) cells in the old age-group, drug treatment led to a significant reduction of the average CD68 particle size in the old animals. In young animals, neither the number of CD68\(^+\) expressing microglia, nor the average particle size were affected by montelukast.

Addressing more specifically the pattern of neuroinflammatory gene expression, we made use of the BV-2 microglia cell line. First, BV-2 cells showed immunoreactivity for the target receptors of montelukast, the leukotriene receptor CysLT\(_1\) and the GPR17 receptor (Fig. 2h). Second, we treated BV-2 cells for 24 h with 100 nM of the receptor ligand leukotriene D\(_4\) (LTD\(_4\)), with 15 \(\mu\)M montelukast, or with 100 nM LTD\(_4\) together with 15 \(\mu\)M montelukast, and analysed mRNA expression of inflammatory and anti-inflammatory genes (Fig. 2i). A 24-h treatment with LTD\(_4\) led to a significant upregulation of the expression of the pro-inflammatory enzyme NOS2. 24 h co-stimulation with montelukast counteracted this effect and significantly reduced NOS2 mRNA expression. Moreover, in the presence of LTD\(_4\) montelukast downregulated the expression of the monocyte-recruiting cytokine CCL2, whose expression is elevated in ageing\(^3^0\). Also, mRNA expression of the neuroprotective cytokine TGF-beta1 was significantly elevated after 24 h treatment with montelukast in the presence of LTD\(_4\). In summary, these findings further add to the established mode of action of montelukast as an anti-inflammatory drug.

Next, as the BBB is disrupted in neuroinflammation and in ageing\(^3^0\), we analysed BBB integrity using the tight junction protein claudin-5. Endothelial cells in the hippocampus of young (4 months) vehicle-treated rats showed strong and continuous immunostaining with an anti-claudin-5 antibody, illustrating an intact BBB (Supplementary Fig. 2). In contrast, claudin-5 expression in endothelial cells of old (20 months) vehicle-treated rats was diffuse and weak, which points toward a disrupted BBB. The old montelukast-treated rats showed more intense, more continuous and better-defined endothelial claudin-5 staining compared with age-matched vehicle controls, indicating that montelukast improved BBB integrity in old rats.

Montelukast restores hippocampal neurogenesis in old rats. Further, we investigated if the 6-week treatment with montelukast had any effects on DG neurogenesis. Proliferation (number of PCNA\(^+\) cells in the subgranular and granular zone) was approximately three-times lower in old compared with young animals. Montelukast treatment significantly enhanced proliferation, and this effect was specific for the old individuals (Fig. 3a). The surplus in PCNA\(^+\) cells in the old montelukast-treated animals was apparently not due to an enlargement of the neural stem cell pool, as the number of subgranular Sox2\(^+\) cells was unchanged by the drug treatment (Fig. 3b). By contrast, it was the pool of DCX\(^+\) young immature neurons that was significantly expanded by the montelukast treatment (Fig. 3c). The additional newly generated cells in the old montelukast-treated rats survived for at least 4 weeks as indicated by the number of cells that had incorporated BrdU two weeks after onset
of treatment (Fig. 3d). The effects of montelukast on proliferation and survival resulted in a significant increase of BrdU+ newly generated cells in the DG of old animals after the 6-week treatment. The fate of the newly generated cells (percentage of BrdU+ cells co-labelling with NeuN for mature neurons or with GFAP for astrocytes) was in the expected range of 60–80% neuronal and ~15% glial differentiation and was neither affected by age nor by treatment (Fig. 2e). As a net result, montelukast treatment significantly increased the number of newly generated neurons in the DG of old rats (215.97 ± 60.04 BrdU+/NeuN+ cells) compared with age-matched controls (131.48 ± 44.16 BrdU+/NeuN+ cells).

Since ageing is associated with loss of presynaptic terminals8,9 and altered neuronal functionality31, we explored the possibility that the montelukast-induced effect on cognitive function was related to these parameters. Indeed, synaptic density...
The synaptophysin positive area was significantly reduced in the hippocampus of old rats, but only slightly and not significantly in the cortex. Montelukast treatment did not restore the synaptic density in the hippocampus (Supplementary Fig. 3a) implying that the cognitive improvements in old rats were not related to changes in synaptic densities in the DG. In the same context, the 6-week montelukast treatment did neither change the DG immunohistochemical expression pattern of c-Fos, an immediate early gene and marker for activated neurons (Supplementary Fig. 3b), nor did montelukast and/or leukotrienes alter neuronal network activity in rat hippocampal cultures (Supplementary Fig. 3c–g).

Taken together, montelukast treatment reduced microglia activation, elevated neurogenesis, and restored cognitive function.
in old rats. Regression and correlation analyses showed that learning did not correlate with microglial soma size \((R^2 = 0.001)\) and that the montelukast-induced learning improvement was independent of the changes in microglia morphology in the old animals \((R^2 = 0.027)\) (Fig. 4a). In contrast, correlation coefficients of \(R^2 = 0.306\) for learning/number of PCNA\(^+\) cells in the DG, and of \(R^2 = 0.225\) for learning/number of BrdU\(^+\) cells in old vehicle-treated rats, let rather suggest that the learning success of old rats might depend on the rate of neurogenesis (Fig. 4b,c). Moreover, montelukast treatment provoked a stronger correlation between learning and cell proliferation \((R^2 = 0.843)\) and led to a steeper slope of the regression line, and also strengthened the correlation between learning/number of BrdU\(^+\) cells \((R^2 = 0.601)\). This might allow the hypothesis that montelukast not only increases the generation of new neurons but also might improve the functional use of these neurons.

**Leukotriene signalling in the ageing brain.** Since montelukast was developed as a leukotriene receptor inhibitor, we hypothesised that leukotriene production might correlate with the herein described age-associated brain changes. First, we analysed the mRNA expression of 5-LOX, the key enzyme in leukotriene synthesis, in different brain regions of young and old rats. We found increased levels of 5-LOX mRNA in the neurogenic regions (hippocampus and subventricular zone), but not in the cortex, of old rats (Fig. 5a). Age-related elevation of 5-LOX expression was also evident at the protein level as illustrated by

**Figure 4** | Montelukast-mediated cognitive improvements in old rats correlate with increased neurogenesis. (a) Correlation analysis showed that the individual learning scores of old vehicle-treated and old montelukast-treated rats were independent of the soma size of Iba1\(^+\) cells in the DG (vehicle: \(R^2 = 0.001; y = 81.79 + 0.034\,*x\); montelukast: \(R^2 = 0.027; y = 137.35 + 0.368\,*x\)). (b) The correlation coefficient between learning success and number of PCNA\(^+\) cells revealed a tendency towards correlation in the old vehicle-treated rats \((R^2 = 0.361; y = 63.75 + 0.063\,*x)\). Montelukast provoked a stronger correlation and led to a steeper slope of the regression line \((R^2 = 0.843; y = 80.8 + 0.129\,*x)\). (c) Similarly, learning and the number of BrdU\(^+\) cells showed a trend for correlation in vehicle-treated old rats \((R^2 = 0.225; y = 64.11 + 0.111\,*x)\), and again, this correlation was strengthened in montelukast-treated rats \((R^2 = 0.601; y = 69.01 + 0.261\,*x)\). N per group: 6 (old vehicle), 6 (old montelukast). Correlation analysis was performed using the Pearson product moment correlation test. DG, dentate gyrus.

**Figure 5** | 5-LOX expression is upregulated in the hippocampus of old rats and elderly humans. (a) 5-LOX mRNA expression was significantly elevated within the neurogenic regions (hippocampus: 2.4-fold; SVZ: 1.8-fold) of old (20 months) compared to young (4 months) rats \((n = 3\) per group). (b) 5-LOX immunoreactivity was strongly increased in the DG of old \((\ast\ast)\) compared with young \((\ast)\) rats. Although in young rats 5-LOX staining was predominately allocated to granular neurons \((\ast)\), in old rats also Iba1\(^+\) microglia \((\ast\ast\ast)\) arrowheads expressed 5-LOX. Pictures illustrate representative 5-LOX stainings; five rats per group were analysed. (c) In elderly humans \((> 60\) years \((\ast)\), intensity of 5-LOX immunostaining in the DG was clearly upregulated compared with young persons \((<35\) years \((\ast)\) lower panels show higher magnifications of the dentate gyri depicted in c,\(\ast\)) Images in c illustrate the DG of a 27 year old human person \((\ast)\), representing the young age group (hippocampi from five persons < 35 years analysed), and the DG of a 67-year-old person \((\ast)\), representing 5-LOX expression in the elderly group (hippocampi from five persons > 60 years analysed). Data are shown as mean ± s.d.; \(\ast\ast\) indicates \(P < 0.01\); Student’s unpaired t-test (a). Scale bars, 100 \(\mu m\). (b,c). DG, dentate gyrus; HC, hippocampus; SVZ, subventricular zone.
immunohistochemistry. 5-LOX immunoreactivity was more intense in the hippocampus of old compared with young rats (Fig. 5b) and was localised to DG granular neurons and to some microglia (Fig. 5b). Importantly, 5-LOX immunoreactivity was also strongly enhanced in the DG of elderly humans (> 60 years) (Fig. 5c) compared with young ones (< 35 years) (Fig. 5c').

Regarding the targets of montelukast, we and others recently demonstrated CysLTR and GPR17 mRNA expression in adult neurosphere cultures. Here, we analysed immunoreactivity of GPR17 and CysLTR1 in the hippocampal DG of old rats (Fig. 6) and in adult hippocampal neurosphere-derived cells (Supplementary Fig. 4). GPR17 was localised to progenitors and mature cells of the oligodendroglial (Olig2, RIP) and neuronal (DCX, NeuN, betaIII-tubulin) lineage, as well as to some microglia (Iba1). We excluded GPR17 expression in neural stem cells (subgranular Sox2) and in astrocytes (GFAP). In contrast, CysLTR1 expression was predominantly observed on astrocytes (GFAP), on microglia (Iba1) and on some oligodendroglial cells (Olig2, RIP). CysLTR1 was only rarely detected on neural stem cells (Nestin, Sox2) and was absent on cells of the neuronal lineage (DCX, NeuN, betaIII-tubulin).

Hence, both in vitro and in vivo, CysLTR1 expression was primarily found on non-neuronal cells, while GPR17 was predominantly present on cells of the neuronal lineage, suggesting that the montelukast-induced effects on neurogenesis might most likely be mediated through inhibition of GPR17 and not CysLTR1.

Since (i) the montelukast-induced improvement in cognition correlated best with the elevation of neurogenesis, and (ii) neuronal progenitors and differentiated neurons were devoid of CysLTR1 but expressed GPR17, we focussed the further analysis on GPR17. It is reported that GPR17 is a target of FoxO transcription factors, and that GPR17 expression requires FoxO1. Here, we first used FoxO1,3,4fl/fl mouse-derived neurospheres that were recombined with GFP/Cre retrovirus and FACS sorted for GFP+ cells to get first insight into a potential role of GPR17 in neurogenesis. We could demonstrate a drastic reduction of GPR17 mRNA and protein intensity in the recombined cells compared with control–infected cells (Fig. 7a,b). Further, in line with our present immunohistochemical observations and our previously published findings, neither control nor FoxO1,3,4/C0/C0 neurospheres expressed CysLTR1.
Figure 7 | GPR17 knockdown and knockout in neurospheres induce hyperproliferation and abolish the effects of montelukast. (a) GPR17 mRNA expression in mouse neurospheres derived from adult FoxO1,3,4/fl/fl mice and recombined with GPR17/Cre retrovirus (FoxO1,3,4/fl/fl NPCs), compared with neurospheres infected with GFP-only retrovirus (control; CTR NPCs). CysLTR1 mRNA was not expressed in mouse neurospheres and not affected by the FoxO1,3,4 knockout. BV-2 cells served as positive control for CysLTR1 primer specificity. (b) GPR17 immunoreactivity in GFP+ cells of FoxO1,3,4/fl/fl neurospheres compared with control neurospheres. (c) Overexpression of FoxO1 by lentiviral transfection upregulated GPR17 mRNA expression in FoxO1,3,4/fl/fl neurospheres (FoxO1(fl/fl) NPCs + FoxO1) to 60%. (d) Proliferative activity of FoxO1,3,4/fl/fl neurospheres assessed by a neurosphere bulk assay. Cell numbers were 6× higher in FoxO1,3,4/fl/fl neurospheres compared with controls. A 7-day montelukast treatment (10 μM) significantly increased cell numbers in control neurospheres, but did not affect FoxO1,3,4/fl/fl neurospheres. FoxO1 overexpression significantly reduced cell numbers in FoxO1,3,4/fl/fl neurospheres; Montelukast treatment provoked a significant elevation of cell numbers in the FoxO1 overexpressed FoxO1,3,4/fl/fl neurospheres. (e) In a single-cell neurosphere assay, neural stem cells with FoxO1,3,4 deletion generated significantly more neurospheres than control cells. A 7-day montelukast treatment (10 μM) significantly elevated the number of neurospheres in control, but not in FoxO1,3,4/fl/fl cells. FoxO1 overexpression decreased neurosphere numbers in vehicle-treated FoxO1,3,4/fl/fl NPCs. Montelukast treatment increased the numbers of neurospheres in FoxO1 transfected FoxO1,3,4/fl/fl NPCs compared with vehicle. (f) In neurospheres isolated from adult GPR17+/- GPR17−/− mice, high numbers of GFP+ cells are present, indicating active transcription of the GPR17 gene locus in these cells. (g) Optical density (OD) in an MTS assay was 97% increased in GPR17−/− neurospheres compared with wild-type (WT) neurospheres. A 7-day treatment with 10 μM montelukast significantly elevated OD absorbance in wild-type NPCs, but did not affect GPR17−/− cells. (h) In the single-cell neurosphere assay, neural stem cells from GPR17−/− neurospheres generated more neurospheres than control cells (P=0.0561). Montelukast (10 μM, 7 days) significantly elevated neurosphere numbers in control, but not in GPR17−/− NPCs. Three independent experiments were done in triplicates. Data are shown as mean ± s.d. (a, c-e, g, h). * indicates P<0.05, ** indicates P<0.01. Student’s unpaired t-test (a) and Two-way ANOVA (c-e, g, h) with Bonferroni post hoc tests were performed. Scale bars (b, f), 50 μm. MTK, montelukast; NPC, neuronal progenitor cells; VEH, Vehicle.
activities and psychiatric behaviours were not affected by the drug as a result of the physiological ageing process. Other general leukotriene receptor antagonist montelukast restores learning and memory function40. For example, the baseline production of pro-inflammatory cytokines like IL-1beta, TNF and IL-6 is increased and microglia have shortened and less motile processes and an enlarged cell soma in the brains of aged rodents2–5. Further, aged microglia are discussed to be impaired in their most important effector function, that is, phagocytosis6, which is for example illustrated by an age-dependent accumulation of CD68 immunoreactive phagosomal/lysosomal vesicles6,7. Here, we demonstrate that montelukast reduced age-associated neuroinflammation, in particular microglia activation. Montelukast treatment reduced the soma size of microglia in old rats, suggesting a less reactive phenotype. Interestingly, montelukast also reduced the size of CD68 immunoreactive particles in these aged microglia. Although the precise function of CD68 is not well understood9, its accumulation in the aged brain could be interpreted in the sense that aged microglia either have a higher rate of phagocytic uptake or a reduced capacity to process the incorporated material, and importantly, montelukast might modulate such activities.

Discussion

The present work demonstrates that the anti-asthmatic drug and leukotriene receptor antagonist montelukast restores learning and memory function in old rats, in which cognition is compromised as a result of the physiological ageing process. Other general activities and psychiatric behaviours were not affected by the drug treatment in the old animals.

Remarkably, montelukast serum levels of the rats used in this study (young: 288 ± 57.18 ng ml⁻¹; old: 365 ± 56.45 ng ml⁻¹; Supplementary Fig. 1) were almost identical to the maximum plasma concentrations in humans after oral administration of the clinical dose of 10 mg montelukast daily (385 ± 85 ng ml⁻¹)27, illustrating that the animals were treated with montelukast in a dose that pharmacologically resembles the one that is approved for its use in humans. A critical issue for montelukast to be developed as a CNS drug is, of course, its CNS pharmacokinetics. Although montelukast was so far always considered as a drug with only limited CNS penetration, careful re-analysis of the original pharmacokinetic report on montelukast27 reveals that one hour after i.v. drug administration, a substantial amount of radioactive equivalents of [C14] montelukast (~1/10 of the plasma levels) had reached the brain (Supplementary Fig. 1b). Most remarkably, while in plasma (and most other organs, for example, lung and muscle) montelukast levels strongly decreased within 24 h, the amount of montelukast in the brain increased. As a consequence, 24 h after drug injection, montelukast levels in the brain were even higher than in plasma (Supplementary Fig. 1b), suggesting the existence of an active transport mechanism for montelukast through the BBB. Indeed, montelukast is taken up from the intestine into the blood stream by the organic anion transporting polypeptide (OATP)2B1, a transporter that is expressed also by endothelial cells of brain capillaries44. Also, the majority (99%) of montelukast in plasma is bound to proteins, mainly albumin27, providing a BBB transport mechanism as albumin has been shown to act as a carrier through the BBB35. The potential of montelukast to enter the CNS is further strongly supported by our present pharmacokinetic results obtained from rats (Supplementary Fig. 1a). Strikingly, montelukast was also detected in the CSF in a human asthma patient, who was on the approved 10 mg per day dose of montelukast, and levels in serum and CSF were almost identical to the concentrations found in rats treated with 10 mg kg⁻¹ montelukast (Supplementary Fig. 1a). Entry of montelukast into the CNS is further supported by the plethora of preclinical data on the effects of systemic montelukast treatment on brain structure and function. In various animal models of neurodegenerative diseases, including a model of kainic acid-induced loss of memory function, an acute Huntingtong’s disease model of quinolinic acid and malonic acid injection-induced degeneration of striatal neurons, and a β-amyloid injection model of Alzheimer’s disease, treatment with montelukast attenuated behavioural deficits36–38, which was accompanied by structural brain changes such as inhibition of neuroinflammation and reduced neuronal cell death37,38. Neuroprotective effects of montelukast have also been demonstrated in animal models of acute CNS injuries and stroke. For example, in the middle cerebral artery occlusion model of stroke, pre-treatment with montelukast significantly attenuated neurological deficits, infarct volumes, brain oedema, loss of neurons and BBB disintegrity39. In a rat model of spinal cord injury, montelukast treatment resulted in neuroprotection, reduction of neuroinflammation and in improved motor function40.

Physiological brain ageing is accompanied by the appearance of specific histopathological and molecular hallmarks of neuroinflammation. For example, the baseline production of pro-inflammatory cytokines like IL-1beta, TNF and IL-6 is increased and microglia have shortened and less motile processes and an enlarged cell soma in the brains of aged rodents2–5. Further, aged microglia are discussed to be impaired in their most important effector function, that is, phagocytosis6, which is for example illustrated by an age-dependent accumulation of CD68 immunoreactive phagosomal/lysosomal vesicles6,7. Here, we demonstrate that montelukast reduced age-associated neuroinflammation, in particular microglia activation. Montelukast treatment reduced the soma size of microglia in old rats, suggesting a less reactive phenotype. Interestingly, montelukast also reduced the size of CD68 immunoreactive particles in these aged microglia. Although the precise function of CD68 is not well understood9, its accumulation in the aged brain could be interpreted in the sense that aged microglia either have a higher rate of phagocytic uptake or a reduced capacity to process the incorporated material, and importantly, montelukast might modulate such activities.
So far, the beneficial functional effects of montelukast in animal models of neurological diseases have been attributed almost exclusively to its anti-neuroinflammatory action. For example, montelukast prevented the release of the pro-inflammatory cytokines IL-1beta and TNF in BV-2 microglia cells and in primary microglia cultures.\textsuperscript{41} Also, montelukast reduced the Abeta1–42 injection-induced elevation of pro-inflammatory signalling mediators and cytokines such as NF-kappaB p65, TNF-alpha and IL-1beta \textit{in vivo}.\textsuperscript{25,30} In the present study, we reveal that montelukast elevated the expression of the anti-inflammatory cytokine TGF-beta1. Moreover, it reduced the expression levels of Nos2, a pro-inflammatory enzyme that is increased in the ageing CNS, and of the chemokine and peripheral monocyte/macrophage chemotactic CCL2, which has been identified in a proteome screen as a blood-derived ageing factor.\textsuperscript{41} Interestingly enough, these inflammatory markers were reduced to levels even lower than those of untreated controls or of cells exposed to montelukast alone. This suggests that when cells are co-stimulated simultaneously, as in the present experiments, with both LTD4 and montelukast, montelukast does not merely antagonize the activation induced by LTD4, but very likely tunes the inflammatory systems to differential responses. This, in turn, indicates that montelukast ‘primes’ the system towards an anti-inflammatory phenotype, and that it does so preferentially in the presence of pro-inflammatory signals suggesting a rather specific protective spatio-temporal effect only where and when inflammation is activated.

Alterations of the peripheral innate and adaptive immune system during ageing are well documented,\textsuperscript{42} and there is accumulating evidence that the bidirectional crosstalk between the peripheral immune system and the CNS plays a decisive role in shaping brain functions.\textsuperscript{43} Age-associated impairments of the peripheral immune system are under discussion to alter the highly coordinated interactions between the immune system and the brain, and to contribute to age-related cognitive declines.\textsuperscript{42} Since montelukast was originally developed to inhibit peripheral inflammatory reactions in asthmatic patients, and has been further reported to modulate innate immune cell functions in the periphery, it is very likely that this drug might, besides its direct effects on CNS microglia activity, exert a number of anti-inflammatory responses in the CNS indirectly by modulating the peripheral immune system, for example by downregulating CCL2 in peripheral organs.

Besides its anti-inflammatory action, montelukast stabilizes the BBB, as suggested by the claudin-5 immunohistochemistry illustrated in the present study. This is further supported by the literature, as montelukast reduced the infiltration of inflammatory cells and BBB permeability in the experimental autoimmune encephalomyelitis animal model of multiple sclerosis,\textsuperscript{44} decreased pentylenetetrazol-induced BBB dysfunction,\textsuperscript{25} and reduced BBB permeability in an animal model of traumatic brain injury.\textsuperscript{45} Considering the potential of montelukast to restore the BBB, it is even more important to emphasize that montelukast was detected in the brain and CSF after oral administration.

Here, we are demonstrating for the first time that montelukast promotes hippocampal neurogenesis, in particular progenitor cell proliferation, which results in an increased number of newly generated neurons. The effect on neurogenesis was, like the anti-inflammatory activity, specific for the old rats. Thus, montelukast might stimulate neural progenitor proliferation only in situations in which neurogenesis is compromised. Montelukast might liberate progenitors from age-associated inhibitory mechanisms, which most likely include elevated levels of leukotrienes. Obviously, the extrapolation of these results from normal ageing to neurodegenerative diseases is intriguing, and some of the beneficial effects of montelukast in animal models of neurodegeneration might well be attributed to enhanced neurogenesis. The fact that montelukast did not stimulate cell proliferation in the young animals is an important safety feature, as a hyperstimulation of neural progenitors might increase the risk to generate brain tumours.

An important issue to address is the mode of action of montelukast and if the drug-induced improvement in cognition is mediated through elevation of neurogenesis. Regression and correlation analyses suggested a causal link between cognition and neurogenesis in the old rats, and montelukast even fostered the correlation between neurogenesis and cognition in these animals. In contrast, learning success showed no correlation with the anti-inflammatory effects of montelukast. Thus, the montelukast-induced elevated neurogenesis rather than the anti-inflammatory action of montelukast might be the prime causal factor related to the improved cognition in old rats. This, however, requires further experimental evidence, for example through genetic, physical or pharmacological modulation or inhibition of adult hippocampal neurogenesis. Nevertheless, a correlation between cognitive performance and DG proliferation has already been reported previously in aged rodents.\textsuperscript{46} Moreover, pharmacologic stimulation of neurogenesis by countering age-associated inhibitors of neurogenesis has been shown to improve cognitive functions in ageing.\textsuperscript{47}

In general, a clear dissection between neurogenesis- and neuroinflammation-mediated effects on cognition is not straightforward as neurogenesis and neuroinflammation strongly influence each other. For example neural progenitors induce microglia proliferation and activation,\textsuperscript{48} and vice versa, microglia regulate adult hippocampal neurogenesis.\textsuperscript{49} We still lack detailed knowledge on the exact interrelations between specific patterns of neuroinflammation and neurogenesis. Pharmacological modulation of neuroinflammation with minocycline altered hippocampal inflammatory cytokine gene expression and significantly improved learning in the water maze paradigm in aged mice, but did not change the rate of neurogenesis in these animals, while it elevated neurogenesis without affecting cognition in young animals.\textsuperscript{50} Hence, the minocycline-induced effects on cognition in the aged animals appear to be independent of adult hippocampal neurogenesis modulation. This further suggests that minocycline and montelukast, although both being anti-inflammatory agents, might address different types of neuroinflammation with specific consequences on neuroprotection and regenerative responses such as neurogenesis.

As one of the potential mechanisms of montelukast action, we identified elevated leukotriene production in the brains of old rats and elderly humans, and the montelukast targets CysLTR1 and GPR17 being expressed in the neurogenic niche. Indeed, montelukast might interact with CysLTR1 and with GPR17 in the brain, as we detected montelukast after oral delivery in the brain parenchyma and in the CSF. Since GPR17 is, besides being expressed in oligodendroglia, also expressed in the neuronal lineage, and since genetically mediated reduction of GPR17 (either through deletion of FoxO1,3,4 or through direct knockout of the GPR17 gene) increased the neural progenitor proliferation \textit{in vitro}, we suggest that the effects of montelukast on neurogenesis and cognition are most likely mediated through inhibition of GPR17. Indeed, FoxO1,3,4 as well as GPR17 knockout neurospheres did not respond to montelukast in terms of cell proliferation, and responsiveness in FoxO1,3,4 knockout cells was rescued through viral mediated expression of FoxO1, which also restored GPR17 expression. FoxO plays a crucial role in regulating neuronal stem cell proliferation and self-renewal, as FoxO1,3,4 knockout animals show increased brain size and proliferation of neural progenitor cells during early postnatal life leading to an exhaustion of the stem cell pool and to low
neurogenesis in adulthood. This has been attributed to the effects of a number of FoxO targets, such as genes that cooperatively act as soluble inhibitors of the Wnt pathway to repress Wnt signalling in neural stem cells. GPR17 signalling might be one of such FoxO regulated pathways that are involved in neural stem and progenitor modulation, in particular in the aged brain. Targeting GPR17 might further be relevant for acute CNS lesions such as spinal cord injury, since GPR17 has been identified in spinal cord ependymal stem cell line cells, and the in vivo knockdown of GPR17 by antisense oligonucleotides in a spinal cord injury model reduced tissue damage and motor deficits.

What are the upstream signalling components relevant for the observed montelukast effects? Montelukast is an antagonist of CysLT1 and of GPR17, favouring the hypothesis that leukotrienes are the relevant ligands that cause neuroinflammation and reduce neurogenesis in the brains of old animals. The leukotriene signalling pathway is currently under discussion to contribute to brain inflammation associated with age-related dementia and neurodegenerative diseases. In the CNS, 5-LOX mRNA levels are elevated in the hippocampus in an Alzheimer's disease transgenic model and in the hippocampus of Alzheimer's disease patients. In addition, the concentrations of 5-LOX transcripts and of leukotrienes are increased specifically in the hippocampus of old rodents, as shown in the present work and by others. Furthermore, intracerebral infusions of LTD4 in adult mice lead to an accumulation of Aβ 1–40 and Aβ 1–42 in the hippocampus and cortex as well as to memory deficits, which could be inhibited by pre-treatment with the CysLT1 antagonist pranlukast. Although leukotrienes mediate and/or exaggerate pathogenesis in the CNS, it appears that these effects of leukotrienes are context-dependent. For example, leukotrienes alone did not induce neuronal cell death in rat cortical neurons in vitro. Besides leukotrienes, other ligands and agonists for GPR17 have been described, which might well contribute to the activation of the receptor in ageing and in neurodegenerative diseases. For example, UDP-glucose, which is elevated in the brain after damage, has been described as GPR17 agonist, and UDP-glucose stimulates oligodendroglial progenitor migration and differentiation by activating GPR17. Also, oxyestrols, which act as inflammatory mediators, have recently been identified as potential GPR17 ligands and agonists. Nevertheless, more recent studies using GPR17 overexpression in different cell lines reported that neither UDP-glucose nor leukotrienes were ligands for GPR17, leaving the question of the physiologic ligands of GPR17 still open for future discoveries.

Similarly, the exact downstream signalling components that mediate the beneficial effects of montelukast in the old rats are not fully deciphered at present, and leukotriene receptor-independent effects cannot be excluded. In this context, Yamagami et al. suggested that voltage-gated calcium channels mediated neuroprotective effects of the leukotriene receptor antagonist pranlukast in an in vitro model of brain ischaemia. Moreover, a network-based interference approach has recently identified montelukast as a potential inhibitor of dipeptidyl peptidase-IV, a proteolytic enzyme and target of a number of approved anti-diabetes drugs which increase the release of insulin. Of interest in the context of the present study, such anti-diabetic drug treatment could attenuate pathogenesis in animal models of Alzheimer's disease, Parkinson's disease and in an animal model of multiple sclerosis.

In summary, we demonstrate for the first time the possibility of using montelukast to functionally rejuvenate the aged but otherwise healthy brain. Oral treatment with montelukast restored learning and memory in old rats, which was significantly impaired in comparison to young animals. Considering the various beneficial effects of montelukast on CNS functions in different animal models, this illustrates that leukotriene receptors and their underlying signalling mechanisms might contribute to the development of many neurological deficits, and that montelukast, by targeting these mechanisms, might be able to modulate and to improve a number of neurological functions in various CNS diseases.

Methods

Methods that were required to generate Supplementary Data are provided in the Supplementary Material.

Animals. For the in vivo experiments, young (4 months) and old (20 months) male transgenic F344/D radix rats were used. They express the fluorescent reporter DiR2ed under control of the neuronal precursor cell-specific promoter of the doublecortin (DCX) gene and were originally generated at the transgenic mouse facility of the University of Heidelberg, Germany, and previously described. For all other studies on rat tissue, young (4 months) and old (20 months) male wild-type Fisher 344 rats (Charles River, Germany) were used. All rats were bred and housed in the animal facility of the PMU Salzburg under standard conditions of a 12 h light/dark cycle with food and water ad libitum. All animal care and use was in accordance with the European Community Council Directive (86/609/EEC) and approved by the Federal State Land Salzburg, Austria (20091-TVG/406/6-2010). The numbers of rats used to reach statistically significant differences were calculated using G*Power 3.1.7 software. Standard power analyses with α = 0.05 and a power of 0.8 were performed to calculate power and sample sizes. For neuropsychology culture experiments, FoxO1/3/4± mice were obtained from Ronald A. DePinho (University of Texas MD Anderson Cancer Center, Houston, TX) and GPR17−/− mice (2 months old, male, N = 10 animals) were provided by Douglas Fields (NICHHD, Bethesda, USA). Mouse tissue was collected in conformity with the Austrian Federal Law for experiments on living animals (TVG2012/28).

Montelukast treatment and BrdU injections. Montelukast sodium powder (Sigma), dissolved in ethanol for maximum solubility and then further diluted (1:9 ratio) with a 0.9% saline (NaCl) solution, was administered daily per oral gavage (0.1 g of body weight for 42 days for adult (4 months) and old (20 months) rats (N = 7–10 rats/group). Age-matched control rats received oral gavage of the vehicle solution (10% ethanol in 0.9% NaCl). For the analysis of cell survival and cell differentiation, all rats received intra-peritoneal injections of BrdU (Sigma) at 50 mg kg−1 of body weight dissolved in 0.9% saline solution on days 14, 15, 16 and 17. Weight and general appearance of the rats was recorded daily during the course of the experiment. Criteria for early termination of treatment were obvious signs of pain, apathy, and loss of weight more than 15%. During the course of this experiment, one vehicle-treated rat (20 months old) was killed because of a weight loss of more than 15% for unknown reasons.

Behavioural tests. 28 days after starting the montelukast treatment, several standardised behavioural tests were carried out. All parameters analysed were recorded via the use of the video tracking software Ethovision 9.0 (Noldus Information Technology).

In the open field test (day 29 after the first montelukast administration), spontaneous changes in general locomotor activity were detected. The open field was a black circular plastic plate with a diameter of 1 m and a 5 cm high wall, set in the middle of the testing room. Before a new trial was begun, the open field was cleaned with 70% ethanol. Each rat was placed into the centre of the plate and was allowed to explore the apparatus for 5 min. The total distance moved was recorded.

The elevated plus maze (performed on day 30 after the first montelukast administration) was used to test anxiety of the rats. The wooden maze had four arms of the fore arms at opposite dimensions (30 cm) and was situated above the floor. Two opposite arms were enclosed by 20 cm high opaque walls (closed arms). To reduce olfactory influences, the maze was cleaned with 70% ethanol after every trial. The rats were allowed to perform trials with 10 min of free exploration starting in the central intersection facing an open arm. The camera software recorded the time spent in the closed arms, in the open arms, and in the centre zone, and the overall distance moved. The forced swim test was performed on day 33 to analyse depressive-like behaviour. Rats were forced to swim for 10 min in a square plastic tank (40 cm side length) filled to a depth of 30 cm with tap water (20 ± 1 °C). During the forced swimming session, the behaviour was recorded by a video system and scored by a trained observer, quantifying absolute time measurements. The behaviour of the animals was assigned to one of the following behavioural categories: (1) ‘struggling’, defined as movements during which the forelimbs broke the surface of water; (2) ‘swimming’, defined as movement of the animal induced by movements of the fore and hind limbs without breaking the water surface; and (3) ‘immobility time’, defined as the behaviour during which the animal used limb movement just to keep its equilibrium without any movement of the trunk. After the 10 min
swimming session, animals were gently dried using a towel and returned to their home cage.

The Morris water maze test (days 35–41) was used to assess the ability of spatial learning and memory. The apparatus consisted of a circular swimming pool built of black plastic (170 cm diameter, 30 cm height), filled with 21 °C ± 1 °C tempered water. The tank was virtually divided into four equal quadrants, with a submerged hidden 10 × 10 cm flagstone platform placed 3 cm below the water surface in the middle of the target quadrant. The position of the platform was kept unaltered throughout the learning sessions. In the testing room, several black cue symbols were put on each wall for spatial orientation. The water maze task was carried out twice a day for 5 consecutive days. One day before starting the learning experiment (day 0), each rat was put into the water and was allowed to locate the submerged platform for 60 s. If the animal failed to find the platform within the 60 s, it was guided onto the platform and allowed to remain there for 10 s. For the learning tasks on days 1–5, each rat was put into the water at one of four starting positions, the sequence of which was selected randomly. In each trial, a ceiling time of 60 s to find the platform was defined. The escape latency time to locate the platform and the distance moved during the trial were recorded with the camera software as indices of spatial learning. On day 6, after the learning phase of the experiment, a probe trial was performed. Here, the platform was removed and each animal was allowed to explore the pool for 60 s. From the probe trial, several parameters were recorded as indices of memory: time spent in platform quadrant, number of crossings of the former platform location, number of crossings of ‘zone P’ (a defined circular area with 20 cm diameter enclosing the former platform location) and time spent in zone P. A learning index was calculated for every rat based on the individual learning curve and was used as a measure of spatial learning paradigm. For this, the value of the area under the curve between days 0 and 5 was calculated and subtracted from 300, which represents the value for the worst possible performance. Thus, high values indicate successful learning, while low learning indices illustrate poor spatial learning abilities.

For the object location memory test, a test of hippocampus-dependent memory functions, a different cohort of old (20 months) male Fisher 344 rats was used. Animals received montelukast and vehicle administration as well as BrdU treatment, the object location memory test was performed. Prior to training, rats habituated to the circular apparatus (a 20 × 20 × 40 cm open top cylinder) for 5 min day−1 in the absence of objects for 3 days. During the training period, rats were placed into the box with two identical objects (beverage cans; 13.5 × 5.2 cm) and allowed to explore for 15 min. During the long-term retention test, that is, 24 h following the training period, one of the object locations was shifted and the rats were allowed to explore the experimental apparatus for 10 min. Exploration was scored when a rat’s head was oriented toward the object within a distance of 2 cm or when the nose was touching the object. The relative exploration time (t) of the newly positioned object was recorded and expressed as a novelty index (NI = 1 – tnew/ttotal (× 100)).

### Perfusion and tissue processing

On day 42, all rats were deeply anesthetized using a ketamine (20.36 mg ml−1), xylazine (5.38 mg ml−1) and acepromazine (0.29 mg ml−1) mixture (compounded per mg kg−1 body weight) injection. The animals were intracardially perfused with 0.1 M NaCl solution, followed by a 4% paraformaldehyde, 0.1 M sodium phosphate solution (pH 7.4). The brains were dissected and post-fixed in 4% paraformaldehyde, 0.1 M phosphate-buffered saline (pH 7.4) for 20 min. Tissues were then transferred to 30% sucrose solution. Brains were cut into 40 μm sagittal sections using a sliding microtome on a rotation microtome and stored at 4 °C. The brains were subsequently processed into liquid paraffin. All tissue samples were cut at 5 μm and mounted with Neo-Mount (Merck).

### Immunohistochemistry

Free-floating sections were treated with 0.6% H2O2 in tris-buffered saline (TBS: 0.15 M NaCl, 0.1 M Tris-HCl, pH 7.5) for 30 min, followed by three washes with TBS. For immunological detection of PCNA and BrdU, sections were incubated in 0.3 M NaCl/30 mM citrate buffer (pH 7.0)/50% triis-buffered saline (TBS: 0.15 M NaCl, 0.1 M Tris-HCl, pH 7.5) for 30 min, rinsed in 0.1 M borate buffer (pH 8.5) for 10 min and again rinsed in TBS. Sections were blocked with a solution composed of TBS, 0.1% Triton X-100, 1% BSA and 0.2% teleostean gelatin (Sigma) for 20 min, and incubated overnight at 4 °C with a rabbit anti-5-LOX antibody (1:50; 610695, BD Pharmingen) diluted in the blocking solution. After incubation, the sections were washed in TBS, incubated with a biotinylated rabbit anti-mouse antibody (1:500, Jackson Immuno Research) for 1 h, washed in TBS, and incubated for 1 h in a peroxidase-avidin complex solution (Vectastain Elite ABC kit; Vector Laboratories). The peroxidase activity of immunocomplexes was revealed with a solution composed of 0.03% hydrogen peroxide and 0.03% diaminobenzidine (Vector Laboratories), 0.01% H2O2, and 0.04% NiCl2. The following antibodies and final dilutions were used. Primary antibodies were added, vortexed and centrifuged (15 min at 12,000 g). After transferring the aqueous phase into a new tube and adding 350 μl of ethanol, RNA extraction was performed using the ‘Analyse Particles’ function of ImageJ 1.45 s (ImageJ website: http://imagej.nih.gov/ij/). For each animal at least 40 microglial cells were assessed. The corresponding tissue area was measured and multiplied by 40 μm to obtain the tissue volume. To assess cell densities, the total number of cells counted was divided by the sample volume and represented as cells per mm3. Because of the low level of neurogenesis in old animals, in case the total number of BrdU-positive cells remained below 50, every BrdU-labelled cell detected was examined. For assessment of possible alterations in the activation state of microglia within the hippocampus, the somas of the Iba1- cells within the DG were determined using the ‘Analyse Particles’ function of ImageJ 1.45 s (ImageJ website: http://imagej.nih.gov/ij/). For each animal at least at least 40 microglial cells were assessed. For immunolabelling experiments to ensure specificity of the antibody staining, Chromogenic immunodetection was performed using a 1:3,3-diaminobenzidine (Vector Laboratories), 0.01% H2O2, and 0.04% NiCl2. Sections were mounted with Neo-Mount (Merck).

### Human post-mortem brain tissue

Human hippocampal tissue sections from autopsy samples of young adult (< 35 years, N = 5) and elderly (> 60 years, N = 5) humans with a post-mortem interval < 24 h were used. Human post-mortem tissue was obtained from the collection of the Department of Neuropathology at the University Hospital Erlangen (Germany). Written informed consent was obtained from the patients’ next of kin. The use of these specimens for scientific purposes was in accordance with institutional ethical guidelines and was approved by the ethics committee of the University of Erlangen (Germany). All samples used were obtained from individuals without any neurological or psychiatric diagnoses. After tissue extraction, the brain samples were fixed overnight in 10% formalin and subsequently processed into liquid paraffin. All tissue samples were cut at 5 μm on a rotation microtome and stored at 4 °C.

### Formalin-fixed paraffin-embedded immunohistochemistry

Formalin-fixed paraffin-embedded human hippocampal tissue sections were deparaffinized by incubation in xylene, rehydrated by a graded series of ethanol and rinsed in distilled water for 5 min. For antigen retrieval, the slides were steamed in 0.01 M sodium citrate buffer, pH 6.0 at 100 °C for 20 min, followed by three washes in TBS. Endogenous peroxidases were quenched with 0.3% hydrogen peroxide for 20 min. Sections were blocked with a solution composed of TBS, 0.1% Triton X-100, 1% BSA and 0.2% teleostean gelatin (Sigma) for 20 min, and incubated overnight at 4 °C with a rabbit anti-5-LOX antibody (1:50; 610695, BD Pharmingen) diluted in the blocking solution. After incubation, the sections were washed in TBS, incubated with a biotinylated rabbit anti-mouse antibody (1:500, Jackson Immuno Research) for 1 h, washed in TBS, and incubated for 1 h in a peroxidase-avidin complex solution (Vectastain Elite ABC kit; Vector Laboratories). The peroxidase activity of immunocomplexes was revealed with a solution composed of 0.025 mg ml−1 3,3-diaminobenzidine (Vector Laboratories), 0.01% H2O2, and 0.04% NiCl2. Sections were mounted with Neo-Mount (Merck).

### RNA extraction and quantification of 5-LOX mRNA

To detect 5-LOX mRNA levels in different brain regions of young and old rats, total RNA was extracted from rat hippocampus, subventricular zone and cortex from 4-month- and 20-month-old Fisher 344 wild-type rats. Animals were decapitated and the tissues of interest were dissected. Brain samples were homogenized in 1 ml of Trizol (TRI Reagent, Molecular Research Centre). The homogenate was added, vortexed and centrifuged (15 min at 12,000 g). After transferring the aqueous phase into a new tube and adding 350 μl of ethanol, RNA extraction was performed using a new column and eluted with 30 μl of RNase-free water.
corresponding to the number of counted cells were added and the cells were cultured in a 15-ml Falcon tube and centrifuged at 120 g for 5 min. The pellet was resuspended in 2 ml culture medium. Virus particles, which were transduced with a GFP-expressing retrovirus, and which had been transduced with a GFP/Cre-Recombinase-expressing retrovirus, were used in all experiments.

Retroviral transduction of neural stem cells. Mouse neurospheres from young C57BL/6 mice were used as a control. The neurospheres were dissociated into single cells. Total numbers of cells were counted and normalized to untreated control cells.

Analysis of cell proliferation—MTS-assay. After passing, cells were seeded in 96-well plate cultures at a concentration of 5 × 10^4 cells/ml in a volume of 100 μl NB/B27 medium under proliferative conditions (with 20 ng/ml EGF and FGF-2). Montelukast was added to a final molarity of 10 μM on days 0, 2, 4 and 6 (control cells received medium only). After 7 days, neurospheres were harvested and dissociated into single cells. Total numbers of cells were counted and normalized to untreated control cells.

Neurosphere bulk assay. A total of 5 × 10^4 cells were seeded in a 24-well culture flask in 5 ml medium under proliferative conditions (with 20 ng/ml EGF and FGF-2). Montelukast was added to a final molarity of 10 μM on days 0, 2, 4 and 6 (control cells received medium only). After 7 days, neurospheres were harvested and dissociated into single cells. Total numbers of cells were counted and normalized to untreated control cells.

For quantitative gene expression analyses, total RNA was isolated from control and FoxO1/3/4 KO neurospheres (with and without FoxO1 rescue) by using the RNeasy Mini-Kit (Qiagen) according to the manufacturer’s protocol. Possible genomic DNA contamination was eliminated by on-column DNAse treatment using the RNAse-free DNAse-Set according to the manufacturer’s protocol. cDNA was transcribed using the Fermentas RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) according to the manufacturer’s instructions.

Quantitative PCR with reverse transcription was performed using the StepOnePlusTM Real-Time PCR System. Brilliant II Fast SYBR Green quantitative PCR (qPCR) Master Mix was used for PCR reactions according to the manufacturer’s protocol. qPCR primers were designed using the software’s Primer3 (http://primer3.sourceforge.net) and NetPrimer (http://www.premierbiosoft.com). Amplicon sizes ranged from 100–250 bp. Suitability of qPCR primer was analysed by evaluation of melting curves and by determination of the efficiency via a standard curve. For quantitative expression analysis, the delta delta Ct method was applied to determine the relative quantity of target sequence using a reference sample (control) and an endogenous control target sequence. RPL27 was used as an endogenous control target. Primers for qPCR were as follows: RPL27: forward, CTTGACTGGCCTCTCTCTTT; reverse, CGCAGGTGAGCTAGAGAGGA. CysLTR1: Forward, CCTCTCCGFTGTGCTTATATCAT; reverse, ACGCGAAGAAAGCTGTTGGCT.

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was performed on a confocal scanning laser microscope (LSM 710, Zeiss) with LSM software (ZEN 2011).

**Statistical analysis.** Histological and behavioural experiments were randomized and performed blinded. Groups were unblinded at the end of each experiment before statistical analysis. Statistical analyses were performed using the GraphPad Prism software (GraphPad Software) and IBM SPSS Statistics 20® software (IBM Corporation). Data were tested for normality using the Kolmogorov–Smirnov or Shapiro–Wilk test, and equality of variance was confirmed using the F-test. Means between two groups were compared by the two-tailed unpaired Student’s t-test or, in case of non-gaussian distribution, by using the two-tailed Mann–Whitney U-test. Data from multiple groups were analysed by one-way ANOVA, and two-way ANOVA, followed by the appropriate post hoc tests (as indicated in the figure legends) when necessary. Learning index correlation analyses were performed with the ‘Pearson Product Moment Correlation test’.

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Author contributions

L.A., I.M. and S.C.-D. designed the studies. L.A. and J.M. analysed the data and wrote the manuscript. J.M. and C.S. performed the in vivo experiments. J.M., M.R. and M.C. performed the montelukast pharmacokinetics studies. J.M., B.K., M.J. and M.B. performed histological studies. J.M., B.K. and P.R. performed the in vitro BV-2 experiments and PCR analysis, J.M., F.J.R. and L.G. performed the in vitro neurosphere experiments and ICC stainings, and I.S. and D.C.L. performed the in vitro Foxt studies. R.G. and S.I. performed multi-electrode array analyses. R.C. and I.B. provided human hippocampus tissue. F.R.L., F.J.R., M.P.A., N.S., H.C.B., S.C.-D. and U.B. supported in discussing and editing the manuscript.

Additional information

Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

Competing financial interests: L.A., S.C.-D. and J.M. are inventors on the patent application WO 2014090990 A1 ‘Leukotriene pathway antagonists for the treatment of dementia, cognitive deficits in parkinson’s disease and/or learning and memory deficiencies in parkinson’s disease’. All other authors declare no competing financial interests.

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