Loss of Cln5 causes altered neurogenesis in a mouse model of a childhood neurodegenerative disorder

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ABSTRACT

Neural stem/progenitor cells (NPCs) generate new neurons in the brain throughout an individual’s lifetime in an intricate process called neurogenesis. Neurogenic alterations are a common feature of several adult-onset neurodegenerative diseases. The neuronal ceroid lipofuscinoses (NCLs) are the most common group of inherited neurodegenerative diseases that mainly affect children. Pathological features of the NCLs include accumulation of lysosomal storage material, neuroinflammation and neuronal degeneration, yet the exact cause of this group of diseases remains poorly understood. The function of the CLN5 protein, causative of the CLN5 disease form of NCL, is unknown. In the present study, we sought to examine neurogenesis in the neurodegenerative disorder caused by loss of Cln5. Our findings demonstrate a newly identified crucial role for CLN5 in neurogenesis. We report for the first time that neurogenesis is increased in Cln5-deficient mice, which model the childhood neurodegenerative disorder caused by loss of Cln5. Our results demonstrate that, in Cln5 deficiency, proliferation of NPCs is increased, NPC migration is reduced and NPC differentiation towards the neuronal lineage is increased concomitantly with functional alterations in the NPCs. Moreover, the observed impairment in neurogenesis is correlated with increased expression of the pro-inflammatory cytokine IL-1β. A full understanding of the pathological mechanisms that lead to disease and the function of the NCL proteins are critical for designing effective therapeutic approaches for this devastating neurodegenerative disorder.  

KEY WORDS: Neurogenesis, Neuronal ceroid lipofuscinoses, Batten disease, Lysosomal storage disease, Stem cells, CLN5

INTRODUCTION

Neurogenesis occurs throughout life in the mammalian brain (Altman, 1962), and is correlated with learning and memory functions. During this complex process, new neurons are generated in specific neurogenic niches in the brain, including the subgranular zone of the hippocampal dentate gyrus and the subventricular zone of the lateral ventricles. The newly generated neurons are derived from neuronal stem/progenitor cells (NPCs), which proliferate and produce neuronal and glial cells under physiological conditions. This dynamic process requires coordinated cell proliferation, apoptosis, migration, differentiation and functional integration. The regulation of neuronal development in the postnatal period is intricate and involves numerous intrinsic and extracellular factors, including growth factors and cytokines (Faigle and Song, 2013).  

Neurogenic impairments occur in the aging brain and in several neurodegenerative diseases. During aging, the proliferation of NPCs is reduced, resulting in a reduction in the production of new neurons. However, the survival and differentiation of the limited number of newly born cells is thought to remain unaltered during the aging process (Shruster et al., 2010). There is increasing evidence demonstrating that alterations in neurogenesis occur in neurodegenerative disorders, including Alzheimer’s disease, Parkinson’s disease and many others (Winner and Winkler, 2015). In addition to a gradual loss of existing neuronal populations, impairments in the endogenous capacity for cell renewal are a common pathological feature of these diseases. In most instances, the exact mechanisms underlying neurogenic defects remain unknown. Moreover, contradictory reports exist as to whether neurogenesis is increased or reduced during neurodegenerative diseases.  

Neuronal ceroid lipofuscinoses (NCLs) form the most common group of neurodegenerative disorders that primarily affect children. This group of inherited disorders is characterized by progressive blindness, difficulties in learning, motor impairment and premature death. NCL is caused by loss of the ceroid-lipofuscinosis neuronal (CLN) proteins, 13 of which have been identified to date (Mole and Cotman, 2015). Depending on the affected CLN protein, the age of disease onset and rate of progression varies, yet the ultimate outcome is the same. Pathological features of the NCLs include accumulation of lysosomal storage material, neuroinflammation and neuronal degeneration. The CLN5 disease form of NCL (Santavuori et al., 1973) has a mean age of onset of 5.6 years (Xin et al., 2010) and is caused by mutations in the CLN5 gene. The CLN5 protein is a glycoprotein that does not share any apparent homology with other proteins. It is associated with lipid metabolism, myelination, protein transport and endosomal sorting (Carcérol-Trullols et al., 2015). Recently, it was shown that CLN5 might be important during embryonic development (Fabritius et al., 2014). However, the exact function of CLN5 remains unknown.  

The present study sought to examine neurogenesis in a neurodegenerative disorder caused by loss of Cln5. We demonstrate for the first time the importance of CLN5 for neurogenesis. Neurogenic alterations are manifested by reduced proliferation, altered differentiation and impaired function of NPCs. Furthermore, increased neurogenesis was found to be associated with high levels of interleukin-1β (IL-1β) in the diseased brains, and
altered IL-1β signaling was observed in NPCs in vitro. Our results suggest a newly identified role for CLN5 in neurogenesis.

RESULTS

Cln5 deficiency increases hippocampal neurogenesis

To assess whether the disease caused by loss of Cln5 is associated with neurogenic alterations in vivo, brains from Cln5-knockout (KO) and wild-type (WT) mice at various ages were processed for immunohistochemistry. Quantification of the expression of doublecortin (DCX), a marker expressed by neuronal precursor cells and immature neurons, revealed a 23% increase in the number of newly born neurons in the hippocampal dentate gyrus at the age of 5 months (Fig. 1A) and a 29% increase at the age of 7 months (Fig. 1B). However, changes in the number of DCX-positive cells

Fig. 1. Neurogenesis is increased in Cln5-deficient brains. (A) Representative DCX immunostaining images from hippocampal dentate gyrus of 5-month-old WT and Cln5-KO mice. The graph shows the numbers of DCX-positive cells counted from 4-6 brain sections at 400-μm intervals from 6 animals of each genotype. **P<0.001. Scale bar: 200 μm. (B) Representative DCX immunostaining images from hippocampal dentate gyrus of 7-month-old WT and Cln5-KO mice. The graph shows the numbers of DCX-positive cells counted from 4-6 brain sections at 400-μm intervals from 6 animals of each genotype. **P<0.001. (C) The immunoreactive area occupied by DCX staining was quantified from 4-6 brain sections at 400-μm intervals from 7-10 mice of each genotype at the age of 3 months. (D) The number of BrdU-positive cells was calculated from 4-6 brain sections at 400-μm intervals of the hippocampal dentate gyrus of 5-month-old WT and Cln5-KO mice after 7 days of intraperitoneal BrdU injection. N=7/genotype. (E) The number of DCX-positive cells in the hippocampal dentate gyrus was calculated following 7 days of BrdU injection and is shown proportional to the number of BrdU-positive cells. N=7/genotype. (F) The immunoreactive area occupied by Ki-67 staining was quantified from 7-month-old WT and Cln5-KO lateral wall of the lateral ventricle. N=6-7/genotype. *P<0.05. The data in this figure are expressed as mean number of cells or the mean area occupied by the immunoreactivity in the dentate gyrus of the hippocampus±s.d. Statistical method: 2-tailed t-test.
were not observed at the age of 3 months (Fig. 1C). Quantification of hippocampal dentate gyrus bromodeoxyuridine (BrdU) labeling following a 7-day BrdU administration revealed that the numbers of proliferating cells were similar in WT and Cln5-KO dentate gyrus at the age of 5 months (Fig. 1D). The ratio of DCX-positive cells to BrdU-labeled cells trended towards an increase in the hippocampal dentate gyrus (Fig. 1E). In the subventricular zone of the lateral ventricles of Cln5-deficient mice, the number of Ki-67 (marker of proliferation)-positive cells was increased by 38% at the age of 7 months (Fig. 1F). This indicates that cell proliferation is increased in the subventricular zone in Cln5-KO mice. Taken together, these results indicate that Cln5 deficiency alters neurogenesis in a time-dependent fashion, and that the number of newly forming precursors and immature neurons is significantly increased in Cln5-KO mice.

**Proliferation is increased in Cln5 deficiency**

Increased neurogenesis can occur through several mechanisms, including accelerated proliferation. To understand whether the observed increase in neurogenesis in Cln5-KO brains was related to the increased proliferation of cells, we next applied an *in vitro* approach to study the phenotype of NPCs isolated from Cln5-KO hippocampi. NPCs cultured from embryonic hippocampi grow as neurospheres and serve as an *in vitro* model for the study of neurogenesis. To examine the effect of Cln5 deficiency on the proliferation of NPCs isolated from the hippocampi, we cultured single NPCs in the presence of growth factors and assessed sphere size and BrdU incorporation by pulse labeling. Cln5-deficient NPCs formed larger neurospheres in comparison to WT neurospheres (Fig. 2A,B). Quantification of the sphere size revealed that Cln5-KO spheres were approximately 38% larger than the WT controls after 5 days in culture (Fig. 2C). BrdU pulse labeling experiments also demonstrated that the number of proliferated Cln5-deficient cells was approximately 32% higher compared to WT cells (Fig. 2D). Taken together, these results demonstrate that Cln5 deficiency causes increased proliferation in the neurogenic niche *in vivo* and of NPCs *in vitro*.

**Loss of Cln5 does not increase apoptosis in the hippocampi**

To ensure that the observed increase in neurogenesis in Cln5-deficient mice was not related to alterations in the degree of apoptosis, hippocampal caspase-3 levels were assessed by immunohistochemical stainings and quantitative real-time polymerase chain reaction (qRT-PCR). The mRNA expression level of caspase-3 was not altered in the hippocampi of 7-month-old Cln5-KO mice (Fig. 3). Similarly, the degree of cleaved caspase-3 immunoreactivity or TUNEL staining was not altered in the hippocampi when WT mouse brain sections were compared to those of Cln5-deficient brains (data not shown). These results suggest that the observed alterations in neurogenesis are not related to changes in hippocampal apoptosis caused by Cln5 deficiency.

**Cln5 deficiency alters the phenotype of neuronal progenitors**

To further characterize the effects of Cln5 deficiency on the phenotype of NPCs, we next examined the expression of Cln5 in NPCs, the migration and differentiation of NPCs, and NPC neurite length.

It has been reported that Cln5 mRNA expression is approximately 4-fold higher in microglia than in neurons (Schmiedt et al., 2012). However, the expression of Cln5 in hippocampal NPCs has not been measured. To quantify the expression level of Cln5 mRNA in NPCs, we compared NPC expression to that of microglia using qRT-PCR. These analyses showed that Cln5 is expressed at a similar level in NPCs compared to microglia (Fig. 4). The expression level of Cln5 in NPCs suggests an important role of Cln5 in neurogenesis.

To determine the effect of Cln5 loss on the phenotype of hippocampus-derived NPCs, we first assessed NPC migration. In the absence of growth factors, single NPCs began to migrate away from the edges of the neurosphere and differentiate. The migration of NPCs was determined by measuring the distance traveled from the edge of the neurosphere by individual cells (Fig. 5A). In comparison to WT cells, the migration of Cln5-deficient NPCs was reduced by close to 40% at 7 days after plating (Fig. 5B). This reduction in the migration of Cln5-KO cells was also observed at 3 days after plating (data not shown).

In order to further understand the role of Cln5 in neurogenesis, we next assessed the effect of Cln5 loss on the differentiation of NPCs. NPC differentiation was assessed by immunocytochemical staining for DCX, neuron-specific class beta III tubulin (TuJ-1; a marker of mature neurons) and glial fibrillary acid protein (GFAP; a marker of astrocytes). Fig. 6 demonstrates the proportion of cells that are positive for each of the markers at 3 and 7 days after plating. After 3
days of differentiation, the proportion of immunoreactivity occupied by Tuj-1 staining is approximately 70% lower than that of DCX (Fig. 6B). Four days later, Tuj-1 staining is higher than that of DCX, indicating the maturation of the neuronal cells (Fig. 6C). The majority of the cells are positive for GFAP at both 3 and 7 days after differentiation. Comparison of the differentiation of WT NPCs to that of \( {\text{Cln5}} \)-deficient NPCs revealed that the proportion of both DCX- and Tuj-1-expressing cells was higher in the \( {\text{Cln5}} \)-KO cultures after 3 days of differentiation (Fig. 6D). The expression of DCX was increased 1.6-fold in the \( {\text{Cln5}} \)-deficient NPC cultures. The expression of Tuj-1 appeared to also be increased in the \( {\text{Cln5}} \)-deficient cells, but this difference was not statistically significant. Loss of \( {\text{Cln5}} \) did not alter the proportion of GFAP-positive cells.

Given that \( {\text{Cln5}} \) loss appeared to increase neuronal differentiation, but reduce cellular migration, we next explored the appearance of the cells in more detail by measuring the numbers and lengths of neuronal processes in Tuj-1-positive WT (Fig. 7A) and \( {\text{Cln5}} \)-KO (Fig. 7B) cells. In comparison to WT controls, the \( {\text{Cln5}} \)-deficient cells had an approximately 3-fold increase in the number of processes per nucleus (Fig. 7C). However, the mean length of each \( {\text{Cln5}} \)-KO neurite was reduced by over 40% (Fig. 7D). These data suggest that, even though the number of cells of the neuronal lineage and the lengths of neuronal processes are increased in \( {\text{Cln5}} \) deficiency, loss of \( {\text{Cln5}} \) results in impairment of the formation of mature neurons with long processes. In support of this data, an altered neuronal phenotype was observed in the hippocampi of \( {\text{Cln5}} \)-KO mice. Immunohistological staining for microtubule-associated protein 2 (MAP-2; a marker of neuronal cytoskeleton) in 7-month-old mouse brains revealed that the morphology of MAP-2-positive neuronal processes in the hippocampi was altered under \( {\text{Cln5}} \) deficiency (Fig. 7E,F).

Taken together, these results demonstrate that loss of \( {\text{Cln5}} \) induced the NPCs to differentiate towards the neuronal lineage, yet the migration and formation of neuronal processes was impaired.

**Absence of \( {\text{Cln5}} \) alters \( {\text{Ca}}^{2+} \) responses of differentiating neuronal progenitors**

To test the hypothesis that the phenotypic changes observed in \( {\text{Cln5}} \)-deficient cells are concomitant with a functional impairment, we analyzed intracellular calcium (\( {\text{Ca}}^{2+} \)) transients from the differentiated NPCs by using confocal \( {\text{Ca}}^{2+} \) imaging. Depolarization-induced intracellular calcium transients were analyzed from neurospheres that had been differentiated for 3-4 days. Neurospheres were treated with high-potassium solution to induce depolarization of neuronal cells. The majority of cells responded to depolarization-induced changes in calcium concentration and there were no genotype-related differences observed (Fig. 8A). The number of cells responding to GABA (Fig. 8B) and serotonin (Ser, Fig. 8C) were reduced upon \( {\text{Cln5}} \) deficiency. There was no genotype difference in the number of cells responding to ATP, acetylcholine or glutamate (data not shown). Previously, it has been shown that the calcium responses of NPCs...
may alter depending on the distance they migrate away from the edge of the neurosphere (Achuta et al., 2014). Therefore, for the analysis, we next divided the cell migration area into three zones starting from the edge of the neurosphere and reaching to the outermost migrated cells (Fig. 8D). When the data was analyzed separately for each migratory zone, the results for both GABA- and Ser-induced changes in intracellular calcium transients remained similar to that in Fig. 8B,C.

Neurogenesis impairment in Cln5 deficiency is associated with increased expression of IL-1β

Numerous molecules, growth factors and cytokines are involved in the regulation of neurogenesis. To assess whether the increased level of neurogenesis observed in Cln5-deficient brains occurs concomitantly with alterations in the expression of these factors, qRT-PCR analyses were carried out for components of the Wnt signaling pathway (catenin beta-1 and axin-1); the trophic factors nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), insulin growth factor 1 (IGF1) and tumor necrosis factor (TNF); and the cytokines interleukin (IL)-6 and IL-1β. The expression levels of the majority of factors measured remained unaltered in Cln5 deficiency (data not shown). A modest, but statistically significant, 0.6-fold reduction was observed in IL-6 expression in the 9-month-old Cln5-KO hippocampi (data not shown). Likewise, a small, 0.3-fold, reduction in NGF expression was observed in the Cln5-deficient brains (data not shown). Interestingly, the expression of IL-1β was robustly increased in the hippocampi of both 7- and 9-month-old Cln5-KO mice (Fig. 9A,B). The increase of IL-1β in Cln5-deficient hippocampi was confirmed by ELISA (data not shown). The expression of IL-1β was not changed at the age of 3 months (data not shown), the age at which DCX expression was also unaltered in Cln5-KO mice. Loss of Cln5 also did not alter the mRNA expression levels of the IL-1β receptor (IL1R1) or the receptor accessory protein in the hippocampi (data not shown). This finding of robust IL-1β elevation in Cln5-KO hippocampi that occurs concomitantly with...
neurogenesis alterations prompted us to further investigate the role of IL-1β in neurogenesis in CLN5 disease.

To characterize the effects of IL-1β in NPCs in vitro, IL-1β levels were first measured by qRT-PCR and cytometric bead array (CBA) assay from WT and Cln5-KO neurospheres, and by enhanced-sensitivity CBA assay from the cell culture media. However, IL-1β levels were too low and below the detection limit of both methods. Nevertheless, loss of Cln5 increased the expression level of IL1R1 in neurospheres (Fig. 9C), suggesting that this signaling pathway is altered in Cln5-KO NPCs. The expression of the IL-1 receptor accessory protein was not altered (data not shown). Next, to further investigate the relationship between IL-1β and neurogenesis in CLN5 disease, we treated WT and Cln5-KO NPCs with recombinant IL-1β, or with an IL-1β blocking antibody. Treating WT NPCs with recombinant IL-1β for 7 days did not affect the number of DCX-positive cells (Fig. 10A).
However, a significant increase in the number of DCX-positive cells was observed in Cln5-KO cultures (Fig. 10B). Treatment with the IL-1β blocking antibody had no effect in either WT or Cln5-KO cultures. Treatment of NPCs with recombinant IL-1β or the anti-IL1β antibody did not affect sphere size in either WT (Fig. 10C) or Cln5-KO (Fig. 10D) cultures.

**DISCUSSION**

Our findings demonstrate a newly identified role for Cln5 in neurogenesis. We report for the first time that neurogenesis is increased in Cln5-KO mice, which model the childhood neurodegenerative disorder caused by CLN5 deficiency. These results demonstrate that, during Cln5 deficiency, proliferation of NPCs is increased, NPC migration is reduced and NPC differentiation towards the neuronal lineage is increased concomitantly with functional alterations in the NPCs. Moreover, the observed impairment in neurogenesis is correlated with increased expression of the pro-inflammatory cytokine IL-1β.

To our knowledge, this paper is the first description of impaired neurogenesis of the mammalian brain in NCL. The increase in neurogenesis did not occur at the age of 3 months, but was observed starting at the age of 5 months. At this age, it is known that Cln5-KO mice show such pathological features as glial activation and defective myelination (Schmiedt et al., 2012). A previous study has shown that secondary neurogenesis is impaired in a zebrafish model of CLN2 disease (Mahmood et al., 2013), yet information about NCL-related alterations in mammalian neurogenesis is completely lacking. Recently, Fabritius et al. described that, during embryonic brain development, Cln5 is strongly expressed in the hippocampi and ventricular region (Fabritius et al., 2014). This high expression in those brain regions where neurogenesis is known to occur continued into the early postnatal period and suggested that Cln5 may have a role in the generation of new neurons during embryonic development. Our results are in agreement with this suggestion and demonstrate that loss of Cln5 impairs neurogenesis in vivo and has striking effects on the phenotype of NPCs in vitro.

In support of our in vivo and in vitro results of increased numbers of neuronal precursor cells in Cln5 deficiency, alterations in cell proliferation have also been demonstrated to occur in the brains of other NCL mouse models. For example, Weimer et al. showed that, in the early postnatal cerebella, the proportion of proliferating cells is increased in Cln3 deficiency (Weimer et al., 2009). In addition, neuronal progenitors derived from the ventricular wall of Cln1-deficient mice were also shown to proliferate more than WT cells, whereas alterations in their ability to differentiate were not observed (Ahtiainen et al., 2007). Given that impaired proliferation of...
precursor cells is observed in at least three forms of NCL, it is possible that these alterations are a common disease feature of the NCLs. Further studies are clearly required to assess how various aspects of neurogenesis are affected in multiple forms of the disease.

The maturation of neurons has previously been shown to be reduced in NCL caused by loss of the CLN6 protein (Benedict et al., 2009). Using primary hippocampal neurons from the nclf mouse model of CLN6 disease, Benedict et al. demonstrated that cellular and synaptic connections were reduced in Cln6-deficient neurons. The neurons also displayed an abnormal morphology with thinner processes when compared to WT cells. Interestingly, neuronal growth cone and axon guidance pathways are affected in the Cln5-KO mouse model (von Schantz et al., 2008). Our results support these findings of altered neuronal morphology. The neuronal processes of differentiated Cln5-KO cells were shorter than those of WT cells, yet the number of processes was increased in the Cln5-deficient cells. In addition, the migration of Cln5-deficient cells was reduced and their differentiation towards the neuronal lineage was increased. It may well be that our observed defects are related to impaired neuronal maturation and at least partly explain the increase...
in neurogenesis under Cln5 deficiency. It is possible that increased NPC proliferation and differentiation towards the neuronal lineage is a compensatory mechanism to counteract the loss of fully functional, mature neurons in the Cln5-KO brain. Our results also suggested that alterations in neurogenesis that are caused by Cln5 deficiency are not related to changes in hippocampal apoptosis. However, in addition to apoptosis, other mechanisms, such as autophagy, have been reported to modulate stem cell development and neurogenesis (Wu et al., 2016; Li et al., 2016). Given that autophagy impairment is a central feature of the NCLs, it is possible that alterations in neurogenesis and autophagy are linked and should be further investigated.

The idea that proliferation and differentiation of neural stem cells are a convergence point for several neurodevelopmental disorders has gained support in recent years (Ernst, 2016). It has been hypothesized that many genes associated with these disorders may function as brakes on stem cell differentiation, which leads to hypothesized that many genes associated with these disorders may function as brakes on stem cell differentiation, which leads to hypothesized that many genes associated with these disorders may function as brakes on stem cell differentiation, which leads to hypothesized that many genes associated with these disorders may function as brakes on stem cell differentiation, which leads to hypothesized that many genes associated with these disorders may function as brakes on stem cell differentiation, which leads to hypothesized that many genes associated with these disorders may function as brakes on stem cell differentiation, which leads to hypothesized that many genes associated with these disorders may function as brakes on stem cell differentiation, which leads to hypothesized that many genes associated with these disorders may function as brakes 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We demonstrate for the first time that neurogenesis in the mammalian brain is altered in NCL and show the importance of the CLN5 protein for this process. Increased neurogenesis is associated with high levels of IL-1β in the diseased brains, and alterations to IL-1β signaling in NPCs in vitro. A full understanding of the pathological mechanisms that lead to disease and the function of the NCL proteins are critical for designing effective therapeutic approaches for this devastating neurodegenerative disorder.

MATERIALS AND METHODS

Animal models
All experimental procedures were carried out according to the national regulation of the usage and welfare of laboratory animals and approved by the Animal Experiment Committee in State Provincial Office of Southern Finland. Cln5-KO mice of both genders with a deletion of exon 3 in the Cln5 gene (Kopra et al., 2004), kindly provided by Dr Anu Jalanko, were used for the study. Age-matched WT C57BL/6J RecI Hsd mice were used as controls.

BrdU administration
BrdU (Sigma-Aldrich, Louis, MO, USA) was administered to 5-month-old Cln5-KO and WT mice intraperitoneally (i.p.) at a dose of 50 mg/kg body weight every 12 h during 7 consecutive days. The BrdU stock solution was prepared in phosphate buffered saline (PBS), and the pH was adjusted to 7.1-7.4 with 10 M NaOH. Mice were sacrificed and the brains further processed for histology as described below.

Immunohistochemistry
The mice were deeply anesthetized with avertin and perfused with heparinized saline. The brains were collected, post-fixed with 4% paraformaldehyde (PFA) overnight and cut to 20-µm sections with a cryostat (Leica Microsystems GmbH, Wetzlar, Germany). Following blocking with 10% normal goat serum (NGS); Chemicon International, Merck Millipore, Billerica, MA, USA), 5-7 sections 400-µm apart were incubated overnight with DCX (Cell Signaling, Danvers, MA, USA), MAP-2 (Merck Millipore, Billerica, MA, USA) and Ki-67 (Abcam, Cambridge, UK) primary antibodies at 1:200 or 1:500 dilution. Following washing, sections were incubated for 2 h with an Alexa-Fluor-488 or Alexa-Fluor-568 secondary antibody (Molecular Probes, Invitrogen, Eugene, OR, USA). For BrdU staining, sections were permeabilized in an ethanol–acetic-acid mixture, after which DNA denaturation was carried out by treatment in 2N HCl for 30 min at 37°C. HCl was neutralized by treatment in 0.1 M borate buffer pH 8.5 for 10 min at room temperature (RT). After washing with PBST, the sections were treated with mouse IgG blocking reagent (Vector Laboratories, CA, USA) followed by blocking in 0.1% bovine serum albumin/5% NGS. PBST-rinsed sections were then incubated with BrdU primary antibody (Roche, Basel, Switzerland) at 1:50 dilution overnight at RT. Following washing, sections were incubated for 2 h with an Alexa-Fluor-568 secondary antibody (Molecular Probes, Invitrogen). Stained sections were visualized using an Olympus AX70 microscope (Olympus Corporation, Tokyo, Japan) equipped with a digital camera (Color View 12 or F-Fiew; SoftImaging Systems, Gmbh, Munster, Germany). The numbers of immunoreactive cells were counted and/or the immunoreactive areas were quantified using ImagePro Plus software (Media Cybernetics, Rockville, MD, USA).

Quantitative real-time polymerase chain reaction
Total RNA was isolated using RiboPure Kit or Trizol reagent (both from Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer’s instructions. The purity and concentration of RNA was measured with a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific). Five-hundred nanograms of total RNA were used for cDNA synthesis using random hexamer primers (Promega, Madison, WI, USA) and Maxima reverse transcriptase (Fermentas, Thermo Fisher Scientific). The relative expression levels of mRNA were measured according to the manufacturer’s protocol by qRT-PCR (StepOnePlus; Life Technologies Carlsbad, CA, USA) using TaqMan chemistry and specific assays-on-demand target mixes (Life Technologies, Carlsbad, CA, USA). The expression levels were obtained by normalizing the target gene to ribosomal RNA or to GAPDH, and presented as fold change in the expression or in arbitrary units using the 2^−ΔΔCt method; Ct is the threshold-cycle value.

NPC culture
The hippocampi were dissected from the brains of WT and Cln5-KO mice at E18 and cultured as described (Kärkkäinen et al., 2014) as free-floating neurospheres in the presence of epidermal growth factor (EGF) and basic fibroblastic growth factor (FGF). Cells were grown in culture medium containing DMEM/F12 (Gibco, Thermo Fisher Scientific), 1 M HEPES (Sigma-Aldrich), 100 U/ml penicillin, 100 µg/ml streptomycin, B27 supplement (all from Gibco, Thermo Fisher Scientific), 20 ng/ml EGF (PeproTech, Rocky Hill, NJ, USA) and 10 ng/ml basic FGF (PeproTech). Cells were cultivated at 37°C in 5% CO2. Fresh medium as well as EGF and FGF were added to the cells every 3 days.

Characterization of NPC proliferation, migration and differentiation
To quantify NPC proliferation, cells were plated at a density of 30,000 cells in 6-well plates in culture media supplemented with EGF and FGF. After 3-7 days, neurospheres were imaged and the average size of neurospheres was measured using ImageJ (Wayne Rasband, National Institutes of Health, Bethesda, MD, USA) software. In addition, proliferation was assessed by BrdU labeling. NPCs were incubated in culture media supplemented with EGF, FGF and 10 µM BrdU (Sigma-Aldrich) for 24 h. After incubation, cell clusters were dissociated using TrypLE (Gibco, Thermo Fisher Scientific) and single NPCs were applied onto poly-DL-ornithine (Sigma-Aldrich)-coated 48-well plates overnight in culture media without growth factors. On the next day the cells were fixed with 4% formaldehyde solution and immunostained as described below. Cells were imaged with an Olympus IX71 microscope with MT10 illumination system attached with the DP70 digital camera, running DP software (all from Olympus, Tokyo, Japan) and BrdU-positive cells were counted from replicate wells.

To assess NPC migration, 10-15 neurospheres were collected and plated onto the bottom of poly-DL-ornithine (Sigma-Aldrich)-coated plates and grown for 7 days without growth factors. For the evaluation of cell migration, neurospheres were imaged as above and the average migration distance from the edge of the neurosphere was measured using ImagePro Plus software (Media Cybernetics, Rockville, MD, USA) or ImageJ (Wayne Rasband, National Institutes of Health, Bethesda, MD, USA) software.

To measure the differentiation of NPCs, neurospheres were plated onto the bottom of poly-DL-ornithine-coated plates as above and grown for 3 or 7 days without growth factors. Neurospheres were then fixed with 4% formaldehyde solution and immunostained for GFAP (Dako, Glostrup, Denmark), Tuj-1 (Covance, Princeton, NJ, USA) or DCX (Cell Signaling) as described below. Samples were imaged and quantified using ImagePro Plus software (Media Cybernetics, Rockville, MD, USA). Fiji (an image-processing package based on ImageJ) and neurite tracer plugin were used for the neurite analyses.

Treatment of NPCs
To investigate the relationship between IL-1β and NPC proliferation and differentiation, WT and Cln5-KO neurospheres were dissociated to single cells and then grown for 6 days in the presence of anti-IL-1β or control IgG antibody (both 1.0 µg/ml, Abcam, Cambridge, UK). In another set of experiments, NPC’s were grown in the presence of 50 ng/ml recombinant IL-1β (PeproTech) for 6-9 days. Assessment of the effect of IL-1β blockage and treatment with recombinant IL-1β on NPC proliferation and differentiation were assessed as above.

Primary microglia culture
Primary mouse microglia were isolated and prepared from mouse pups at the age of P0-P1 as described previously (Haniisch et al., 2004). After brain isolation, meninges were removed and brain tissues were digested with 0.05% trypsin (Sigma-Aldrich) and ethylenedinitrilotetraacetic acid
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Author contributions
Conceptualization: P.T., A.R.W., T.M., J.K., K.M.K.; Methodology: E.S., Y.S., H.K., K.L., L.M.S., A.G., V.K., V.K.-G., N.N.; Formal analysis: E.S., Y.S., H.K., K.L., L.M.S., A.G., V.K., N.N.; Investigation: E.S., Y.S., H.K., A.G., V.K.; Resources: K.M.K.; Data curation: E.S., K.L., V.K.-G.; Writing - original draft: E.S., Y.S., H.K., K.L., L.M.S., A.G., V.K., V.K.-G., N.N., P.T., A.R.W., T.M., J.K., K.M.K.; Supervision: P.T., A.R.W., T.M., J.K., K.M.K.; Project administration: K.M.K.; Funding acquisition: P.T., J.K., K.M.K.

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Confocal calcium imaging
Calcium imaging was performed as previously described (Mutikainen et al., 2016). Following 3-4 days of differentiation and growth on poly-lysine-coated coverslips, NPCs were incubated with 5 μM of Fluo-4-acetoxymethyl (AM)-ester (Thermo Fisher Scientific), an intracellular calcium indicator, at 37°C for 30 min. Then, coverslips with attached neurones were transferred to the recording chamber (Cell MicroControls) with flow rate approximately 1-2 ml/min. For perfusion, preheated and oxygenated (37°C, 100% O2) external saline solution containing 125 mM NaCl, 5.5 mM KCl, 2 mM CaCl2, 0.8 mM MgCl2, 10 mM HEPES, 24 mM glucose and 36.5 mM sucrose (pH 7.3) was used. Measurement was carried out after a period of 20 min to allow de-esterification of the dye. Measurement was performed with a confocal inverted microscope Fluoview 1000 (Olympus, Japan). To measure calcium signals, the cells were excited at 488 nm, the emitted light band was 500-600 nm through a 20× objective lens, and frame-scan mode was used at resolution 1000×1000. When calcium signal was stable, measurement was performed for 2-5 min with 10 Hz frame rate. The raw signals were adjusted with baseline and fitted with a smooth line to determine fluorescence intensity changes. The raw data were exported and analyzed using GraphPad Prism software (GraphPad Software Inc., La Jolla, USA). 

Statistical analyses
The data were analyzed using t-test or 1-way ANOVA with Tukey’s post hoc test as appropriate in GraphPad Prism software (GraphPad Software Inc., La Jolla, USA). A P-value of <0.05 was considered significant. Data are shown as mean±s.d. and mean±s.e.m. for NPC in vitro data.

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