Microglia in Health and Disease
Learning from the zebrafish

Nynke Oosterhof

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Chapter 1

Introduction

Introduction

The main cell types in the brain parenchyma were first described in the late 19th and early 20th century, and are known as neurons, astrocytes, oligodendrocytes and microglia. Neurons were first described by the Italian scientist Camillo Golgi who developed a silver-impregnation based method that allowed him to visualize the complex branched structure of brain cells. These same methods were used to further study the structure of the brain by Santiago Ramon y Cajal, who is considered to be the father of modern neuroscience, leading to the hypothesis that the neuron is the functional unit of the nervous system. As a result, Golgi and Ramon y Cajal received the Nobel prize for their work on neurons in 1906[1,2]. Ramon y Cajal, in addition to his interest in neurons, also developed an interest in the non-neuronal cells in the brain, the glial cells, which at that time were simply thought to be the glue holding the brain together. In addition to star-shaped glial cells, now known as astrocytes, he noticed other cells, which he called the “third element”. In 1919, the Spanish scientist Pio del Rio-Hortega discovered that Ramon y Cajal’s third element consists of two cell types now known as oligodendrocytes and microglia. The microglia, he described as highly ramified cells with a small cell body. He also noticed that these small ramified microglia displayed macrophage properties and were able to transform into highly phagocytic amoeboid cells, upon injury[2]. We now know that microglia are versatile cells, that have important physiological functions in brain development and in the adult brain.

After the discovery of microglia by del Rio-Hortega, it took several decades to prove experimentally that microglia are tissue-resident macrophages, which originate from macrophages on the embryonic yolk sac that colonize the brain during early embryonic development[2-5]. Tissue resident macrophages are multifunctional immune cells that support their host tissue and defend it against for example pathogens. Unlike most other tissue-resident macrophage populations, microglia completely rely on self-renewal by proliferation to maintain a proper density[4,6-9] (Fig 1). Before becoming mature microglia, yolk sac macrophages go through a stepwise differentiation program that is characterized by distinct gene expression profiles[10]. The timing of the different steps of microglia differentiation, which most likely facilitate specific microglia functions, is crucial for normal brain development. Disruption of developmental microglia activity and differentiation, for example through maternal inflammation or by genetic mutations, can have lasting effects on adult brain function or even cause severe embryonic and post-natal brain defects[10,11]. The steps of microglia differentiation are most likely driven by a changing microenvironment, and consistent with that microglia, and other tissue macrophages, are highly sensitive to changes in their microenvironment. In fact, several studies have shown that the identity of mature tissue-resident macrophage populations, including microglia, is largely determined by the host tissue[10,12,13]. However, the exact cues driving microglia development, differentiation and identity are still unclear.

In both the developing and adult brain, microglia clear waste, dead cells and pathogens from the host tissue and support tissue growth and repair via the secretion of trophic factors[14-16]. Growth factors like brain-derived neutrophic factor (BDNF) and insulin-like growth factor 1 (IGF1), stimulate neurogenesis, as well as primary myelinogenesis, respectively[15,17]. In the developing brain microglia also prune synapses (removal of neuronal connections). Defects in synaptic pruning have been postulated to be a potential cause of neurodevelopmental disorders including autism spectrum disorder (ASD)[16,18], which further indicates that microglia can have a profound effect on

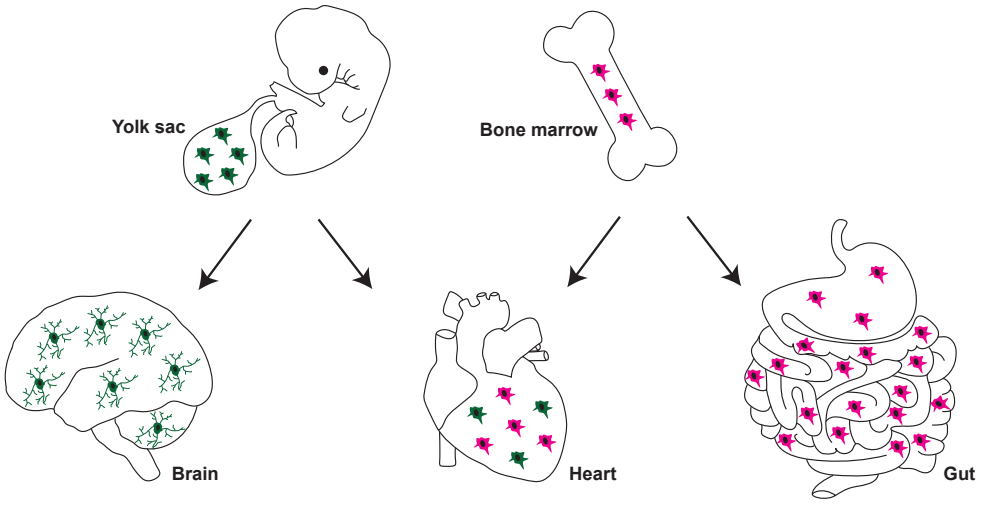

Fig 1. Tissue macrophages have different origins. Microglia are completely derived from embryonic yolk sac (YS) macrophages, whereas the macrophages in the gut originate in the bone marrow (BM). Macrophages in the heart have distinct origins. Some subpopulations come from the yolk sac, while others have a bone marrow origin.

brain development.

About half of the human brain consists of white matter, which is rich in myelin, produced by oligodendrocytes to facilitate fast and efficient communication between neurons. Intriguingly, most currently known genetic disorders caused by microglia defects are characterized by white matter degeneration, which indicates that, in the adult brain, microglia play a crucial role in white matter homeostasis[19-21]. Recently, it was shown that both white matter degeneration and defective post-phagocytic processing by microglia can result in the accumulation of myelin inside microglia[22]. Upon excessive accumulation of myelin in microglia, microglia may transform into foam cells, which are rounded macrophages filled with lipid-rich material, that are likely unable to perform normal microglia functions[23]. Additionally, microglia appear to be involved in the regulation of the numbers of oligodendrocyte precursor cells by currently unknown mechanisms[24]. Although it is still unclear how microglia contribute to white matter homeostasis, this indicates that maintenance of white matter integrity may be one of the main functions of microglia in the adult brain.

Despite the importance of physiological microglia functions for brain development and homeostasis, the primary focus in microglia research has been on their inflammatory response in the context of brain disease. Chronic aberrant inflammatory responses of microglia are thought to exacerbate a variety of brain diseases, including neurodegenerative diseases, stroke and multiple sclerosis[25]. Under pathological conditions, microglia become activated. Activated microglia have an amoeboid morphology, often show increased expression and secretion of inflammatory cytokines. For years, the categorization of microglia activation states was based on macrophage activation stereotypes observed in vitro. Continuously improving gene expression profiling techniques, however, have revealed that the stereotypical microglial activation states observed in vitro do not reflect microglia activation states in vivo[26,27]. Microglia

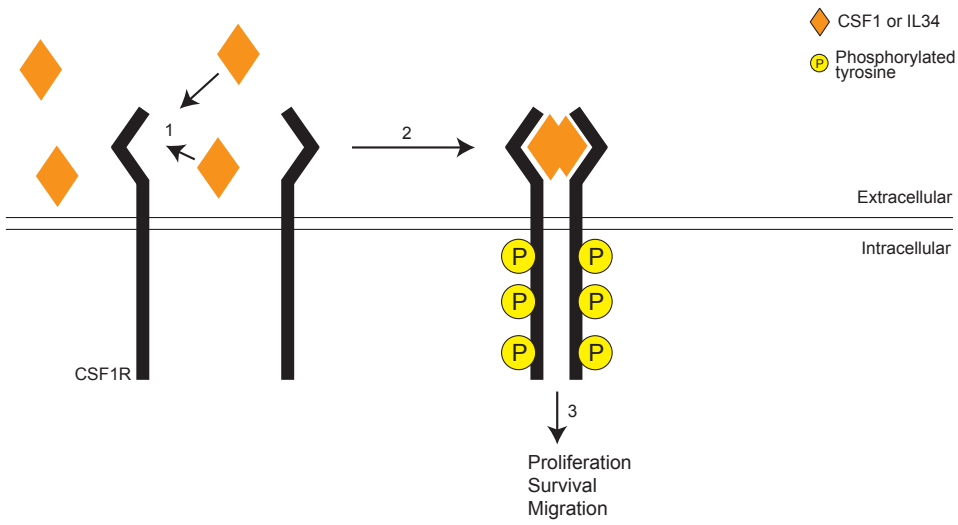

Fig 2. CSF1R is a receptor tyrosine kinase. CSF1 and IL34 are the ligands for CSF1R. Bind- ing of either one of these ligands to the receptor causes CSF1R to form stable dimers. Subse- quent autophosphorylations of the tyrosine residues in the intracellular domain of the receptor results in activation of downstream signaling pathways involved in cell proliferation, survival and migration.

appear to have many different activation states that are highly time dependent, and rely on the activating stimulus and on the microenvironment[27-30]. Because of the high dependence of microglia on their microenvironment, microglia lose their identity and specific properties quickly after they are taken out of the brain. This makes it extremely difficult to study microglia functions and responses in vitro, which underscores the need to study microglia function and responses in their native environment.

New insights suggest that the contribution of microglia to brain disease is not limited to potentially pathogenic inflammatory responses. Genetic studies indicate that microglia defects can contribute to, and even cause, different types of brain diseases, including neurodegenerative, neurodevelopmental and psychiatric disorders[16]. For example, dominant loss-of-function mutations in the important macrophage regulator, colony-stimulating factor 1 receptor (CSF1R) result in a rapidly progressing white matter disease called adult onset leukoencephalopathy with axonal spheroids (ALSP)[19,31] (Fig 2). Even though the exact roles of CSF1R in microglia are still unclear, it is known that CSF1R is required for microglia development and survival[32,33]. Despite the fact that loss-of-function mutations in CSF1R cause brain disease, CSF1R is currently considered as a putative therapeutic target to treat neurodegenerative diseases, such as Alzheimer's disease and amyotrophic lateral sclerosis (ALS)[34-36]. Another known microgliopathy, Nasu-Hakola disease is caused by recessive mutations in the microglia genes TREM2 and TYROBP[20,31]. Intriguingly, variants in TREM2 and TYROBP are associated with increased risk of developing Alzheimer's disease[37-39]. In fact, most risk variants for Alzheimer's disease are found in genes that show much higher expression in microglia than in other brain cell types[40]. However, it is still unknown to what extent loss of normal microglia functions, some of which likely remain to be identified, contributes to

the development and progression of brain disease. Therefore, better understanding of genetic regulation of microglia, their function, their responses to changes in the microenvironment and the contribution of microglia to disease is of utmost importance.

Aim of the thesis

The aim of this thesis was to gain more insight into the genetic mechanisms important for microglia development and the role of microglia in disease. The discovery that microglia-specific gene expression, and by extension likely their function, is largely determined by their microenvironment stresses the need to study microglia in vivo. Therefore, we used zebrafish as an in vivo model organism. Zebrafish (*Danio rerio*) are small tropical fresh water fish that have a high fecundity and are small and transparent during early developmental stages. Like mammalian model organisms such as mice and rats, zebrafish are vertebrates in which most tissues, cell types and genes found in humans are conserved. However, unlike these mammalian model organisms, zebrafish are relatively easy to genetically manipulate and are very well suited for in vivo imaging, because of their transparency, and the many available transgenic fluorescent reporter lines. Taken together with the small size of zebrafish embryos and larvae, zebrafish provide an excellent opportunity to study microglia biology and genetics in vivo.

In **chapter 2** we provided a comprehensive outline on why the zebrafish is a valuable model organism in microglia research. In **chapter 3** we identified the zebrafish microglia gene expression profile using RNA sequencing. We showed that microglia identity is well conserved between zebrafish and mammals, thereby providing a guide for future microglia research in the zebrafish. Additionally, as neuronal death and phagocytic microglia are hallmarks of neurodegenerative disease, we characterized gene expression of microglia acutely responding to neuronal cell death in vivo. We anticipated primarily an increase in the expression of phagocytic and immune-related genes. Instead, we mainly observed increased expression of genes involved in cell proliferation, which was in line with the increased proliferative response we observed in vivo. In **chapter 4** we present a scalable reverse genetic screening pipeline to identify new genes important for microglia development and function using CRISPR/Cas9 technology. We developed a software tool to automatically quantify the numbers of microglia labelled with the vital dye neutral red. Using our screening strategy, we identified interleukin 34 (*il34*) as an important regulator of microglia development in the zebrafish. In mammals, IL34 is a ligand for colony-stimulating factor 1 receptor (Csf1r), which is required for microglia development. This showed that our genetic screening pipeline is an effective strategy to further unravel microglia genetics. In **chapter 5,** we studied how loss of *csf1r* affects microglia development during early embryonic stages, by applying RNA sequencing and in vivo imaging to embryonic yolk-sac macrophages. This revealed that, although in zebrafish lacking *csf1r* yolk sac macrophages were formed and differentiated properly, they failed to colonize embryonic tissues including the brain. In **chapter 6** we studied the role of *csf1r* in adult microglia, thereby gaining insight into the rare familial brain disorder ALSP. We showed that CSF1R regulates microglia density both in zebrafish and humans. Additionally, partial loss of CSF1R resulted in aberrant microglia distribution and widespread microglia depletion. Both in zebrafish and humans the depletion of microglia was observed in regions without apparent white matter pathology. Given that white matter degeneration is a hallmark of ALSP, this indicates that local loss of microglia is an early event which could contribute

to ALSP pathogenesis. **Chapter 7** provides a detailed description of the conditional neuronal ablation protocols we used to study the phagocytic response of microglia to dying neurons (in **chapters 4** and **6**). In **chapter 8** our findings are discussed in the context of recent literature on microglia development and function, their contribution to disease, and how our data will facilitate future microglia research.

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Chapter 2

Immune cell dynamics in the CNS: learning from the zebrafish

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Glia, 2015

Abstract

A major question in research on immune responses in the brain is how the timing and nature of these responses influence physiology, pathogenesis or recovery from pathogenic processes. Proper understanding of the immune regulation of the human brain requires a detailed description of the function and activities of the immune cells in the brain. Zebrafish larvae allow long-term, non-invasive imaging inside the brain at high-spatiotemporal resolution using fluorescent transgenic reporters labeling specific cell populations. Together with recent additional technical advances this allows an unprecedented versatility and scope of future studies. Modeling of human physiology and pathology in zebrafish has already yielded relevant insights into cellular dynamics and function that can be translated to the human clinical situation. For instance, in vivo studies in the zebrafish have provided new insight into immune cell dynamics in granuloma formation in tuberculosis and the mechanisms involving treatment resistance. In this review, we highlight recent findings and novel tools paving the way for basic neuroimmunology research in the zebrafish.

Key words: Microglia, brain disease, neurodegeneration, neuroinflammation, live imaging, immune cell behavior

Introduction

It is well established that the immune system plays an important role in brain homeostasis and most conditions affecting the brain, including neurodegenerative diseases, psychiatric diseases and neurodevelopmental disorders[1-3]. Our current understanding of neuroimmunology, the complex interactions of the immune system with the central nervous system (CNS), however, is limited and many fundamental questions are still unanswered. Basic questions concerning the ontogeny of the brain's immune cells and glia, their functional phenotypes, the life-span of brain immune cells and the effect of aging remain to be answered and are essential for a better understanding of the role of the immune system in CNS health and disease[4-6].

A prerequisite for a more comprehensive description of immunological processes in the brain is a thorough characterization of the function of the different types of immune cells involved. This can be achieved in animal model systems by long-term visualization and mapping of neuroimmune cellular dynamic interactions in the living brain. In the last decade, the zebrafish has gained substantial popularity as a model for basic research as well as translational biomedical research. Also in neuroscience research, the use of zebrafish as a model is now quickly gaining momentum. Recent technical advances including genome editing[7-9], optogenetics[10,11], fluorescent imaging tools[12,13] and high-throughput behavioral screens have highlighted the use of zebrafish in understanding brain development and function and sped up the discovery of novel psychoactive drugs[14,15].

An important use of zebrafish in biomedical research, is the very powerful possibility for in vivo high-resolution imaging of dynamic cellular and even subcellular pathogenic mechanisms in transparent larvae. This has yielded detailed in vivo mechanistic insight into developmental and disease processes, including tissue regeneration[16,17], various types of cancer[18-21] and infectious disease[22-24]. Although a similar level of in depth genetic, cellular and molecular understanding in neuroimmunology is still far away, this is clearly feasible. Comparable imaging of in vivo cell biology at micron-scale level, is hard to achieve in the human brain or in classical mammalian models. Clearly, knowledge about these cellular dynamics may be essential to better understand and eventually treat brain diseases.

The main purpose of this review is not as much to give a summary of recent studies but to illustrate the unique application of the zebrafish for detailed long-term monitoring of motile immune cells in the healthy CNS as well as in a disease context. To do so, we will make a case for the specific niche the zebrafish occupies as a discovery platform in the field of neuroimmunology and brain disease research to help resolve specifically in vivo mechanisms that are unlikely to be studied in another way. Data derived from these zebrafish studies are generally complementary to studies in other animal models rather than redundant, help to generate hypotheses, provide unexpected novel in vivo insights, and sometimes provide very rapid preclinical development.

First, we will provide background on the conservation of the zebrafish CNS and immune system, relevant to consider the strengths and weaknesses of zebrafish as model for neuroinflammation. Subsequently, we will describe illustrative examples of studies using zebrafish showing how conceptual questions can be addressed yielding unexpected in vivo insight in disease-related mechanisms, with sometimes direct clinical relevance. We conclude by consolidating recent technical advances to illustrate what this has yielded so far, and provide several examples already showing the tremendous

potential and technical possibilities to address some of the main questions related to functions and origins of immune cells in the brain.

Hereby this review should provide convincing arguments to apply the zebrafish as a tool, allowing an unprecedented view into functions of cellular behavior and contribution to pathogenic mechanisms with the realistic possibility of extrapolating basic findings to patients. Although linear translation of a human CNS disease to zebrafish and back may often not be possible, relevant insights into immune cell dynamics related to such CNS diseases as well as discovering novel concepts that can be translated to more clinical models is very well achievable.

The zebrafish as an in vivo model for human disease

The zebrafish, a small teleost fish native to streams of the south eastern Himalaya, was first introduced as a model organism for developmental biology by George Streisinger in the late 1960s, mainly because of its rapid, completely external embryonic development[25]. Within 24 hours after fertilization several organs and cell types, have formed, and are already functioning including the heart, circulation and early innate immune cells capable of ingesting dying cells and bacterial pathogens[26]. Behaviors requiring interactions between different neuronal circuits, such as hunting and capturing prey animals, also develop within the first days of development[27-29]. Because of their transparency, small size, and rapid development of organs and tissue including the CNS and the immune system zebrafish embryos and larvae have been used extensively for in vivo imaging studies of organ development and behavioral research.

The zebrafish brain

In vertebrates, including the zebrafish, the embryonic development of the CNS involves formation of the neural tube, which subsequently folds in an intricate manner, creating more distinct fore-, mid- and hindbrain regions. Eventually, by cell-migration and differentiation these regions further develop into the cerebrum, thalamus and hypothalamus, tectum, tegmentum, cerebellum, pons and medulla[30,31]. The main vertebrate cell types in the mammalian CNS, including neurons and glial cells, such as oligodendrocytes, microglia (Fig. 1B,D, Table 1) and astrocytes, have been identified in the zebrafish, as well as specialized barrier structures such as the blood-brain barrier (BBB) and the choroid plexus[32-37]. Main inhibitory as well as excitatory neurotransmitter systems, including GABAergic, glutamatergic, cholinergic, dopaminergic and serotonergic neurotransmission are highly conserved at all levels in the zebrafish[38].

Although most basic anatomical regions, cells, and neurotransmitters are conserved between zebrafish and mammals, there are a few important differences (Fig. 1A,B; Table 1, 2). For example, fish have nociceptive pathways and respond to noxious stimuli but it is still unclear whether fish can perceive pain, which is considered to be a higher cognitive process[39]. The neocortex, arguably the most distinguishing feature of the human brain, is thought to be exclusively found in mammals. Fish only have a primitive cerebral brain structure lacking these regions important for higher cognitive functions. However, higher cognitive processes have been described in birds and reptiles suggesting there likely is functional conservation located elsewhere. Similarly, the dorsal pallium in non-mammalian vertebrates is thought to provide functions equivalent to the mammalian hippocampus, for example with regard to spatial navigation[40].

Another major difference is the high abundance of neurogenic zones in the adult

	Mammals	Zebrafish
CNS		
Main structures		
Forebrain (cerebrum, thalamus, hypothalamus)	+	+
Midbrain (tectum, tegmentum)	+	+
Hindbrain (cerebellum, pons, medulla)	+	+
Blood brain barrier	+	+
Meninges	+	+
Choroid plexus	+	+
Ventricular system	+	+
CNS cell types		
Neurons	+	+
Oligodendrocytes	+	+
Astrocytes	+	+/-
Microglia	+	+
Major neurotransmitter systems		
Amino acids (glutamate, GABA, glycine)	+	+
Monoamines (dopamine, serotonin)	+	+
Peptides (somatostatin, opioids)	+	+
Other (acetylcholine)	+	+
Immune system		
Main structures		
Thymus	+	+
Bone marrow	+	-
Lymph nodes & antigen presentation	+	+/-
Lymphatic system	+	+
Leukocytes		
Mononuclear phagocytes (monocytes, macrophage, APCs)	+	+
Mononuclear phagocytes (microglia)	+	+
Granulocytes (neutrophils, eosinophils)	+	+
Lymphocytes (T cells, B cells, NK cells)	+	+/-
Molecular components		
Myeloid differentiation (PU.1, IRF8, CSF1R)	+	+
PRRs (Pattern recognition receptors e.g. TLRs)	+	+
Complement cascade	+	+
Transcription factors (NFκB)	+	+
Antigen presentation (MHC II)	+	+
Cytokine signaling (IL-1α, IL-4, IL-10, IL-6, TGFα, IFNγ, TNFα)	+	+
Chemokine signaling	+	+

Table 1. Conservation of the CNS and immune system between mammals (e.g., human) and teleosts (e.g.,zebrafish) Major structures, cell types and molecular pathways in the CNS and immune system share a high level of conservation between mammals and teleosts (e.g. zebrafish). Some of the differences indicated in this table, are in fact not as distinct when viewed from a different perspective. Although zebrafish have no hematopoietic bone marrow, hematopoiesis occurs largely in the kidney marrow, which is at least to a large extent functionally equivalent to the mammalian bone marrow. The same applies to lymph nodes: although lymph nodes are absent in fish, antigen presentation, the main function of lymph nodes, occurs by antigen presenting cells but elsewhere. Although teleosts exhibit a blood brain barrier, the meninges is structurally different from mammals. In mammals the meninges consists of three layers, whereas in teleost only one membranous layer is present known as the primitive meninx. With regard to genetic conservation, it is important to note that a an ancient genome duplication occurred in teleosts. Therefore, it is estimated that 30% of genes have a duplicate variant, that may or may not exhibit redundancy and share the same function. Second, although main immune signaling pathways are highly conserved even across invertebrates, in particular the situation for chemokines is more complex, as they differ quite extensively[66,67]. In fact even among mammals several chemokines are not conserved at the sequence level, and functional homologs remain to be identified.

Organisation/Resource	Website
Zebrafish Information Network (ZFIN)	ZFIN.org
Zebrafish International resource center (ZIRC)	zirc.org
European zebrafish resource center (EZRC)	ezrc.kit.edu
National BioResource Project Zebrafish	http://www.shigen.nig.ac.jp/zebra/index_en.html
Zebrafish Disease Models Society	www.zdmsociety.org
Zebrafish Mutation Project (Sanger Institute)	sanger.ac.uk/Projects/D_rerio/zmp/
Zebrafish Brain Atlas	zebrafishbrain.org
Zebrafish Atlas	zfatlas.psu.edu/index.php
Zebrafish Genome (Ensembl)	www.ensembl.org/Danio_rerio/Info/Index
Zebrafish Genome (Sanger)	sanger.ac.uk/resources/zebrafish/genomeproject.html
Zebrafish Genome (NCBI)	ncbi.nlm.nih.gov/genome?term=danio%20rerio

Table 2. Online zebrafish resources

zebrafish brain and their high regenerative capacity, which in the adult mammalian CNS is thought to be mainly restricted to the subventricular zone (SVZ) and subgranular zone (SGZ)[41]. One interesting line of thought, suggests that the strong regenerative capacity of the non-mammalian vertebrate CNS may have become repressed in mammals during evolution[42]. An interesting finding, which supports this idea is a study by Powell and colleagues, who showed correlation between DNA demethylation and regeneration-associated gene expression in a zebrafish model for retinal injury. In particular methylated promoter regions of genes important for regeneration were demethylated in regenerating retina, whereas the same regions in mammals were not[42]. This suggests that the zebrafish could be suitable for identifying regenerative programs that could possibly be switched on in adult mammals as a potential therapeutic option in traumatic brain injury (TBI), brain damage arising from stroke, and other diseases involving loss of neurons.

Zebrafish immunity

Most major human immune cell lineages, such as macrophages, neutrophils, lymphocytes -B and T cells- have been identified in the zebrafish[43-45]. The development of zebrafish immune cells follows a similar differentiation trajectory and is controlled by a similar transcriptional program as found in mammals, involving waves of primitive and definitive hematopoiesis[46]. During primitive hematopoiesis, cells of the intermediate cell mass, the zebrafish equivalent of the primitive blood islands in the mammalian yolk sac, become either myeloid lineage cells or erythroid cells. This decision depends on Pu.1 and Gata1-dependent transcriptional activity, respectively, similar to mammals[46,47]. During definitive hematopoiesis, starting 1 day post-fertilization, hematopoietic stem cells (HSCs) are formed which expand into erythroid lineage cells and other myeloid lineage cells[46,48].

The adaptive branch of the immune system develops at a later stage which allows a

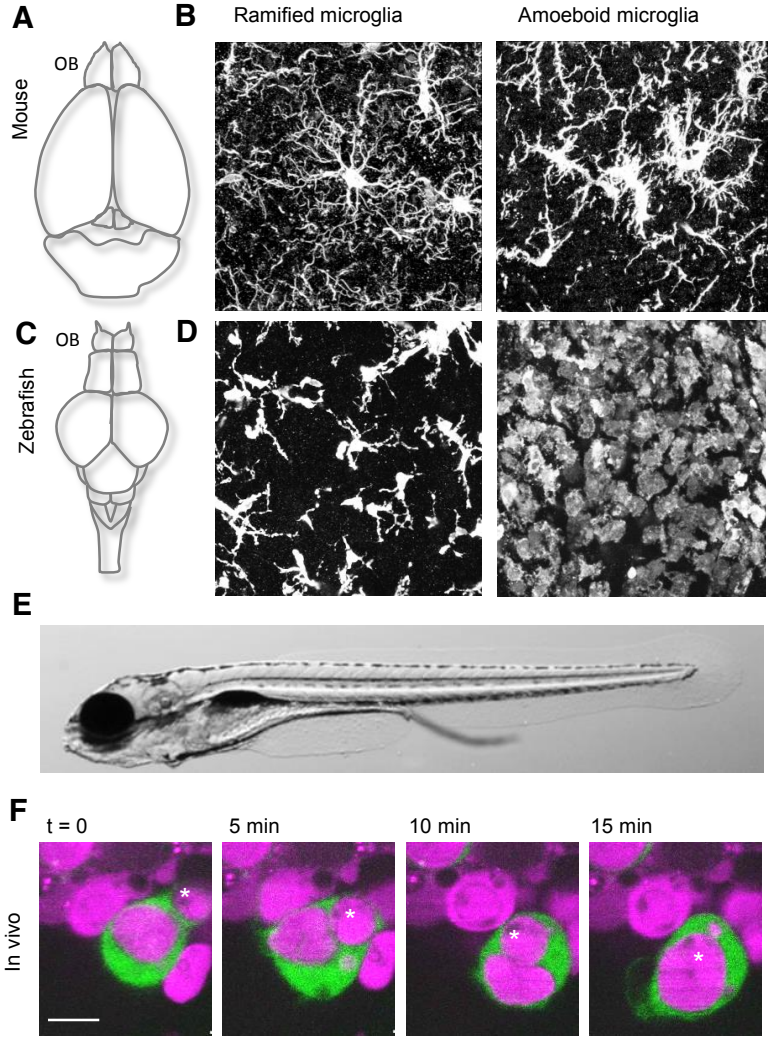

Fig 1. Conserved anatomic features of the brain in vertebrates and conserved microglial morphology and behavior. A, **C** Schematic representations of the mouse (top) and zebrafish brain (bottom). FB = Forebrain, OB = Olfactory bulb, Tel = Telencephalon, MB = Midbrain, HB = Hindbrain, CB = Cerebellum. **B** Mouse brain sections showing Iba1 antibody-stained microglia in ramified (left) and activated state (right). **D** Zebrafish brain sections showing L-plastin antibody-stained microglia (left) in ramified and activated, amoeboid state (right). **E** 5-day-old zebrafish larva. **F** Stills of confocal microscopic time-lapse recording showing engulfment of an apoptotic neuron (magenta, neuronal red fluorescent protein) by a microglia cell (green, microglial green fluorescent protein) in the brain of a 3-day-old zebrafish larva.

window in which the innate immune system can be studied with involvement of the adaptive system. In zebrafish generation of lymphocytes -lymphopoiesis- starts around 3 days after fertilization[49-51]. One major difference between the fish and mammalian adaptive immune system is the absence of lymph nodes in fish species in general[52]. However, zebrafish do have a repertoire of MHC expressing antigen presenting cells, B- and T-lymphocytes and a lymphatic system, showing that functional components of the adaptive immune system are present[44,49,53,54]. Quintana and colleagues demonstrated that the zebrafish has active mechanisms of self-tolerance by showing that the zebrafish ortholog for mammalian Foxp3 (zFoxp3), which is involved in the regulation of self-tolerance, controlled expression of IL-17, which is associated with autoimmune pathology in mammals[55]. Furthermore, zFoxp3 induced a regulatory phenotype on mouse T-cells, indicating that zFoxp3 has a similar function as mammalian Foxp3[55]. This suggests that the adaptive immune system in the zebrafish has fundamental similarities with mammalian adaptive immunity.

In addition to immune cell types, many of the mammalian immune receptor classes (e.g. TLRs, NLRs), signaling pathways and inflammatory mediators (e.g. interleukins, complement) are conserved in the zebrafish [56-59] (Table 1, 2). Furthermore, cellular responses to different immune challenges such as pathogens [60-62], wounding [63], and cancer [64] are similar. With regard to genetic conservation, it is important to note that an ancient genome duplication has occurred in teleosts. Therefore, it is estimated that 30% of genes have a duplicate variant, that may or may not exhibit redundancy and share the same function [65]. Second, although several main immune signaling pathways are highly conserved even across invertebrates, for example TLRs were discovered in fruit flies, the situation for chemokines in particular is a bit more complicated. Chemokine signaling molecules differ quite extensively between mammals and teleosts[66,67]. In fact even among mammals several chemokines are not conserved at the sequence level, and many functional homologs remain to be identified in the zebrafish.

Zebrafish CNS immune cells

Microglia are the resident immune cells of the CNS. They develop from primitive yolk sac-derived macrophages which colonize the CNS during embryogenesis and differentiate into microglia in a Spi1/Pu.1-transcription factor dependent manner[26,34]. Interestingly, this process was identified in the zebrafish about a decade before an analogous Spi1/Pu.1-dependent process, was identified in mammals[68,69]. Similar to mammalian microglia, zebrafish microglia are ramified cells (Fig. 1C,D) with dynamic processes that are scanning their environment. Upon infection or injury they can immediately respond by migrating to the relevant site, and are capable of efficiently phagocytizing bacteria and neuronal debris[26,34,37]. Pioneering studies by Philippe Herbomel showed that colonization of the CNS by early macrophages, which are microglial precursors, during early development involves a tyrosine kinase receptor named macrophage-colony stimulating factor receptor (CSF1R), a key regulator of the myeloid lineage. In a zebrafish mutant for the Csf1r gene these early macrophages initially fail to reach the CNS, although later colonization of the CNS by microglia appears to take place. Because zebrafish have two homologs of Csf1r, it is possible that the second receptor is sufficient for eventual microglial development[34]. In Csf1r knock out mice microglia fail to develop indicating that Csf1r-dependent microglia development is conserved between fish and mammals[68]. It is unclear how Csf1r signaling exactly

controls microglial development, as this appears to differ from development of other myeloid lineages. Additionally, a recent study shows the requirement for Csf1r signaling in microglial proliferation in mouse disease models[70]. Although the role of Csf1r in microglial proliferation has not been investigated in adult zebrafish this highlights the potential strength of zebrafish as a model to discover concepts directly relevant to mammalian and disease biology[70,71].

Astrocytes are highly abundant glial cells in the mammalian CNS, and although they are of ectodermal origin, they have immunological capabilities as well[72]. They are often identified by their high expression levels of the intermediate filament glial fibrillary acidic protein (GFAP). In the zebrafish brain radial glia are the main GFAP-expressing cells[35,73]. However, they possess properties attributed to astrocytes as well as radial glia in mammals. Whereas mammalian astrocytes are stellate cells with multiple processes, zebrafish radial glia cell bodies are localized at the ventricle with a single long process spanning the brain, more reminiscent of mammalian radial glia. As well, they share the strong neurogenic potential with mammalian radial glia. Typical properties shared with mammalian astrocytes include glutamate re-uptake from the synaptic cleft by the glutamate transporter GLT-1[74]. Additionally, in an adult zebrafish neuronal injury model radial glia respond in a manner similar to mammalian astrocytes[73,75,76]. Thus, although zebrafish radial glia show neurogenic potential and morphology characteristic of mammalian radial glia, they also show functional properties of mammalian astrocytes, suggesting they are partly functional equivalents of mammalian radial glia as well as astrocytes.

In vivo cell biology in the zebrafish

Recent developments in fluorescent imaging technology and the generation of transgenic zebrafish expressing fluorescent proteins labeling specific proteins, organelles and cells of interest make the zebrafish an increasingly powerful model organism (Fig. 1E,F). The development of effective genome editing strategies allows creation of virtually any type of genetic modification. We will highlight how combinations of these developments have led to basic insight in disease related processes, some of which have turned out to be of direct clinical use. This will illustrate the specific advantages of this model system and the expected benefit of using these models in the context of CNS disease.

Transgenic zebrafish have been very useful for functional genetic research by determining the effect of tissue-specific overexpression of genes of interest. Additionally, transgenic fluorescent reporter lines, labeling specific cell types or tissues using various fluorescent protein (FP) derivatives, is revolutionizing our understanding of in vivo cell biology. Initial experiments were directed at expressing green fluorescent protein (GFP)-tagged proteins to label a particular cell type, including different immune cell types allowing live imaging of their function in vivo[77-79]. For example, myeloperoxidase driven GFP (mpx-GFP) labels neutrophils, macrophage-expressed gene 1 (mpeg1)-driven GFP labels all mononuclear phagocytes including microglia, whereas apolipoprotein E (ApoE) more specifically labels microglia[37,80,81].

To target transgenes to specific cell types one can search and optimize a minimal promoter region yielding potent expression in the cell type of choice. Such promoters often lack all up and downstream regulatory elements to achieve a faithful representation of the actual expression pattern of the gene -or cell type of choice. Other approaches have been developed making it feasible to increase the reporter repertoire

substantially. BAC-recombineering takes advantage of recombination in bacteria to insert a sequence of choice into a BAC-clone. By targeting an FP directly behind the ATG translational start site of the gene of choice in a BAC containing the promoter but also more distant regulatory sites, the physiological expression of this gene can be captured[82].Hereby the need for initial identification of the required promoter region of the gene is circumvented. Additionally BAC-recombineering allows relatively straightforward creation of multiple reporter genes rapidly. Therefore this would facilitate the creation of new lineage or activation-state specific reporter lines, for example for newly identified microglial specific genes for which minimal essential promoter regions have not yet been identified[83].

Nowadays many types of FPs are available with diverse spectral properties, and localization signals, allowing quantitative in vivo visualization of organelles, subcellular processes including ionic fluctuations, activity of signal transduction pathways and macromolecular structural alterations[84-86]. For example genetically encoded calcium indicators (GECIs), such as Gcamp, have been optimized and used to study neuronal circuits in the zebrafish[84,87,88]. Recently, Hochbaum et al., have developed voltage indicators, which can be used for optical electrophysiological studies[89]. In addition to 'normal' FPs, there also is a repertoire of photo-convertible proteins available that allow switching colors of FPs from green to red (e.g. kaede, dendra) or from a dark state to an active fluorescent state (photoactivatable GFP, PA-GFP)[90,91]. This allows for spatiotemporal lineage tracing, which will be discussed later. Zebrafish with several of these transgenic markers can be crossbred relatively easily. This yields a virtually endless combination of transgenic reporters. By using combinations of nuclear-, membrane- and cytoplasmic FPs, with only three colors, 9 different cell types could in principle be labeled at the same time, and imaged at high spatiotemporal resolution in the living brain[82,92]. Alternatively, several subcellular processes could be imaged in the same transgenic fish. In all, the development of FPs with diverse properties allows spatiotemporal labeling in vivo at the structural, cellular, subcellular and functional level.

Substantial knowledge on human neuropathology has come from immunohistochemistry using formaldehyde-fixed, paraffin embedded tissue stained for hematoxylin/eosin (H&E) or antibodies thus marking disease specific hallmarks. On the other hand, what we learn about in vivo mechanisms in zebrafish is generally based on monitoring of cell-specific expression of fluorescent proteins. Using live imaging one can monitor events as they occur in vivo. It is sometimes unclear what these features would look like in fixed tissue, preventing direct comparisons and extrapolation of possibly relevant findings in vivo. Thus biological events that are rarely found in a specific snapshot in fixed tissue, are more easily identified by long-term video microscopy[93,94]. Additionally, by correlated microscopy imaging live events and static images can be combined thus allowing the extrapolation of biological processes to conventional histopathology[95]. This makes it easier to address more basic fundamental questions in in vivo models and translate new meaningful hypotheses to disease mechanisms.

In vivo studies can yield unexpected insight and discoveries that would likely be missed in studies using immunohistochemistry or other techniques that do not provide information about dynamic processes. We will highlight insights and discoveries related to immune cell development and function, to indicate the type of mechanistic insight that can be derived. Second, we will highlight how zebrafish models can lead to clinically applicable concepts and potential drugs for translational medicine approaches. As

mentioned in the introduction, the zebrafish has proven its value as a model in various aspects of neuroscience, however, these are beyond the scope of this review. Examples of these aspects include modeling behavior and complex behavioral brain disorders in adult zebrafish, optogenetics, understanding neurogenesis and neuroregeneration, as well as high throughput behavioral and neuroactive drug discovery[96-101].

New insight in infection and immunity from in vivo studies in zebrafish

In vivo studies in the zebrafish have resulted in new, unexpected insights into basic immune responses. For instance, a zebrafish tuberculosis (TBC) infection model has allowed visualization of basic disease mechanisms in vivo with regard to pathogen propagation, treatment resistance and genetic vulnerability[62,102,103]. A hallmark of TBC is the formation of granulomas, which were generally thought of as static structures formed as a protective mechanism by the host organism. Zebrafish studies, however, revealed that these granulomas are highly dynamic structures used by the bacteria for propagation, potentially changing the putative disease mechanism[102]. Another example shows very basic immune cell behavior that has been previously unrecognized, and is currently validated in mammalian disease models. In a zebrafish wounding model using transection of the tail fin, resolution of inflammation was accompanied by reverse migration of neutrophils away from the site of injury[104,105]. Since then, this phenomenon has been shown in mammalian model systems and may be relevant for human disease biology[106,107]. These examples, although they partly remain to be investigated in mammals, indicate the type of unexpected insights found by unbiased in vivo imaging experiments that could alter the view on the roles of behavior of immune cells in disease.

A recent elegant example in which in vivo imaging was used to elucidate a complex multi-organ feedback loop driving the increased production of granulocytes in response to a cerebral infection was is the study by Hall et al[108]. By genetic and pharmacological manipulation, and live imaging they showed that in zebrafish larval brains infected with Salmonella bacteria, macrophages secrete granulocyte colony stimulating factor (Gcsf) into the blood. Subsequently they showed that Gcsf receptor activation in the hematopoietic tissue induces expression of the transcription factor C/ebpâ, which then drives expression of nitric oxidase Nos2a, controlling proliferation of hematopoietic stem cells required for the differentiation of new granulocytes[108]. Although these effects were known already as separate processes, the use of in vivo imaging in this zebrafish experiment allowed for the connection between these separate processes, thereby elucidating a complex multi-tissue signaling mechanism in a single study.

Several immune deficiencies with defects in specific hematopoietic lineages have been modeled using zebrafish mutant for homologs of human disease genes. By imaging leukocyte behavior in these mutants better understanding of the functional effect of these mutations on leukocyte behavior has been achieved[109-111]. Good examples have been provided for Wiskott-Aldrich syndrome (WAS) and X-linked neutropenia (XLN), which are immunodeficiencies caused by mutations in the WASp gene resulting in defective control of the small GTPase Cdc42. Depending on the mutation, this can lead to diverse neutrophil phenotypes ranging from defective generation of neutrophils up to altered chemotaxis and phagocytic capacity. By expression of human disease variants of WASp in a zebrafish background lacking functional WASp, Jones and colleagues observed indeed differential effects on leukocyte functioning, the capacity and generation of neutrophils, chemotactic

responses to wounding, and the phagocytic capacity of macrophages[110]. These studies thus allow testing of clinically relevant mutations and variants of known immunodeficiencies, to gain a detailed in vivo understanding of defects in leukocyte 3-dimensional migratory behavior that may not be apparent in tissue culture models.

Novel insights in microglial phagocytic responses

Phagocytosis is a key function of immune cells in health and disease. Many brain diseases involve extensive cell death, but how dying neurons are cleared from the CNS has proven hard to visualize, partly because the process is thought to be very efficient. Many components of the engulfment machinery have been identified using that are involved in finding, recognizing and engulfing dying cells, and in the subsequent processing of ingested material, but how this machinery precisely operates within the complex brain tissue is poorly understood in vivo[112-114].

In order to study genes involved in control of engulfment in the brain in vivo, we developed a transgenic zebrafish reporter line to label apoptotic cells[115]. Fluorescently tagged annexin V (A5) protein, is widely used to detect cell death, by binding with high affinity to phosphatidylserine (PS), which is exposed on the plasma membrane in apoptotic cells[116]. We created a secreted version of A5 (secA5), to achieve labeling of PS exposure in dying cells in vivo[115]. Subsequently, by intravital microscopy in secA5-transgenic zebrafish we visualized how dying neurons are engulfed by microglia in living brains [117]. Additionally, by visualizing defective engulfment in vivo, we show that the guanine nucleotide exchange factor ELMO1 (*E*ngulfment and Cel*l Mo*tility protein 1) is needed for completion of engulfment after recognition of the dying cell, via regulation of actinomyosin dynamics in the formation of the phagocytic cup [117]. Around the same time ELMO1 was shown to play a role in phagocytic processes required for adult neurogenesis in mice, suggesting this gene may indeed play a role in engulfment under physiological conditions[118]. More recently, Mazaheri and colleagues have used transgenic secA5 expression in live imaging studies as well to visualize PS exposure and clearance of dying neurons[119]. In their study they use secA5 labeling of dying neurons to study the genes bai1 and tim4, previously implicated in vitro in recognition and adhesion of apoptotic cells[120-122]. They show that the in vivo functions of these two genes, is actually different from their function in vitro, as bai1 and tim4 control phagosome formation around dying neurons and stabilization of this phagosome. Together these studies clearly show how in vivo imaging data can assist in achieving a molecular level of understanding in vivo mechanisms involved in engulfment. It will be interesting to determine how the role of these genes extends to mammals. Second, these studies illustrate that the development of new fluorescent reporters can directly facilitate research into cellular and even subcellular processes by gaining truly unprecedented in vivo resolution. Furthermore, unexpected behavior of immune cells provides instructive functional hypotheses for future experiments to test the relevance of phagocytosis behavior in mammalian models.

Before phagocytic cells can perform their task they need to migrate towards the area where they are needed, attracted by chemokines and other cues, and it is critical to understand how and what signaling pathways govern recruitment of these immune cells to specific brain areas . An initial clue of the intercellular signaling mechanisms involved in recruiting microglia to injury comes from a live imaging study in zebrafish by Sieger and co-workers. In a laser mediated neuronal injury model neurons show glutamate

receptor (NMDAR) activation leading to Ca^{2+} influx, which causes ATP-dependent microglial migration through purinergic P2Y12 activation[123]. Similar observations have been made for mammalian microglia recently. Eyo et al., showed that during kainate-induced seizures in mouse brain slices and in vivo, glutamate-induced microglial responses involve NMDA receptor-dependent Ca^{2+}-influx, followed by ATP release and activation of microglial P2Y12[124]. Interestingly, the same issue of the journal contained a second study that demonstrated that NMDAR activity results in ATP-dependent mouse microglial responses[125]. These examples clearly show that fundamental biological functions and processes regarding microglia in the CNS are highly conserved. In addition to these studies on microglia responses to wounding, Li and colleagues used in vivo imaging to show that microglia are induced to contact highly active neurons and decrease their spontaneous firing[126]. Again, these type of conceptual findings may pave the way for more focused studies in mice.

Studying immune responses in zebrafish models for CNS disease

Several studies have shown that zebrafish can be used to study CNS disorders, such as neurodegenerative diseases and epilepsy. For example Paquet and colleagues generated a zebrafish model for frontotemporal dementia (FTD) and Alzheimer's disease (AD) by transgenic overexpression of mutant human tau resulting in cell death and disease related tau-phosphorylation[127]. Polyglutamine inclusions associated with Huntington's disease have also been induced in the zebrafish[128]. More recently loss of function of Tdp-43, involved in amyotropic lateral sclerosis (ALS) and FTD, was shown to result in motor neuron axonopathy in the zebrafish[129]. Additionally, the zebrafish has been used successfully in studies on other brain conditions including genetically inherited epilepsy known as Dravet syndrome, psychiatric disorders and neurodevelopmental disorders[130,131]. Immune cell behavior was not the aim of any of these studies, but it would be interesting to compare in these models how immune cells respond to the different disease contexts.

Recently, we have used nitroreductase (NTR)-mediated ablation to specifically induce neuronal cell death in the zebrafish brain[94,117]. Nitroreductase is a bacterial enzyme known as nsfB, which can convert the antibiotic metronidazole into a DNA crosslinking agent, causing programmed cell death. Transgenic expression of nsfB/NTR has been successfully used in zebrafish to study regeneration after ablation of pancreas, heart and other tissues[132,133]. By applying brain-specific expression of nitroreductase we showed that macrophages and microglia, clear dead neurons at the earliest stages after induction of neuronal cell death whereas only microglia dominate at later stages. Interestingly, animals recover from damage completely after ablation at larval stages, even after multiple ablations. The recovery phase involves programmed cell death of phagocytic immune cells, which are cleared by microglia. This is reminiscent of immune cell behavior upon wounding, where immune cells also undergo programmed cell death in a process known as resolution of inflammation[134]. We also found that all phagocytes that have engulfed neuronal debris are positive for a marker that labels microglia as well as macrophages, whereas only a subset of phagocytes showed activity of the microglia-specific ApoE-promoter[94]. This shows that peripheral macrophages and resident microglia are recruited to brain injury in zebrafish, and reveal these two cell types have different roles during their response to neuronal cell death. Another study used nitroreductase (NTR)-mediated ablation in sensory peripheral neurons and

showed recovery after ablation in these neurons, which seems to require the presence of peripheral glial cells[135]. This suggests that depending on the location of neuronal cell death, different cell types are involved in recovery and that NTR ablation can be used to understand immune maintenance of different types of neuronal tissue.

Several other ways of inducing transgenic targeted cell death include expression of the fluorescent protein KillerRed, which upon irradiation with green light can induce oxidative stress-mediated cell death[10], and thymidine kinase (TK), which can convert ganciclovir into a cytotoxic agent, killing proliferative cells in particular[136]. Such techniques can be used to damage tissues in different ways than NTR, to test if different causes and types of cell death yield the same immunological outcome. Alternatively, these techniques can also be used to ablate specific immune cell lineages, to identify their corresponding functions[137].

Many disease and injury model studies in zebrafish focus on embryos and larvae because of their transparency and small size amongst others. Adult zebrafish models have been developed as well, applying various types of invasive injury including a telencephalic stabwound, spinal cord lesions, and injection of excitotoxic agents[76,138-141]. Alternatively, these paradigms can often be applied to larvae as well. Adult zebrafish are particularly useful to study adult neuroregeneration and may allow assessing whether findings in larval brains apply to adult brain as well, which may better predict their effect in adult mammals. One study using the stab wound paradigm has shown that recovery from a brain stab wound requires an inflammatory response for repair and regeneration to occur[142].

Future perspectives

In view of the recent technological developments the studies mentioned reflect only the onset of the possibilities of the zebrafish as a model for CNS disease. The concept of a genetic model organism is that genes can be manipulated efficiently to study the function of genes for example those involved in immune function. A considerable limitation of application of zebrafish genetics has been the difficulty to generate targeted or conditional knockouts. One useful possibility is the use of TILLING, targeted induced local lesions in genomes, in which ENU mutated fish are screened for mutations in target genes to obtain mutants[143,144]. Recently developed genome editing techniques have made the generation of targeted mutations in the zebrafish much easier. The first of these techniques has been published in 2008 and involves the use of zinc finger proteins[145], followed by recent addition of TALENs and the CRISPR/CAS system[8,9,146]. All these techniques work via the introduction of a double strand break at a specific position in the genome. When targeted at the right position this can block nonsense mutation or truncated transcript, leading to disruption of gene function. Although many papers published on this topic in relation to the zebrafish are in fact proof of concept papers, these techniques will be extensively used in the zebrafish to produce a wide variety of mutants. In addition to directly disrupting gene function, these techniques also allow targeted insertion of DNA elements into the genome[147,148]. Hereby, knock in animals can be generated, expressing for example FPs from a targeted locus, and the generation of conditional mutants should be possible in combination with the Cre-lox system mentioned below. Thus, these developments have established a versatile genetic toolbox well matched to available approaches in mouse genetics.

Although these new reverse genetics techniques allow creation of mutants, the

strength of unbiased forward genetic screening is still appealing. The availability of the sequence of the complete zebrafish genome, the reduced costs and the incredible throughput of massively parallel sequencing approaches greatly facilitates forward screening efforts. An illustrative example of how an ENU mutagenic screen in zebrafish can yield insight in concepts of microglial activity comes from a recent effort to discover new genes essential for microglial function. Shiau and colleagues described that one of the identified mutants showed a complete lack of microglia in the CNS caused by loss of function of the NOD-like receptor nlrc3-like gene[149]. NOD-like receptors (NLRs) are cytoplasmic pattern recognition receptors, with important regulatory function in immune cell activity[150]. Using genetics, live imaging and vital dyes they showed that nlrc3-like normally prevents inflammatory activation in microglia during their journey to the brain[149]. Without functional nlrc3-like, the microglial precursors show an inflammatory, distracted, phenotype preventing them from reaching the brain. Interestingly, the authors did find neutrophils in the brain, consistent with systemic inflammation[149]. One concept derived from recent studies on microglia is that they are under strong inhibitory control, which when lost, can lead to toxicity of microglia towards neurons[151-153]. The study on nlrc3-like very elegantly, although indirectly, shows the concept of a need for inflammatory suppression of immune cells. The homolog of nlrc3-like has not been identified in mammals, but the protein is very similar to human Nlrc3, and it will be interesting to determine the function of mammalian Nlrc3 and other NLRs in microglia. In fact, recent studies linked activation of another NLR, NLRP3, in microglia by amyloid-beta to development of Alzheimer's disease[154].

A key issue in neuroimmune research concerns the individual contributions and roles of different types of immune cells in CNS disease. Fate mapping has been used extensively by embryologists to track distribution of embryonic tissues in older animals by for example dye injections, which allowed important insights in developmental biology. Nowadays, single-cell fate mapping techniques, known as lineage tracing, allow detailed single cell insight into cellular origins and distribution which can be combined with intravital imaging. For example Cre recombinase mediated lox recombination is generally used in mice to generate tissue specific- or conditional genetic knockouts[155]. The bacteriophage gene Cre is expressed in a specific cell type, where it can excise a genetic element based on two flanking genetic elements known as loxP sites. When using Cre, fused to an estrogen receptor fragment (ERT2) the activity of Cre can be induced via treatment with tamoxifen to temporally control Cre activity. In combination with fluorescent proteins CreERT2 can be used to permanently switch on or off expression of a specific fluorescent protein in a given tissue allowing creation of multicolor lineage tracing, as was used in the brainbow mice and zbow zebrafish[156,157]. In addition to fluorescent protein expression, this technique can be used to switch genes on or off in cell type specific manner, allowing the creation of conditional knockouts in zebrafish. An example of how this can be used in the context of neuronal injury is shown a recent study by Kroehne et al[76]. They showed in adult zebrafish that radial glial cells contribute to new neuronal tissue in response to a telencephalic stab wound by using a radial glial expressed CreERT2. In addition to tracking radial glia cells, such a system could as well be used in the zebrafish to study the origin and fate of immune cells upon damage in the brain. This would allow us to address questions related to the individual contributions of separate populations of macrophages and microglial cells.

Additional questions that can be studied using these approaches for example in

combination with an ablation model are related to where specific immune cells come from and where they eventually go to. Is there local or more distant recruitment of microglia, or is there recruitment of peripheral immune cells? Our recent study using in vivo and electron microscopy suggests that peripherally derived macrophages can enter or exit the brain via the olfactory nerve in the nasal cavity[94]. Although this route has been shown in histological sections in mouse models[158], it is virtually impossible to capture using live imaging in mice. Other possible routes, which can be easily imaged, include the zebrafish choroid plexus which is formed within 6 days after fertilization[33] and the BBB, which is formed within the first few days after fertilization[32]. By using lineage tracing techniques, these questions can be conclusively addressed in vivo.

Recently, many comprehensive studies have been published showing transcriptome analyses of microglia in mouse models for neurological diseases using microarrays and RNA sequencing[159-162]. Additionally, genetic studies in humans have identified tremendous numbers of potential disease genes and variants. For both these type of data sets zebrafish provides a great platform to analyze expression patterns and test the effect of manipulating genes on immune function or disease progression before moving to mammalian systems or identifying them as causative variants in disease. For example in situ mRNA hybridization of candidate genes in zebrafish is a relatively straightforward way to identify relevant expression patterns. Similarly, BAC recombineering can be used as an in vivo alternative to in situ RNA detection to identify dynamic expression patterns[82]. In fact BAC recombineering was applied to create a reporter for expression of P2Y12 marking microglia in zebrafish[123]. This gene was later found as a highly enriched microglial gene in several mouse transcriptome studies mentioned[159-161,163]. Subsequently mutants can be created for such a gene, and virtually any combination of the techniques mentioned, including tissue-specific conditional markers or loss of function. Taken together with technical advances mentioned above, this suggests that also for genes found in brain disease models by large scale genetic approaches, functional analysis in zebrafish is becoming a useful starting place for functional genomics and to determine the function of these genes.

Small molecule screening & drug discovery

Another important aspect of zebrafish is their suitability for high-throughput drug screening. Pioneering studies by Peterson and colleagues in 2001 showed in zebrafish embryos that small molecules, efficiently absorbed from the surrounding liquid, can induce very specific developmental phenotypes[164]. Because of their small size, zebrafish embryos and larvae can be kept in 96 well plates. This, in combination with the easiness of treatment with chemical compounds (addition of the compound to the medium) makes them highly suitable for high-throughput drug screens, of which some have already led to direct clinical applications. The prostaglandin derivative dmPGE2, initially identified in zebrafish as a drug that increases the number of hematopoietic stem cells (HSCs) has been approved for phase I clinical trials to enhance engraftment of HSC transplantation in leukemia patients[165,166]. Another compound, lenaldekar (LDK), identified in a zebrafish small molecule screen for selective elimination of immature T cells, may be a new drug for treatment of T cell leukemia[167]. Interestingly, because multiple sclerosis involves an over-activation of T cells as well, they tested this new drug in an MS mouse model (EAE) showing a reduced disease severity due to prevention of expansion of the T-cell population[168]. This also shows that although there are no

zebrafish models for autoimmune diseases -yet-, zebrafish studies can have direct relevance for research on autoimmunity. A recent example of a novel drug found in zebrafish and moving towards clinical application is based on the drug dorsomorphin, which causes dorsalization in zebrafish embryos and inhibits BMP signaling. Because a fatal connective tissue disease fibrodysplasia ossificans progressiva (FOP) is caused by increased BMP signaling, inhibiting this pathway could inhibit the disease process. The effectivity of the dorsomorphin derivative in a mouse model for this disease has led medicinal chemists to develop more potent derivatives of dorsomorphin that may soon be tested in early clinical trials[169,170]. These data clearly indicate that small molecule screens in zebrafish lead to discovery of drugs with high clinical relevance without a priori knowledge of what drug target would be involved. Beyond these screens of disease related phenotypes, and interesting in the context of this review, high-throughput behavioral drug discovery has revealed already many new neuro-psychoactive compounds[14,99]. Although the exact mechanisms of some of the drugs found are unknown, finding new drug classes is an Achilles heel in drug discovery, as most of the currently known drugs were found serendipitously[171].

In the context of this review, small molecule screening could be applied to transgenic fluorescent reporter lines marking brain immune cells to find small molecules that alter intensity or localization of these reporters in vivo. Two recent examples of screens directed at finding drugs that alter immune cell behavior show that such an approach is feasible. In the first study they identified small molecules that inhibit retrograde migration of neutrophils in a zebrafish tailfin wounding model[172]. Interestingly, drugs found in this screen had the same effect on human neutrophils suggesting these responses are highly conserved. In a second study they identified small molecules that inhibits inflammatory recruitment of immune cells to killing of lateral line neuromast cells by copper sulfate[173,174]. Imaging fluorescent phenotypes in the brain in 3D has been difficult in a high throughput fashion, mainly due to out of focus light, and the general low throughput nature of confocal microscopy. Recent developments have also started to overcome these hurdles by designing 96 wells plates to mount larvae in the right position for imaging, building microfluidics systems for automated confocal imaging and automated quantitative analysis of the imaging data[175,176]. Therefore, in addition to using the models described throughout this chapter for annotation of gene function, and elucidation of disease mechanisms, the possibility of small molecule screening in zebrafish has become a mainstay in novel bioactive small molecule discovery[177].

Concluding remarks

A recent zebrafish review has suggested that zebrafish models should prove themselves by discovering new biology instead of merely modeling disease processes[178]. Based on progress described above this step has clearly been taken. Although many aspects of zebrafish biology with regard to neuroimmunology are highly conserved, obviously, there are differences between the human or mammalian and fish immune system. For example zebrafish do not have lymph nodes. On the other hand, zebrafish do have MhcII expressing antigen presenting cells suggesting they have functional equivalents of lymph nodes elsewhere. Also, hardly any of the antibodies used in humans and mice as markers for different immune cell types and inflammatory phenotypes are compatible with the zebrafish. Fortunately, transgenic markers are as versatile as antibodies and can be used for the same purposes equally well. Therefore, for basic questions about

the functioning of the immune system such minor differences in mechanism or approach do not have to be limiting. We argue that recent unexpected discoveries and technical advances create a niche for the zebrafish in the field of neuroimmunology by allowing an unprecedented view into cellular mechanisms of immune-maintenance and repair in the brain with the realistic possibility of eventually extrapolating basic findings to the clinic.

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Chapter 3

Identification of a conserved and acute neurodegeneration-specific microglial transcriptome in the zebrafish

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Abstract

Microglia are brain resident macrophages important for brain development, connectivity, homeostasis and disease. However, it is still largely unclear how microglia functions and their identity are regulated at the molecular level. Although recent transcriptomic studies have identified genes specifically expressed in microglia, the function of most of these genes in microglia is still unknown. Here, we performed RNA sequencing on microglia acutely isolated from healthy and neurodegenerative zebrafish brains. We found that a large fraction of the mouse microglial signature is conserved in the zebrafish, corroborating the use of zebrafish to help understand microglial genetics in mammals in addition to studying basic microglia biology. Second, our transcriptome analysis of microglia following neuronal ablation suggested primarily a proliferative response of microglia, which we confirmed by immunohistochemistry and in vivo imaging. Together with the recent improvements in genome editing technology in zebrafish, these data offer opportunities to facilitate functional genetic research on microglia *in vivo* in the healthy as well as in the diseased brain.

Introduction

Microglia are the resident macrophages of the central nervous system that serve important physiological functions related to neuronal plasticity and connectivity[1-6]. In addition, they play an important role in many neurodegenerative diseases, as scavengers of pathogens, debris and dead cells, and as regulators of immune responses[7-9]. Moreover, several genetic neurological diseases have been found to be caused by microglial defects[10]. Nonetheless, the exact mechanisms by which microglia regulate brain homeostasis and contribute to disease are still unclear. Recently, genome wide gene expression analyses of acutely isolated microglia from mouse brains have revealed many of the genes and pathways that distinguish microglia from other brain and immune cell types[11-15]. However, the role and significance of many of these genes for microglial function remains to be elucidated. Microglial identity is induced by interplay of their developmental ontogeny and their position in the heterogeneous brain tissue, and therefore functional analysis of microglia in healthy and diseased brain is best addressed *in vivo*[16-19].

Zebrafish share high similarity in embryonic development, cell biology and genetics with mammals and they are transparent at larval stages, which makes them highly suitable for non-invasive imaging *in vivo*[20-22]. Analogous to mammalian microglia development, the first zebrafish microglia develop from a subset of early macrophages in the rostral blood island on the embryonic yolk sac that migrate into the brain[15,23-27]. Functions described *in vivo* for zebrafish microglia include the clearance of dead brain cells and debris, the detection and removal of invading pathogens and regulation of neuronal activity[3,26,28,29]. Phenotype driven genetic screens for microglial defects in zebrafish have already yielded new insight in microglial biology[30,31]. Advances made in genome editing technology in zebrafish have now made it possible to perform reverse genetic screens in zebrafish[32-36]. Therefore, the zebrafish appears to be an excellent model to further elucidate *in vivo* microglia gene function in development and in a disease context in a systematic manner by using reverse genetics.

However, as only a handful of zebrafish microglial genes are currently known, it is unknown how zebrafish microglia compare to mammalian microglia at the gene expression level[26,37-40]. To identify genome-wide gene expression in microglia we optimized acute isolation of microglia from zebrafish brains by FACS and used RNA sequencing to compare their gene expression signature to the expression profile of other brain cells. Here, we identified the zebrafish microglia transcriptome, including many orthologs of mammalian microglia-specific genes, indicating conservation of microglia gene expression across vertebrate classes. Additionally, we applied RNA sequencing to study how microglia respond to induced neuronal cell death, and identified that neuronal death induces extensive local proliferation of microglia. These findings will facilitate investigating the genetics of microglial biology and their role in disease.

Results

Acute isolation and RNA sequencing of zebrafish microglia

To identify microglial gene expression in zebrafish, we performed RNA sequencing on acutely isolated adult zebrafish microglia. To label microglia, we used transgenic *mpeg1*-GFP zebrafish expressing GFP specifically in cells of the macrophage lineage, including microglia [49,50]. Approximately 100,000 GFP$^+$ cells were isolated from 5 pooled zebrafish brains, using fluorescence activated cell sorting (FACS) (Fig 1 A, B, S1A). The GFP$^-$ cellular fraction, representing neurons and other glial cells, was used to determine genome wide gene expression in non-microglial brain cells. RNA sequencing was carried out on 3 biological replicates. Principal component analysis based on the expression profile showed that the GFP$^+$ and GFP$^-$ samples formed separate distant clusters (Fig S1B), indicating highly distinctive gene expression patterns.

To confirm that isolated *mpeg*-GFP$^+$ cells are microglia and express previously identified zebrafish microglial genes, we investigated differentially expressed genes in the isolated GFP$^+$ cell fraction. We identified a total of 6511 differentially expressed genes (FDR < 0.01, LogFC > |2|) of which 2411 genes showed significantly higher expression in the GFP$^+$ fraction (Fig 1C,D, Table S1). These 2411 genes included *mpeg1*, on which FACS sorting was based, and other genes previously described in zebrafish microglia including *apoeb*, *csfr1a*, *spi1a*, *slc7a7* and *irf8* (Fig 1E, Table S1)[26,38,39,49]. In contrast, genes mostly expressed in neurons (*snap25a, neurod1)*, oligodendrocytes (*plp1a, mag*) and radial glia (s*lc1a2b, s100b*) were significantly higher expressed in the GFP$^-$ neuronal and glia fraction (Fig 1F). Altogether, we identified previously known, and many novel, zebrafish microglia genes in the isolated *mpeg1*-GFP$^+$ microglia population.

Functional conservation of microglia

To determine whether zebrafish microglia express typical vertebrate macrophage genes and genes related to immune function, we investigated the differentially expressed genes in more detail. Myeloid transcription factors that are essential for macrophage identity and immune function such as *Irf8, Pu.1, Mafb, Cebp/*α and *Jun* showed high expression in zebrafish microglia and were hardly detectable in other brain cells (Table S1). Moreover, Ingenuity Pathway Analysis (IPA) revealed that genes with a significantly higher expression in microglia compared to other brain cells are mainly associated with immune responses, including production of reactive oxygen species (ROS) in macrophages and monocytes, NF-κB and interleukin signaling (Fig 2A). Zebrafish microglia also showed high expression of several Toll like receptors (TLRs) (e.g. *tlr1, tlr7, tlr21*), chemokine receptors (CR) (e.g. *cxcr5, ccr12a, ccr9a*), purinergic receptors (PR) (e.g. *p2rx3a, p2rx7, p2ry12*) and components of the mhc class II complex (e.g. *cd74a, cd74b, mhc2dab*), that were also hardly detectable in other brain cells (Fig 2B, Table S1). Last, we found high microglia-specific expression of components of the complement system, including C1q homologs *C1qa* and *C1qb*, components of the complement receptor 3 (CR3) complex, and progranulin, all of which have been shown to be involved in synaptic pruning in mice[51,52]. Taken together, zebrafish microglia express many of the transcriptional regulators, immune and pathogen recognition receptor repertoire and pruning associated genes found in mammals, indicating that zebrafish microglia show similar functionality as found in mammals.

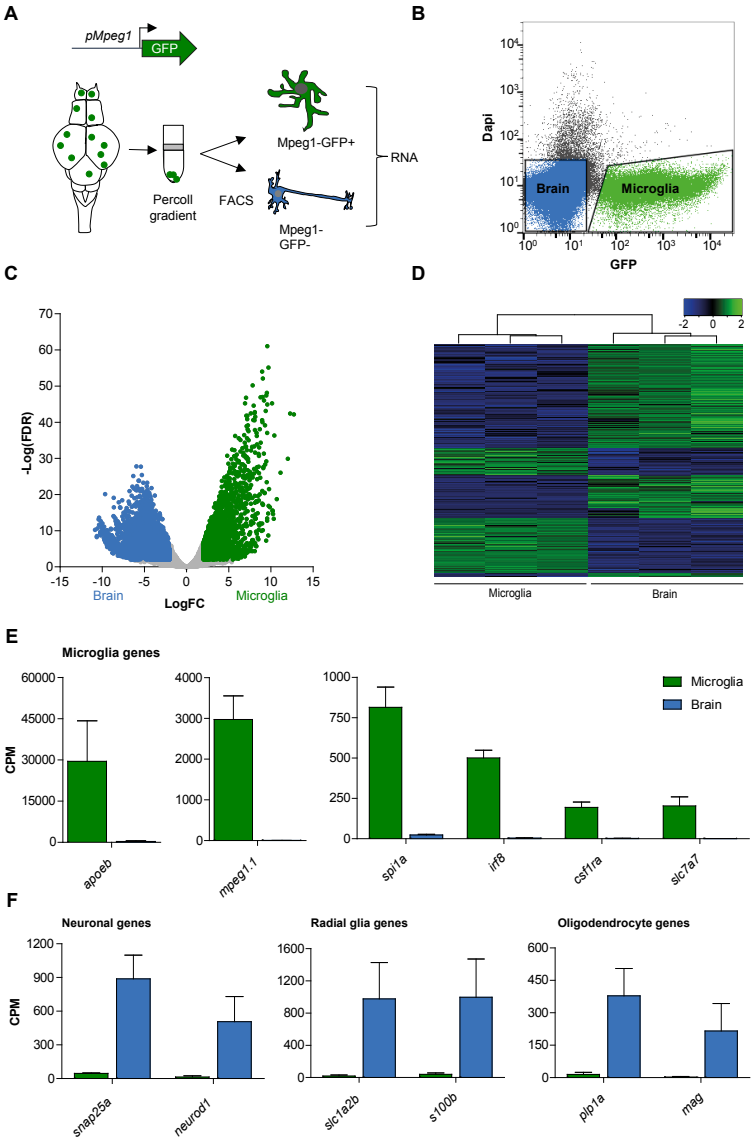

Fig 1. Sequencing of the zebrafish microglia transcriptome. **A** Schematic representation of acute isolation of zebrafish microglia from *mpeg*1-promoter driven GFP transgenic zebrafish (*mpeg1*-GFP). **B** FACS plot showing isolated populations for RNAseq in zebrafish microglia (green) and other brain cells (blue). **C** Differential gene expression (Volcano plot) showing genes significantly higher expressed in microglia (green) and other brain cells (blue). FDR < 0.01, LogFC > |2|, n = 3. **D** Heatmap showing Z-score values of all genes differentially expressed between microglia and other brain cells (6511 genes) (FDR < 0.01, LogFC > |2|). **E-F** Expression values (CPM) for known microglial, neuronal, radial glial and oligodendrocyte genes in GFP$^+$ (microglia) and GFP$^-$ (brain) cells. Values in **E** and **F** represent means of three independent experiments; Error bars in **E,F** represent standard deviation. FDR = False discovery rate. CPM = counts per million.

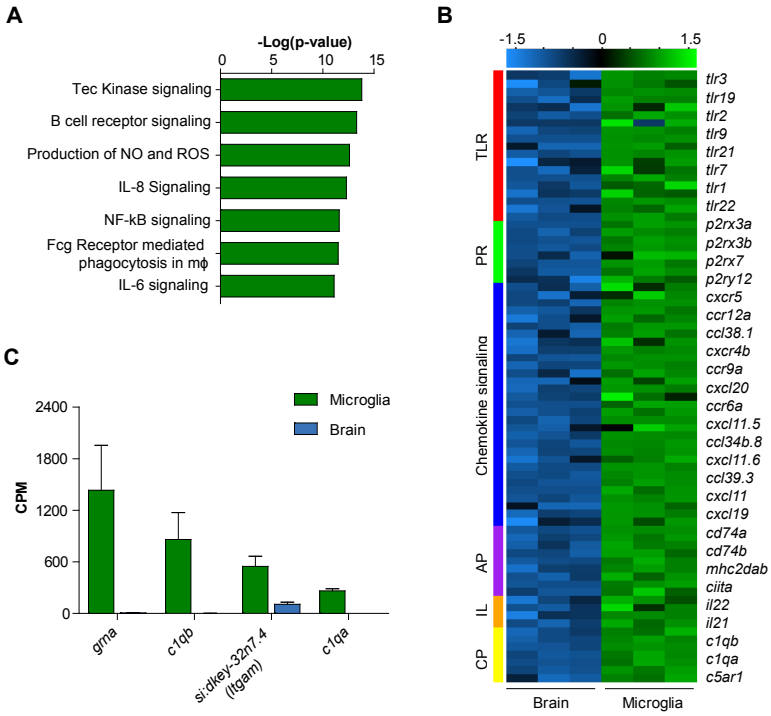

Fig 2. Conserved microglia functions in the zebrafish. **A** Ingenuity pathway analysis (IPA) canonical pathways most significantly enriched in zebrafish microglia. **B** Heatmap showing expression Z-scores of immune genes significantly higher expressed in microglia than other brain cells (FDR<0.01 and logFC>1). **C** CPM values of zebrafish orthologs of genes involved in synaptic pruning in microglia and brain samples. Values in **C** represent mean of three independent experiments; Error bars in **C** represent standard deviation. TLR = Toll-like receptors, PR = purinergic receptors, AP = antigen presentation, IL = interleukins + interleukin receptors, CP = complement.

Identification of a conserved zebrafish microglia gene expression signature

To investigate to what extent the microglial gene expression profile is conserved between zebrafish and mammals, we compared the zebrafish dataset to several previously published mouse microglia transcriptomes[11,13,14]. Zhang et al. (2014) used RNA sequencing and identified 500 significantly enriched genes in mouse microglia compared to other cell types in the brain, including neurons, astrocytes and oligodendrocytes. For these genes we found 361 annotated zebrafish orthologs, of which 163 orthologs showed significantly higher expression in adult zebrafish microglia (e.g. *c1qb, cd68*) compared to other brain cells (FDR < 0.01, logFC > 2) (Fig 3A,B, Table S2). A second study applied direct RNA sequencing on mouse microglia and reported 100 genes encoding mainly cell surface molecules with significantly higher expression in microglia compared to whole brain (Hickman et al., 2013). Comparison with zebrafish microglial genes expression showed that out of 66 identified zebrafish orthologs, 42 orthologs (e.g. *slco2b1* and *gpr84*) are significantly higher expressed in the zebrafish microglia transcriptome (FDR < 0.01, logFC > 2) (Fig 2A,B, Table S2). In a third study, Butovsky et al. (2014) performed quantitative mass spectrometry and gene expression profiling on isolated microglia and showed 106 genes with significantly higher

expression in microglia than in whole brain samples. We found 101 zebrafish orthologs of which 44 are significantly higher expressed in zebrafish microglia compared to other brain cells, including *cmklr1* and *entpd1* (FDR < 0.01, logFC > 2) (Fig 3A,B, Table S2). Taken together, we identified at least 213 mouse genes for which microglia-specific expression is conserved in the zebrafish (Fig 3C). In all, a large fraction of the mouse microglia-specific gene expression signature is conserved in the zebrafish, suggesting evolutionary conservation of processes regulated by these genes across vertebrates from fish to mammals.

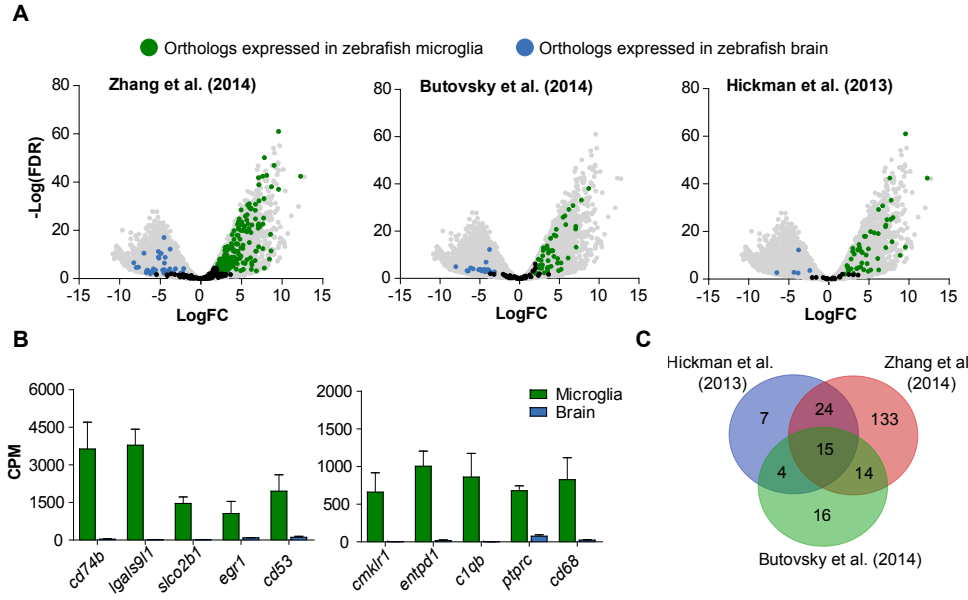

Fig 3. Conserved microglia gene expression in the zebrafish. **A** Volcano plot showing expression of zebrafish orthologs of genes found to be enriched in microglia compared to Zhang et al[14], Butovsky et al[11] and Hickman et al[13], respectively. All zebrafish genes are shown in grey. Differentially expressed orthologs (FDR < 0.01, LogFC > |2|) are shown in green (microglia) or blue (other brain cells). **B** Expression values (CPM) of orthologs for genes found by one or more of the above mentioned studies. **C** Venn diagram showing overlap of microglia-specific orthologs of genes found in three transcriptomic studies [11,13,14]. Values in **B** represent means of three independent experiments; Error bars in **B** represent standard deviation.

RNAseq reveals proliferation as an acute transcriptional microglia response to neuronal cell death

Microglia are involved in many age-related neurodegenerative diseases and there is a widely held view that the microglia state can influence disease outcome. Therefore, transcriptome studies on microglia acquired from mouse models including amyotrophic lateral sclerosis (ALS) and aging have been carried out to identify disease and aging specific signatures. Processes that were identified to be differentially regulated, although highly dependent on the nature of the model or insult, involve inflammation, phagocytosis, lysosomal processing, priming and the inflammasome[12,13].

As virtually all neurodegenerative diseases show extensive degeneration and death of neurons, we wanted to study the microglial transcriptional response specifically to acute degeneration of neurons by conditional neuronal ablation[29,41]. This could allow us

to isolate the microglial processes mostly affected by neuronal cell death in vivo. We have shown previously that addition of the ligand metronidazole (MTZ) to zebrafish larvae expressing a nitroreductase (NTR)-mCherry (neuro-NTR) fusion protein mainly in neurons of the olfactory bulb effectively kills only the transgene expressing cells. Neuronal ablation in zebrafish is accompanied by an increase in number of highly phagocytic microglia, characterized by an amoeboid morphology[53].

To determine whether NTR-mediated neuronal ablation can be used in adult zebrafish to induce neuronal cell death accompanied by microglia activation, we performed immunofluorescence (microglia) and TUNEL (apoptotic cells) labeling in brains of adult Neuro-NTR expressing zebrafish after treatment with MTZ. MTZ-treated animals showed high numbers of TUNEL$^+$ cells (314.8 $\pm$ 44.1) in the olfactory bulb, whereas TUNEL$^+$ cells were rarely observed in DMSO-treated controls (0.6 $\pm$ 0.7) (Fig 4A), showing that NTR-mediated neuronal ablation induces extensive neuronal cell death in the olfactory bulb of adult zebrafish. Additionally, the olfactory bulbs of MTZ-treated animals showed abundant amoeboid microglia, with a significant increase in microglia numbers (DMSO: 7.8 $\pm$ 2.0; MTZ: 44.8 $\pm$ 3.1) (Fig 4A). The NTR-mCherry fusion transgene in neurons allowed us to track the fate of ablated neurons because of persisting mCherry fluorescence, even after engulfment by microglia[29,41]. This revealed accumulation of ablated neurons in microglia of MTZ-treated zebrafish, showing that the induction of neuronal cell death causes microglia to become highly phagocytic (Fig 4A, S2A). These data show that our non-invasive NTR/MTZ-mediated conditional neuronal ablation is an effective strategy to induce neuronal cell death and subsequent microglia activation in adult zebrafish.

To compare genome-wide transcriptional changes accompanying microglial activation upon induced neuronal death, we performed RNA sequencing on isolated GFP$^+$ microglia from Neuro-NTR expressing zebrafish undergoing conditional neuronal ablation for 24 or 48 hours (Fig 4B). Principal component analysis (PCA) and differential gene expression analysis of the transcriptome revealed that the expression profiles of microglia 24 or 48 hours after neuronal ablation are very similar and showed only 2 differentially expressed genes (FDR < 0.01, LogFC > |2|) (Fig S1B, data not shown). Therefore, subsequent analysis was performed on pooled data from 24 and 48 h treated animals, 2 biological replicates each, to increase the statistical power. Differential gene expression analysis of microglia gene expression in fish undergoing conditional neuronal ablation and in control animals revealed 367 differentially expressed genes (FDR < 0.01, LogFC > |2|) of which 125 genes showed increased expression upon NTR-mediated ablation (Fig 4C,D, Table S3). IPA analysis revealed that the upregulated genes upon neuronal death are mostly associated with cell cycle control and DNA replication (Fig 4E). Interestingly, genes previously identified in disease models such as phagocytosis and inflammation did not show significantly increased expression (Fig S2B, data not shown). This suggests that the microglial signature upon detection of extensive neuronal cell death is characterized primarily by upregulation of genes involved in molecular processes related to proliferation.

Microglia proliferate upon the induction of neuronal death

Proliferation of microglia could explain the increase in numbers we observed upon conditional neuronal ablation. However, the expression markers we used to isolate and label microglia, *mpeg1* and *lplastin*, do not distinguish microglia from potentially

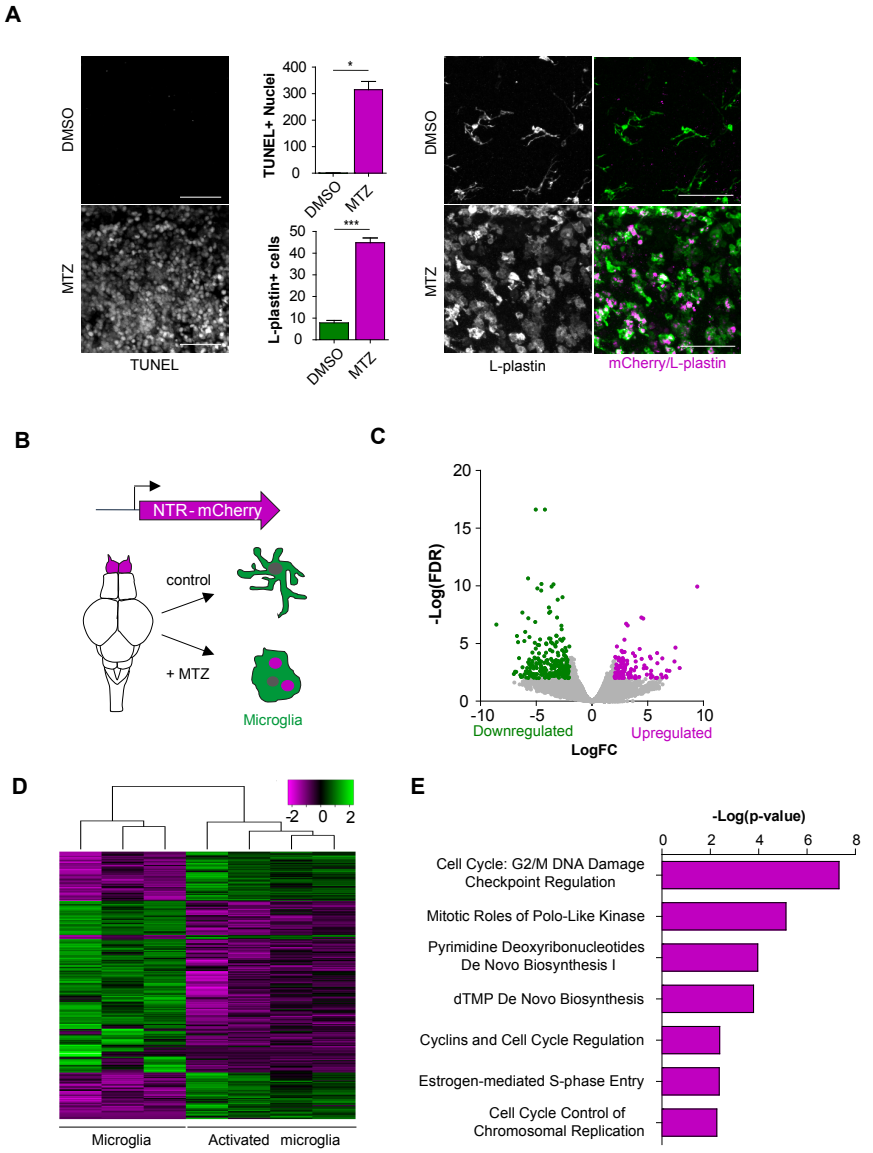

Fig 4. Identification of gene expression changes upon neuronal cell death. **A** TUNEL and L-plastin staining showing increased neuronal cell death upon treatment with MTZ for 48 hours accompanied by microglia activation. mCherry signal represents engulfed neurons. **B** Schematic representation of cells isolated for RNA sequencing on activated microglia. **C** Volcano plot showing differentially expressed genes upon the activation of microglia (FDR < 0.01; LogFC > |2|). **E** Heatmap showing Z-score values of all genes differentially expressed between activated microglia and control microglia (367 genes) (FDR < 0.01, LogFC > |2|).**F** IPA canonical pathway analysis on significantly upregulated genes in microglia upon NTR-mediated ablation (FDR < 0.01;LogFC > |2|). Scale bar = 40 μm in **A**. For quantification in **A** cells were counted in 3 selected volumes in the olfactory bulb (4.0 x 10^{-4} mm^3) per fish (n=3). Error bars represent standard deviation, *p < 0.05 (Student's T-test).

infiltrating macrophages[50]. Therefore, we aimed to address whether infiltration of peripheral monocytes and/or macrophages could explain part of the observed increase in microglia numbers and proliferation. From mouse studies it is known that infiltrating macrophages express high levels of genes encoding MHC class II components, CD45, CD40 and CD44[54,55]. We reasoned that if peripheral macrophages were infiltrating in large numbers this would have been detectable as an increased expression of these genes. However, differential gene expression analysis did not show increased expression of orthologs for these genes and even showed a significantly reduced expression of MHC class II molecules (e.g. *mhc2dab*). Similarly, a large contribution of infiltrating macrophages would likely yield a dilution of microglia, which would be reflected by a decrease in the expression of microglial specific genes. We could not detect a reduction in expression of microglial specific genes. Therefore, taken together, these findings suggests that there is no major contribution of macrophages from the periphery (Fig S3).

To investigate whether the increased expression of cell cycle genes is indeed followed by increased proliferation of microglia, we performed immunofluorescence staining for PCNA (dividing cells) after the induction of neuronal ablation. This revealed a significant increase in the number of PCNA$^+$ microglia (DMSO: 3.3 $\pm$ 2.7; MTZ: 17.8 $\pm$ 8.5) as well as increase in the PCNA$^+$ microglia fraction (DMSO: 0.4 $\pm$ 0.2; MTZ: 0.7 $\pm$ 0.02) in the olfactory bulbs of MTZ-treated animals compared to DMSO-treated controls (Fig 5A). We conclude that proliferation of microglia is an acute response to extensive neuronal death.

To study *in vivo* whether microglia proliferate locally, we performed long-term intravital imaging in transgenic zebrafish expressing *apoeb*-driven GFP after neuronal ablation. Transgenic zebrafish larvae expressing GFP under the *apoeb* promoter, show high GFP expression in microglia, but not in peripheral macrophages[28,41]. We observed occasional mitosis of phagocytic microglia, showing that microglia proliferate locally upon induced neuronal cell death (Fig 5B, Movie S1).Taken together, our transcriptomic, immunohistochemistry and *in vivo* imaging data indicate that neuronal death induces an immediate proliferative response of microglia apparent at the transcriptional as well as cellular level.

Discussion

Zebrafish are highly suitable for *in vivo* microscopic imaging and because of recent advances in genome editing could be an ideal model organism for functional genetic studies of microglia development and function. In this study we used RNA sequencing to map the zebrafish microglia transcriptome, which should aid in elucidating basic microglia biology. We found that, many of the genes expressed in mouse microglia are also expressed in microglia in the zebrafish. Additionally, by using RNA sequencing on microglia in the first stages following neuronal cell death, we showed that proliferation is a very first, and major transcriptional response of microglia to dying neurons. As several neurological diseases are caused by genetic defects of microglia for which the pathogenic mechanisms are currently unknown, it is important to better understand how microglia development and function are regulated genetically. Our gene expression datasets will be a useful tool to facilitate the elucidation of microglia genetic mechanisms relevant to brain development, aging and disease.

The majority of mouse genes for which we could not find homologs encode chemokines, chemokine receptors and genes involved in adaptive immunity (data not

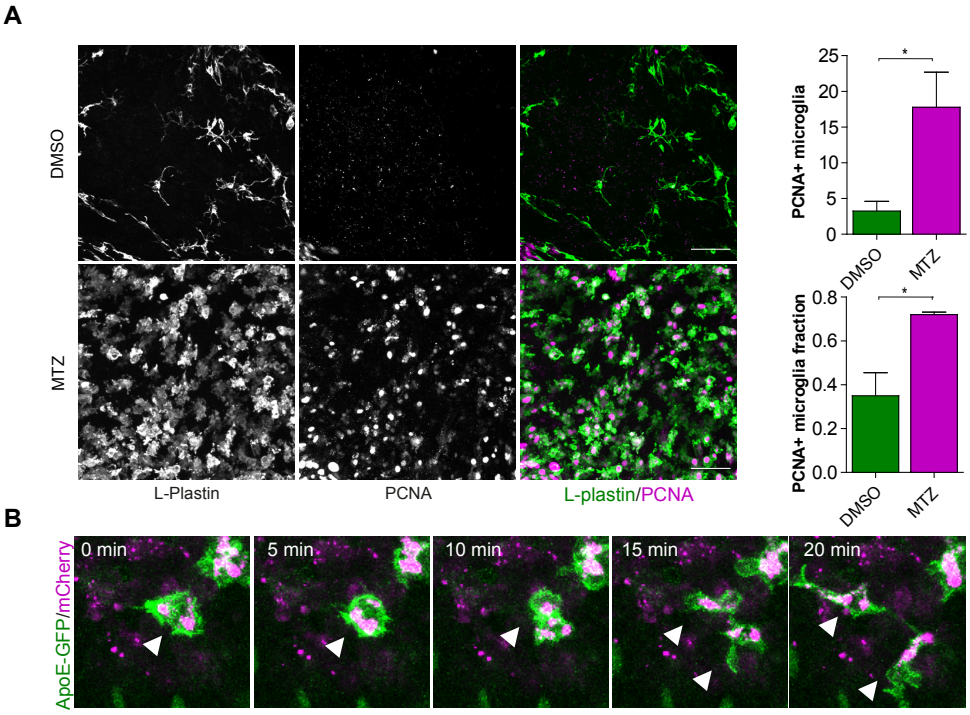

Fig 5. Microglia proliferation upon the induction of neuronal cell death. **A** Immunofluorescence staining in the olfactory bulbs of 3-month old treated MTZ-treated and DMSO-treated (control) fish. **B** Intravital imaging in 7 dpf zebrafish larvae with Apoe-driven GFP undergoing NTR-mediated neuronal cell death, showing the presence of dividing zebrafish microglia upon neuronal death. n=3. Scale bar = 40 μm in **A**. For quantification in **A** cells were counted in 3 selected volumes in the olfactory bulb (4.0 x 10^{-4} mm^3) per fish (n=3). Error bars represent standard deviation, *p < 0.05 (Student's T-test).

shown). It is thought that in general in fish species innate immunity is highly evolved, which may compensate for a less sophisticated adaptive immune system when compared to mammals [56]. Additionally, as chemokines and their receptors are amongst the most rapidly evolving gene clusters, it is difficult to compare them across species[57-59]. Therefore it is not surprising to find non-overlapping gene expression in these particular processes between zebrafish and mouse. However, the differences in species specific microglial gene expression between fish and mammals will be interesting to analyze as this may reveal genetic mechanisms underlying potential microglial adaptations to increasing brain complexity.

The specific identity and gene expression signature, which microglia adopt in the brain, is controlled by environmental factors in conjunction with developmental ontogeny[16,17]. Recently, it has been shown in mice that TGFβ is a main driver of microglia-specific gene expression[11]. Interestingly, upstream regulator prediction analysis on our zebrafish microglia gene expression data, predicted TGFb1 to be one of the main upstream regulators (Table S4). This indicates that beyond transcriptome similarities also the upstream regulation shows important similarities across species, and zebrafish may serve as a powerful model system to address the steps involved in acquiring the unique microglial identity.

Nitroreductase-mediated ablation allowed us to distinguish the very first microglial response specifically to dying brain cells. We did not find increased expression of classes identified in previous studies on microglia gene-expression in mouse models for chronic neurodegenerative diseases. Instead we identified extensive proliferation of microglia as a primary response to induced neuronal death, apparent at both the transcriptional and cellular level. One explanation for this difference could be that previous microglial gene expression studies were performed in models that show a more chronic, gradually developing neurodegenerative process, whereas we observed microglia immediately following neuronal cell death[12,60]. This is corroborated by microglia labeling at 24 hours after treatment which already shows an increase in proliferative gene expression, when they have not all adopted an amoeboid morphology yet or increased in numbers (data not shown). Another possible explanation is that neurodegenerative diseases are associated with a combination of disease-related cues including misfolded proteins and/or dying neurons causing altered immunological activity of microglia. In contrast, NTR-mediated ablation causes a very clean insult consisting only of programmed cell death in the absence of other factors[29,61].

It is clear from several studies including the current that microglia express many genes involved in pathogen recognition, phagocytosis and lysosomal processing of phagocytic cargo[11-14]. Therefore, it may not be surprising that the first upregulated genes are not related to these basic microglia functions. Instead, microglia appear to prepare to counter an immense phagocytic task by locally increasing their numbers by self-renewal as previously described in a mouse model for amyotrophic lateral sclerosis[62]. Although it is as yet unclear whether and when microglial proliferation is beneficial or detrimental in disease, it would be interesting to identify the cues that drive proliferation under these circumstances. In fact, strategies to manipulate microglia production are currently under investigation as potential treatments for patients suffering from neurodegenerative disease[63,64].

In conclusion, with recent advances in scalable genome editing, we anticipate that our study will prove an important guide for functional genetic dissection of microglia activity and behavior, critical to understanding the role of microglia in physiology and brain disease. Our data provide novel insight into the microglial response to dying neurons *in vivo* and, together with the identified zebrafish microglia transcriptome, may accelerate the pace of elucidating molecular mechanisms involved in basic microglia function *in vivo* relevant to brain homeostasis.

Acknowledgements

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Contributions

NO and TVH conceived the study, HVDL, NO, LK, TVH performed experiments, IH, NO and TVH collected and analyzed data, EB and BJLE gave technical support and conceptual advice, NO and TVH wrote the manuscript with contributions from all authors.

Materials and methods

Animals

For all experiments in adult fish we used neuronal nitroreductase (NTR)-mCherry expressing zebrafish incrossed with *mpeg1*-GFP transgenic zebrafish as described previously that were kept on a 14h/10h light –dark cycle at 28℃[41]. They were fed brine shrimp twice a day. During experiments animals were kept in system water under standard water quality parameters. For in vivo imaging we used 6 dpf larvae expressing neuronal NTR-mCherry in addition to expression of *mpeg1*- or *apoeb*-driven GFP expression. Animal experiments were approved by the Animal Experimentation Committee of the Erasmus MC, Rotterdam and UMCG, Groningen.

Acute isolation microglia

For microglial isolation 3-month-old neuro-NTR/*mpeg1*-GFP zebrafish were euthanized in ice water according to animal welfare regulations. The heads were severed behind the gills, followed by removal of the gills, lower jaw and eyes using a watchmaker's forceps. The brains (5 per sample) were taken out of the skull after removal of the skull base and collected in ice cold PBS. Subsequently, the brains were cut using scalpels followed by dissociation in 0,25% trypsin-0,1% EDTA in PBS for approximately 2 hours at 4℃, while re-suspending regularly. Upon complete dissociation of the brain, trypsin was inactivated by adding 1/6 volume of a 6 mM $CaCl_2$ solution in PBS. The cell suspension was run through a 70 μm cell strainer and collected in a 22% Percoll solution[42]. Ice cold PBS was placed on top of the cell suspension while avoiding mixing of the layers, followed by centrifugation at 1000 rcf at 4℃ for 45 minutes. The remaining cell pellet was re-suspended in suspension solution (high-glucose DMEM without phenol red, 0,8 mM $CaCl_2$, 1 % v/v Penicillin/Streptomycin). The suspension was transferred to FACS tubes with 35 μm cell strainer caps, immediately followed by FACS sorting using a MoFlo Astrios cell sorter (Beckman Coulter). DAPI was added to label and exclude dead cells.

RNA extraction and library synthesis

Total RNA extraction was performed using the Qiagen miRNeasy kit according to the manufacturer's instructions (Qiagen) and RNA sample quality was determined using on an Agilent Bioanalyzer 2100 total RNA 6000 Pico series chip (Agilent). Subsequently, cDNA libraries were created using the SMARTer Ultra Low Input RNA Kit for Sequencing - v3 (Clontech). Illumina RNAseq libraries were prepared from cDNA using the Illumina TruSeq™ RNA Sample Prep Kit v2 according to the manufacturer's instructions (Illumina Inc.). In all libraries 50 nucleotide single-end reads (SR50) were sequenced on an Illumina HiSeq2500 sequencer according to the manufacturer's protocol, obtaining 10-20 million reads per sample library. Image analysis and base calling were done using the Illumina pipeline. Reads were aligned to the zebrafish genome (GRCz10) using TopHat (version 2.0.5)[43]. The resulting files were filtered using SAMtools (version 0.1.18) to exclude secondary alignment of reads.

Bioinformatics

The aligned and filtered data was quantified with the Bioconductor package Genomic Ranges[44]. Differential gene expression analysis was performed with Bioconductor package EdgeR[45]. The differentially expressed gene lists were functionally annotated using Qiagen's Ingenuity Pathway Analysis (IPA®, QIAGEN Redwood City,

www.qiagen.com/ingenuity). Data were inspected using MultiDimensional Scaling (MDS) plots, Principal Component Analysis (PCA), and inter-sample correlation plots. The neuronal ablation-associated microglia, 24 h and 48 h after treatment, had very similar transcriptional profiles and were grouped together as neuronal ablation-associated microglia. The RNA-seq data is available via GEO (*www.ncbi.geo*, accession number: GSE86921) and via the Glia Open Access Database (*www.goad.education*)[46]. The Biomart Bioconductor Package was used to annotate the genes, and to identify mouse orthologs. Heatmaps were generated with heatmap.2 of Bioconductor package Gplots[47]. The zebrafish microglia expression profile was compared to recently reported pure mouse microglia expression profiles[11,13,14]. With the Biomart Bioconductor tool, mouse orthologs, with their corresponding gene symbols, were identified for all zebrafish genes. This gene symbol list was intersected with the gene symbol list of genes expressed in mouse microglia and only high-confidence orthologs were selected. For several mouse genes, multiple zebrafish high-confidence orthologs were identified.

Neuronal cell ablation

For neuronal ablation neuro-NTR transgenic zebrafish were used as described previously[41]. 3–month-old zebrafish were placed in system water containing either 0,47 % DMSO (control) or 5 mM MTZ for 48 hours or 0,46 % DMSO for 24 hours followed by 5 mM MTZ for 24 hours. The medium was refreshed after 24 hours of treatment. Fish were kept under 14 h light/ 10 h dark cycles in a temperature controlled incubator (28℃). Fish were fed brine shrimp twice a day during the 48 hour treatment. All experiments were performed according to the animal welfare regulations.

Immunofluorescence staining

Immunohistochemistry was performed as described[29,41]. Briefly, fish were euthanized in ice water, followed by fixation of the brain inside the skull in 4% PFA at 4℃. Subsequently, the brains were carefully removed from the skulls and dehydrated with a 25 %, 50 %, 75 %, 100 % MeOH series and stored at -20℃ for at least 12 hours. After rehydration, brains were embedded in 4 % w/v low melting point agarose in PBS and cut into 80 μm sections using a Microm HM 650V vibratome (Thermo Scientific). Immunostainings on free-floating sections were performed as described [48]. Primary antibodies: PCNA (1:250, Dako), L-plastin (1:1000). Secondary antibodies: DyLight Alexa 488 (1:500), DyLight Alexa 647 (1:500). For nuclear staining Hoechst was used. Sections were mounted in Vectashield mounting medium H1000 (Vector Laboratories).

Combined TUNEL/antibody staining in whole mount brain

For TUNEL staining the Click-iT TUNEL Alexa Fluor 647 Kit (Invitrogen) was used. Fish were euthanized in ice water, followed by fixation of the brain inside the skull in 4% PFA at 4℃. Subsequently, the brains were carefully removed from the skulls and incubated in 11 μg/ml Proteinase K in PBST (PBS containing 0,2% Triton X-100) at room temperature for 40 minutes. Then the brains were incubated in fixative again (4% PFA) at room temperature for 20 minutes. After washing with PBST the brains were incubated in the reaction buffer at room temperature for 30 minutes, followed by overnight incubation in the reaction cocktail at room temperature, according to the manufacturer's instructions. After washing with 3% w/v BSA in PBST the brains were incubated in the Click-iT reaction cocktail at room temperature for 3 hours, followed by washing with 3%

BSA in PBST. Then the brains were incubated in PBST containing 1% v/v DMSO and 1% BSA at room temperature for 2 hours. The brains were incubated with primary antibody (L-plastin, gift from Yi Feng, University of Edinburgh, 1:500) in 5% BSA in PBST at 4℃ for 72 hours. Subsequently, brains were incubated overnight at 4℃ with fluorescently labeled secondary antibody (DyLight Alexa 488 1:250) and Hoechst in PBST containing 2% BSA. Brains were sections as described and sections were mounted in Vectashield mounting medium.

Imaging and quantification
Mounted sections were imaged on a LSM 700 Zeiss confocal system using a 20x dry objective (PlanApo, NA = 0.8) using 405, 488, 555 and 633 laser lines. Confocal z-stack images were acquired. Images were processed with Zen 2012 (Zeiss) and ImageJ/FIJI software. Quantifications were performed on 3 individual 4,0 x 10^{-4} mm^3 volumes within the olfactory bulb in 3 animals. These volumes were chosen in the central areas of the olfactory bulb that contained a similar density of Hoechst-positive nuclei across all imaged brain slices. Average numbers of three individual volumes per fish were quantified. Student's t-tests were carried out on the averages of measurements in at least 3 individual animals (n=3 or n=4) were used to determine p-values.

Intravital imaging
Intravital imaging in zebrafish brains was largely performed as previously described[41]. Briefly, zebrafish larvae were mounted in 1.8% low melting point agarose containing 0.016 % MS-222 as sedative and anesthetic in HEPES-buffered E3. The imaging dish containing the embedded larva was filled with HEPES-buffered E3 containing 0.016% MS-222. Imaging was performed using an Lcl Plan-Neofluar 63×/1.3 lens on the Zeiss LSM780 system. For two-photon excitation of red (mCherry) and green (GFP) fluorophores the laser (Coherent, Santa Clara, CA) was tuned to ~990 nm.

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Supplementary figures

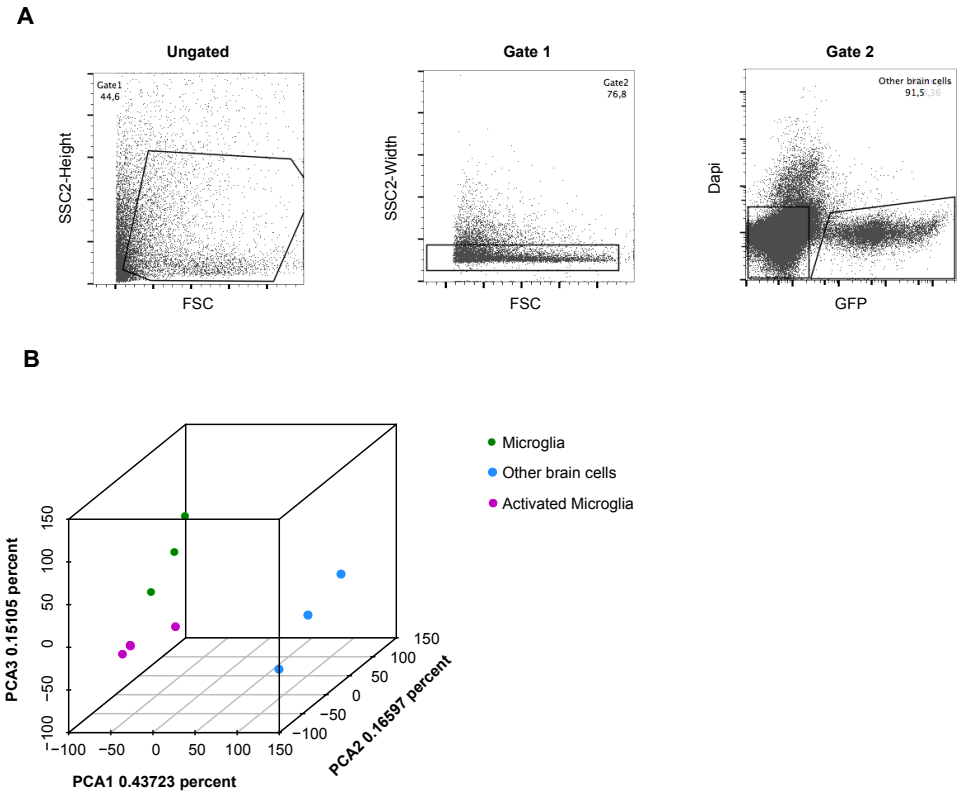

Fig S1. High-throughput sequencing of the zebrafish microglia transcriptome. **A** Gating strategy for FACS-sorting of GFP$^+$ (microglia) and GFP$^-$ (brain) cell fractions. **B** PCA analysis showing GFP$^-$ brain cell samples (blue), GFP$^+$ control microglia samples (green) and GFP$^+$ activated microglia samples (magenta).

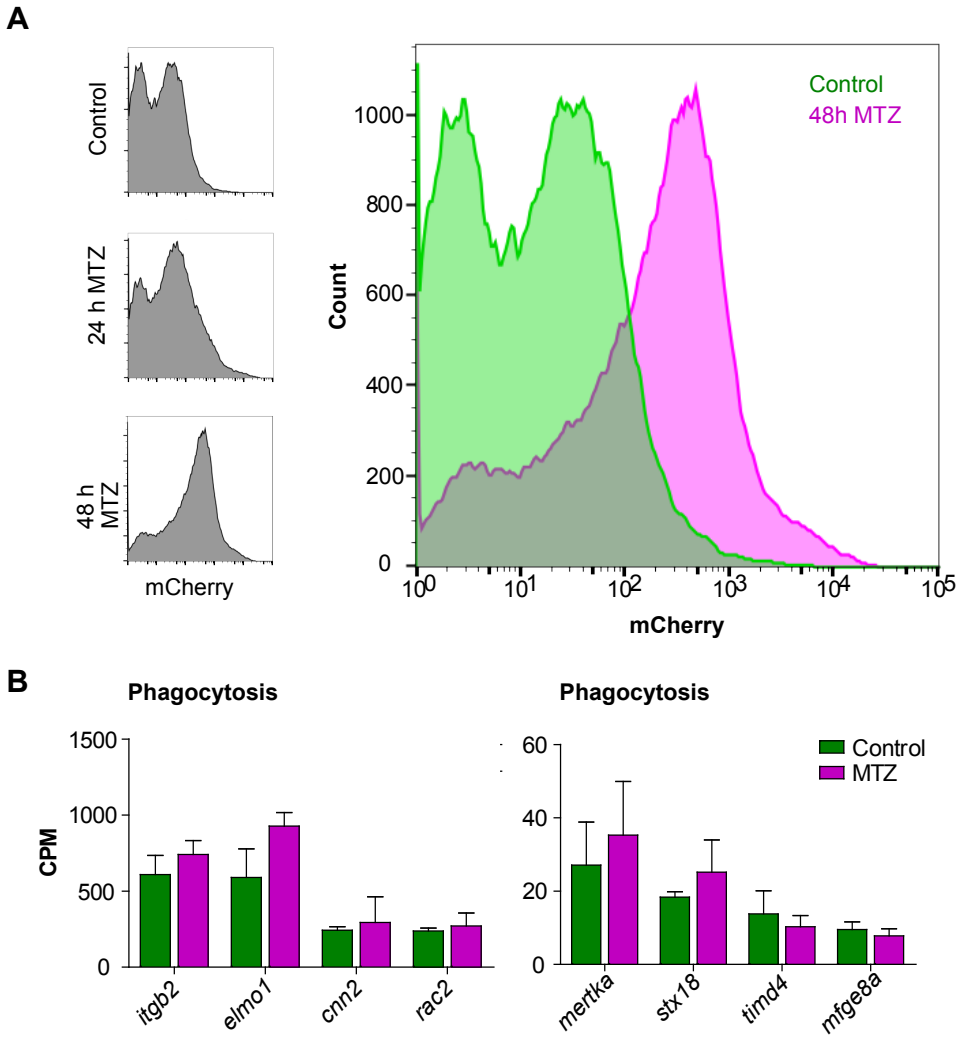

Fig S2. Microglia become phagocytic upon neuronal cell death. A FACS data showing that many microglia are also positive for mCherry signal. mCherry is expressed in NTR-expressing neurons. Therefore, increased numbers of mCherry positive microglia suggest increased phagocytic activity of microglia. **B** CPM values of genes involved in phagocytosis in activated and control microglia. No significant difference in the expression of genes involved in phagocytosis. Values in **B** represent means of at least three independent experiments. Error bars represent standard deviation.

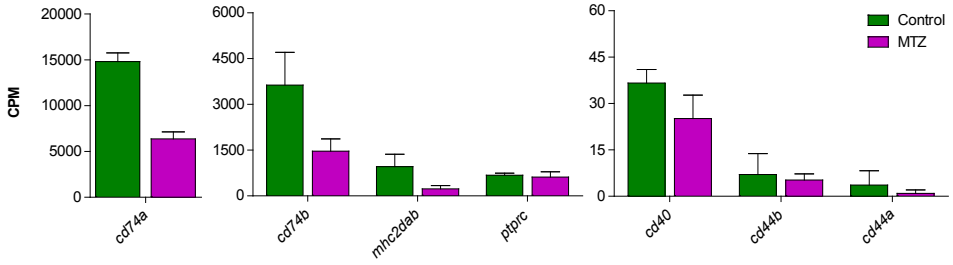

Fig S3. No increased expression of genes associated with infiltrating macrophages. CPM values of genes expected to increase in expression upon infiltration of monocyte-derived macrophages. Values represent means of at least three independent experiments. Error bars represent standard deviation.

For supplementary tables and movie, see online publication

Chapter 4

CRISPR/Cas9-based reverse genetic screen in zebrafish reveals that interleukin 34 regulates early embryonic microglia development

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Abstract

Microglia, the brain's resident macrophages, are crucial for normal brain development, as disruption of normal embryonic microglia development can have lasting consequences for the brain. However, the molecular and genetic mechanisms underlying microglia development and differentiation in vivo are still poorly understood. We recently showed that the zebrafish microglia transcriptome shares extensive homology with mammals. Zebrafish larvae are highly suitable for in vivo genetic screening as they are transparent, have a high fecundity and develop rapidly. Here, we present a CRISPR/Cas9-based reverse genetic screening pipeline to identify new regulators important for microglia development or function using zebrafish. We screened 20 possible microglia regulators, including microglia signature genes and non-cell autonomous factors, by Cas9/gRNA-complex injections, followed by neutral red-based visualization of microglia and their automated quantification in 3-day-old larvae. We identified *il34*, the zebrafish homolog of mammalian interleukin 34 (IL34), as a regulator of microglia development. Previous studies on the role of the colony stimulating factor 1 receptor (CSF1R) ligand IL34 in development of microglia in the mouse were ambiguous. Our data suggest that in zebrafish *il34* is required during the early stages of microglia development. In all, this shows that our reverse genetic screening pipeline in zebrafish can be used for the identification of regulators of microglia development.

Introduction

Microglia are the tissue resident macrophages of the brain, which play a role in brain development and the maintenance of brain homeostasis. Functions of microglia during brain development include the removal of dead cells and debris, modulation of neuronal connectivity by synaptic pruning and maintenance of myelin-producing cells[1-3]. Defects in microglia function have been implicated in neurodevelopmental disorders such as autism spectrum disorder[1]. Genetic microglia defects also cause rare white matter disorders including Nasu-Hakola disease and adult onset leukoencephalopathy with axonal spheroids (ALSP)[4-6]. This strongly suggests that microglia functions are important for both the developing and adult brain. However, the exact genes and mechanisms underlying microglia development and function are still poorly understood.

Microglia originate from mesodermal progenitors known as yolk sac macrophages that colonize the brain during early embryonic development[7,8]. The transition from yolk sac macrophage to mature microglia involves several differentiation stages characterized by distinct transcriptional profiles. The progression through these transcriptional states is synchronised with and most likely driven by the different stages of brain development as microglia gene expression is highly sensitive to changes in the microenvironment and tissue macrophage identity is mostly determined by the host environment[9-12]. As many of the genes specifically expressed in microglia are rapidly downregulated when microglia are taken out of the brain, it is difficult to study microglia in vitro[13,14]. For the majority of microglia signature genes that show much lower or no expression in other macrophage populations or brain cells, the function is still unknown. Therefore, identification of the functions of these genes could provide valuable insights into regulation of microglia development and function in vivo.

Zebrafish embryos are small, transparent, develop ex-utero and are relatively easy to manipulate genetically, which makes them highly suitable for in vivo genetic studies[15]. We recently showed that microglia gene expression is well conserved between zebrafish and mammals[16]. Phenotype-driven, forward genetic screens in zebrafish have identified several microglia mutants with a defect in microglia development or function. Processes affected in these mutants include hematopoiesis, regulation of inflammation, phosphate transport and lysosomal regulation, which implies that these various processes are all critical for microglia development and function[17-19]. However, such forward genetic screens are laborious and relatively low-throughput. A candidate-driven reverse genetic screening approach could lead to identification of additional genes important for microglia. The CRISPR/Cas9-system can be used to create insertions or deletions (indels) in target genes via the repair of Cas9-induced double strand breaks by error-prone non-homologous end joining (NHEJ)[20]. Active Cas9-gRNA ribonucleoprotein complexes injected into fertilized zebrafish oocytes can efficiently induce indels in target genes. The resulting mosaic zebrafish can thereby phenocopy existing loss-of-function mutants (CRISPRants)[21,22]. Using a similar strategy, Shah et al performed a small-scale reverse genetic screen in zebrafish to identify new genes involved in electrical synapse formation[23].

Here, we present a scalable CRISPR/Cas9-based reverse genetic screening pipeline in zebrafish to identify important genetic microglia regulators using zebrafish. In zebrafish larvae, microglia can be visualized by the vital dye neutral red, which is actively taken up by phagocytosis and has been used as an effective readout in forward genetic screens[8,17-19]. We developed an image quantification tool, SpotNGlia, to

automatically count neutral red-positive (NR+) microglia. Out of the 20 putative microglia regulators we targeted by CRISPR/Cas9-mediated reverse genetics, disruption of interleukin 34 (*il34*) showed the strongest reduction in microglia numbers in developing zebrafish larvae. This phenotype could be reproduced in stable il34 mutants, showing that il34 is a regulator of early microglia development in zebrafish. In mammals, Il34 is one of two ligands of the important microglia regulator CSF1R. In all, we here present a scalable reverse genetic screening pipeline to identify new regulators important for microglia development and function.

Results

CRISPants phenocopy existing microglia *csf1r* mutants

Loss of one of several key macrophage regulators, including *spi1b* (*pu*.1), *irf8* and *csf1ra* leads to defects in microglia development in both mammals and zebrafish[8,24-29]. To investigate whether active Cas9-gRNA ribonucleoprotein complexes (RNPs) can be used to induce mutant microglia phenotypes directly, we injected zebrafish oocytes with RNPs targeting either *csf1ra* or *spi1b*. To assess whether CRISPR/Cas9-based targeting of those genes affects microglia development we determined microglia numbers by neutral red (NR) at 3 days post fertilization (dpf). At that age, microglia have just colonized the optic tectum, are highly phagocytic and have low proliferative activity, which makes it an ideal time point to identify genes required for the earliest steps of microglia development. To image microglia, larvae were embedded in low melting point agarose with the dorsal side facing upwards. We quantified NR+ microglia in *csf1ra* CRISPants, in controls and in pre-existing *csf1ra* loss-of-function mutants found in an ENU mutagenic screen (hereafter called *csf1ra*$^{-/-}$)[30]. Similar to *csf1ra*$^{-/-}$ mutants, *csf1ra* CRISPants showed an approximately 80% reduction in the number of NR+ microglia compared to controls suggesting highly effective targeting in F0 embryos (Fig 1A). To assess the targeting efficiency of the *csf1ra* gene we performed Sanger sequencing on the targeted locus of a small pool of *csf1ra* CRISPants and calculated the spectrum and frequency of indels in the *csf1ra* gene using TIDE (tracking indels by decomposition) software[31]. The mutagenic efficiency was >90%, showing efficient mutagenesis (Fig 1B). Similarly, *spi1b* CRISPants showed a strong reduction in the number of microglia and 65-95% mutagenic efficiency (Fig 1C, D). This shows that CRISPR/Cas9-based mutagenesis can be used to reproduce mutant microglia phenotypes in Cas9-gRNA RNP injected zebrafish larvae.

SpotNGlia semi-automatically counts microglia numbers

Manual quantification of NR+ microglia, across z-stack images, is time-consuming and can be subjective. To standardize and speed up quantification, we developed a software tool, SpotNGlia, that automatically counts NR+ microglia in the optic tectum where most microglia are located at 3 dpf. The SpotNGlia tool aligns stacked images of stained zebrafish larvae taken at different axial positions and blends the images into a single 2D image in which all NR+ cells are in focus (Fig 2A). Next, the images are segmented by using polar transformation and dynamic programming to identify the edges of the optic tectum. Finally, NR+ cells are detected and counted by a spot detection technique based on multiscale wavelet products[32]. To test the SpotNGlia software tool, we created and manually annotated a dataset with representative z-stack images of 50 neutral red stained zebrafish larvae. To assess the accuracy of brain segmentation, Jaccard and

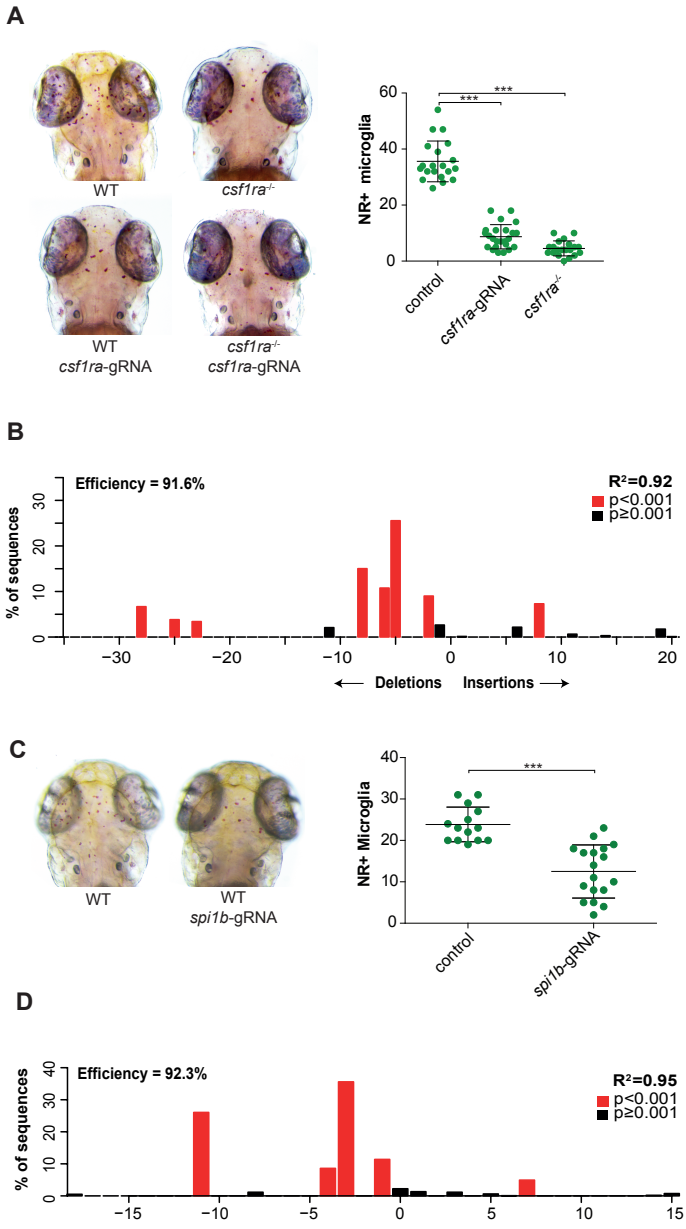

Fig 1. *Csf1r* CRISPants phenocopy existing *csf1r* microglia mutants. **A** Neutral red images and quantification of WT, *csf1ra*$^{-/-}$ and *csf1ra* CRISPant zebrafish larvae at 3 dpf. **B** Indel spectrum of a pool of *csf1ra* CRISPants calculated by tide. **C** Neutral red images and quantification of WT, and *spi1b* CRISPant zebrafish larvae at 3 dpf. **D** Indel spectrum of a representative individual *spi1b* CRISPant calculated by tide. R^2 value represents reliability of the de indelspectrum. ***p < 0.001

Dice indices were determined, revealing indices of 0.86 (Jaccard) and 0.93 (Dice)(Fig 2B, C). To assess the accuracy of microglia detection we determined the precision, recall and F1 scores of the computed annotation, resulting in average scores of 0.85, 0.91 and 0.87, respectively (Fig 2B,C,D). These results indicate that SpotNGlia is able to automatically find the brain region and the microglia in the vast majority of cases. To correct manually for those instances where brain segmentation and microglia detection were not completely accurate (determined by visual inspection), our tool offers the possibility of post-hoc correction. In our experiments we have found that SpotNGlia results in about 80% reduction in the time it takes to quantify NR+ microglia numbers. In all, this indicates that SpotNGlia is a powerful tool for fast quantification of NR+ positive microglia.

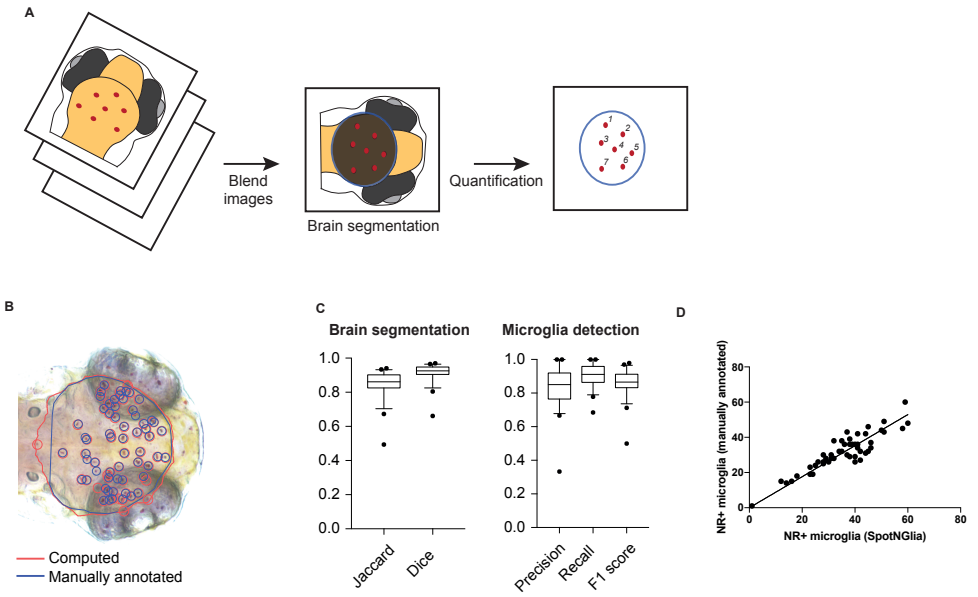

Fig 2. SpotNGlia semi-automatically counts microglia numbers. A Schematic representation of SpotNGlia analysis pipeline. **B** SpotNGlia output of test dataset with both manual (blue) and automated (red) brain segmention and NR+ microglia annotation. **C** Boxplots showing Jaccard and Dice indices for accuracy of brain segmentation and F1, precision and recall scores for the accuracy of NR+ microglia annotation. **D** Correlation between manually and automated microglia quantification after manual correction for segmented brain area.

Reverse genetic screen reveals zebrafish il34 as a regulator of microglia development

To identify new microglia regulators using direct CRISPR/Cas9-targeting and microglia phenotyping by SpotNGlia, we targeted 20 candidate genes individually. These genes were selected based on either our recently identified zebrafish microglia transcriptome (e.g. *slco2b1*, *hcst*/*dap10* and *mrc1b*), an expected effect on microglia development, a connection to brain disease, or genes which could affect microglia in a non-cell autonomous manner (CSF1R ligand encoding genes *il34* and *csf1a*) (Fig 3A, S1). Next, gRNAs were designed to effectively target these genes in one of the first exons of the

gene. Active Cas9-gRNA RNPs targeting candidate genes were injected in fertilized oocytes, after which they were NR stained at 3 dpf, phenotyped and genotyped by Sanger sequencing followed by indel decomposition using TIDE (Table S1). We did not observe any signs of developmental delay, morphological abnormalities or increased mortality upon Cas9-gRNA RNP injections, indicating that the observed microglia phenotypes were not due to Cas9-gRNA toxicity. The gRNAs for 6 of the targeted genes caused a significant reduction in the number of NR+ microglia (Fig 3A). The strongest reduction in NR+ numbers was observed in embryos in which the zebrafish homolog of interleukin 34 (IL34) was targeted (Fig 3A, B)[33]. To confirm that this microglia phenotype is caused by *il34* targeting, we generated a premature stop codon in exon 5 of *il34* by CRISPR/Cas9. Neutral red labelling of homozygous *il34* mutants at 3 dpf revealed a ~60% reduction in NR+ microglia compared to heterozygous and wildtype siblings (Fig 3C, Fig S2). Together, this shows that our methods are an effective strategy to identify new genes involved in microglia development.

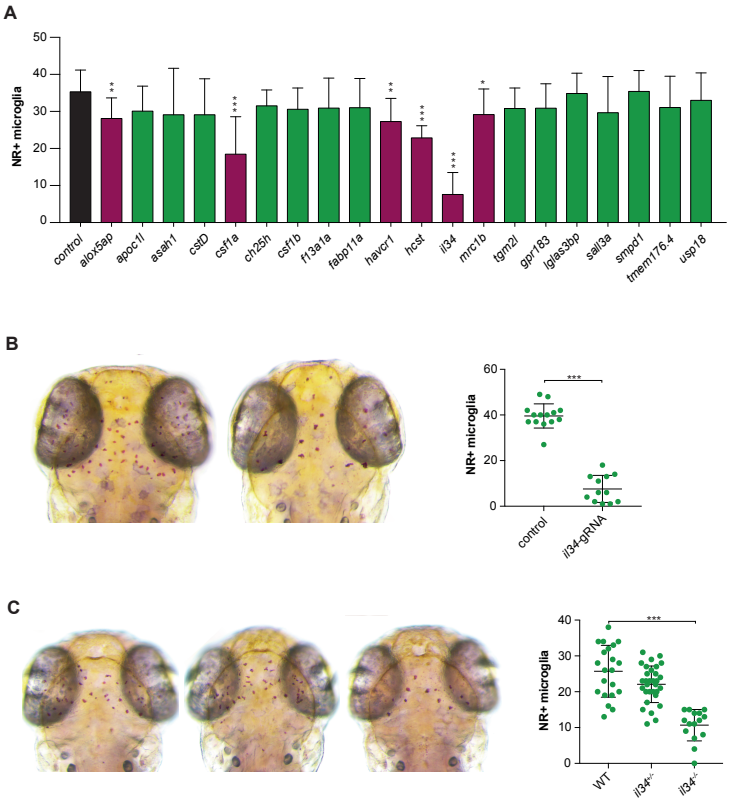

Fig 3. Reverse genetic screen reveals zebrafish *il34* as a regulator of microglia development. A Accumulated data from all gRNA injections showing the number of NR+ microglia as quantified with SpotNGlia. Red bars represent genes which showed a significant reduction in microglia numbers upon CRISPR/Cas9-based targeting. **B** NR+ microglia numbers in 3 dpf zebrafish larvae injected with gRNA-Cas9 RNPs targeting *il34*. **C** NR+ microglia numbers in *il34* mutants with a premature stop codon in exon 5 at 3 dpf. *p < 0.05, **p < 0.01, ***p < 0.001

Discussion

In this study, we developed a scalable CRISPR/Cas9-based reverse genetic screening pipeline in zebrafish embryos using semi-automated image quantification to identify new regulators of microglia biology. We showed that direct genetic targeting of known microglia regulators including *csf1ra* and *spi1b* by Cas9/gRNA injections in zebrafish embryos phenocopies previously identified microglia mutants. We next developed a software tool (SpotNGlia) that allows for automated phenotyping by quantification of neutral red positive microglia. Using this pipeline, we tested 20 candidate genes for a role in microglia development and identified the zebrafish *il34* homolog as a non-cell autonomous regulator of early microglia development. Additionally, we identified 5 other genes with a potential role in microglia development, however, these still await confirmation. This shows that our reverse genetic screening strategy can effectively identify novel regulators important for microglia development.

IL34 is one of two ligands of the colony stimulating factor 1 receptor (CSF1R), a main regulator of development of the macrophage lineage[34]. The precise role of IL34 in microglia development is still unclear. Wang and colleagues, showed that microglia numbers are severely reduced in neonatal *Il34*-/- mice, whereas Greter et al showed normal microglia numbers in *Il34*-/- mice throughout embryonic development[34,35]. Our data revealed a ~40% reduction in microglia numbers in *il34* mutant zebrafish larvae, indicating that *il34* is required for early microglia development. Our data indicates that *il34* deficiency could cause a defect in the production of either yolk sac progenitors or in the colonization of the embryonic brain, as at the time of examination (3 dpf) microglia have just entered the brain and still have a low proliferative rate (Kuil et al., manuscript in preparation). It is as yet unclear why there are two CSF1R ligands, and to what extent they differ in function. In the brain, the expression pattern of *Il34* barely overlaps with that of *Csf1*, indicating that they are differentially regulated and may slightly differ in function[34,36]. Although both CSF1R ligands are important for early microglia development, it remains to be determined what the precise functions of Il34 and Csf1 are in microglia development.

Even though our methods permit analysis of multiple genes, there are several ways to increase the throughput. First, mounting of the injected zebrafish larvae and subsequent image acquisition are the most time-consuming parts of our pipeline. Neutral red stained larvae were manually embedded in low melting point agarose before imaging, which severely restricts the number of animals that can be screened per day. Automated imaging systems that can load zebrafish larvae from liquid medium in multi-well plates and image them in the orientation of interest in glass capillaries could overcome this hurdle[37]. Together with the SpotNGlia tool this would permit a significantly increased screening throughput and efficiency. Additionally, we aimed to achieve maximal CRISPR/Cas9 mutagenic efficiency for individual genes of interest, and therefore targeted individual genes. Shah et al have recently shown a strategy where pools of up to 8 gRNAs are injected simultaneously to target multiple genes at once[23], which could lead to reduced targeting efficiency of the individual gRNAs. Although a pooling strategy could significantly increase the number of genes that can be screened, we observed that, especially for genes with a relatively subtle microglia phenotype, a high mutagenic efficiency increases the chance of detecting the phenotype. Additionally, due to the clonal nature of hematopoietic progenitors cells, including yolk sac macrophages, a high targeting efficiency is likely required, because

non-targeted cells could expand and compensate for mutated cells.

In conclusion, we here present a scalable reverse genetic screening method for the identification of novel regulators of microglia development and function. Microglia are key players in brain disease and there is strong evidence that microglia defects can be a primary cause of brain disease. However, the mechanisms regulating microglia development and function are still largely unknown. Therefore, better understanding of microglial gene functions could be a valuable step in the elucidation of mechanisms underlying microglial biology. As zebrafish larvae have proven their suitability for drug discovery, SpotNGlia automated analysis software in combination with automated imaging systems could also be used to screen for compounds affecting microglia[38]. In all, we here developed a reverse genetic screening pipeline to address genetic regulation of microglia development and function, and identified *il34* as a regulator of microglia development in zebrafish embryos.

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Materials and methods

Fish care

For all experiments Tg(*mpeg1*:*egfp*) fish expressing GFP under the control of the *mpeg1* promotor or Tg(*Neuro-Gal4*, UAS:*nsfB-mCherry*, *mpeg1*:*egfp*) with neuronal specific nitroreductase expression, transgenic zebrafish lines were used. Zebrafish embryos were kept at 28℃ on a 14h/10h light-dark cycle in HEPES-buffered E3 medium. At 24 hpf 0.003% 1-phenyl 2-thiourea (PTU) was added to prevent pigmentation.

sgRNA synthesis

To design sgRNAs the online program CRISPRscan (www.crisprscan.org) was used (Moreno-Mateos et al., 2015). The gRNAs were designed to target exons, except for exon 1, to be as close as possible to the transcription start site and to have no predicted off-target effects. The sgRNAs were generated from primer dimers, containing a minimal T7 RNA polymerase promoter. To generate primer-dimers the FastStart™ High Fidelity PCR System from Sigma was used. A solution was prepared containing 1 mM forward sgRNA oligo, 1 mM reverse oligo consisting of 20 nt overlap with sgRNA oligo and the Cas9-binding part, 0.8 mM dNTPS, 1x FastStart Buffer and 6.25 U / μL FastStart Taq polymerase in 20 μL total volume. Annealed DNA oligo dimers were generated by denaturation at 95℃ for 5 minutes followed by annealing by reducing the temperature by 1℃ per second during 20 seconds to 75℃ f and extension at 72℃ for 10 minutes. The gRNAs were synthetized from annealed DNA oligo's, containing a minimal T7 RNA polymerase promoter, with the mMESSAGE mMACHINE™ T7 ULTRA Transcription Kit (Invitrogen) according to the manufacturer's instructions.

CRISPR-Cas9 injections into zebrafish larvae

The SP-Cas9 plasmid used for the production of Cas9 protein was a gift from Niels Geijsen (Addgene plasmid #62731)[39]. Cas9 nuclease was synthetized as described[39]. 600-900 ng of gRNA was mixed with 4 ng of Cas9 protein to form active gRNA-Cas9 RNPs. Next, 0.4 μL of 0.5% Phenol red (Sigma) and the volume was adjusted with 300 mM KCl to a total volume of 6 μL. Approximately 1 nL of the mix was injected in fertilized zebrafish oocytes. For the creation of the il34 mutant line CRISPants were grown to adulthood and outcrossed to the AB background. Next, Sanger sequencing was used to identify the mutants with a premature stop codon in exon 5 of the *il34* gene.

Neutral red staining and imaging

To label microglia, 3 dpf larvae were incubated in E3 medium containing neutral red (Sigma) (2.5 ug/ml) to for 2 hours at 28 ℃, after which they were rinsed with E3 medium containing 0.003% PTU. Larvae were anaesthetized with 0.016% MS-222 and embedded in 1.8% low melting point agarose in E3 with the dorsal side facing upwards. Serial images (3-6) in the z-plane were acquired with a Leica M165 FC microscope using the 12x dry objective and a Leica DFC550 camera.

Larvae genotyping (Sanger sequencing)

Lysis Zebrafish larvae were euthanized and placed in single tubes containing 100 μL lysis Buffer (0.3% 1M KCl, 1% 1M TrisHCl pH 9.0, 0.1% Triton, 0.15 mg/mL Proteinase K) per larva. The mix was incubated at 55 °C for 10 minutes and 95 °C for 10 minutes. The lysate was centrifuged for 5 to 10 minutes at 4000 rpm. 1 μL lysis was used for PCR.

Sanger sequencing to determine CRISPR/Cas9 targeting efficiency For Sanger sequencing 500 bp long PCR products were obtained. For the sequencing reaction BigDye® Terminator v3.1 Cycle Sequencing Kit from Applied Biosystems was used. The product was placed on Sephadex® columns (Sigma) and centrifuged at 910 rcf for 5 minutes. The ABI 3130 genetic analyzer from Applied Biosystems was used for sanger sequencing. To assess the indel spectrum and frequencies at the target locus we used the program TIDE developed by the Netherlands Cancer Institute (NKI)[31].

SpotNGlia

Preprocessing Images acquired from neutral red labelled larvae (n=50) were used to optimize the algorithm. For each larva, 3-6 images were taken at different depths of focus. Color channels were realigned by finding the translation that maximizes the correlation coefficient[40]. To remove the background the triangle thresholding method was used[41]. Next, we generated an all-in-focus image with extended depth of field[42].

Brain segmentation The orientation of the fish was determined by maximizing the correlation coefficient between the image and a mirrored version of itself, yielding the larvae's rotation angle. The translation parameters were found by directly correlating the image to a template image, which was established by averaging multiple aligned fish. Because of its near-circular shape, the optic tectum was segmented by performing a polar transformation after which the edges of the optic tectum were found by using Dijkstra's algorithm[43,44]. The brain edge becomes an approximately straight line in polar coordinates if it is transformed with respect to the center of the optic tectum which we obtained from the template image. To make it applicable for the shortest path algorithm, the image is correlated with a small image, similar to the average appearance of the brain edge in the polar image. Also priori information of the training set is used to exclude locations where the brain edge cannot be. After Dijkstra's algorithm is applied the found path is transformed back resulting in the brain edge coordinates.

Microglia detection To identify neutral red-positive (NR+) microglia a multiscale wavelets product was computed on the green channel of the image, which contains the highest contrast for the NR signal [32]. Multiple smoothed images from a single fish image were produced with increasing spatial scale. Subtracting adjacent smoothed images resulted in subband images containing different scales of detail present in the image. A product of subband images in the range of the microglia spot size was performed to obtain an image with only high values at the location of the spots i.e. the multiscale wavelet product. A threshold on the multiproduct image was applied to obtain a binary image to determine the spots. The identified spots were discriminated further on typical color and size obtained from the training set, resulting in accurate quantification of microglia numbers.

Statistical analysis

For image processing and quantitative analysis SpotNGlia, ImageJ and Prism (Graphpad) were used. Statistical significance was calculated using the one-way ANOVA with Bonferroni correction. Standard deviations (SD) are shown as error bars and $p<0.05$ was considered significant.

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Supplementary material

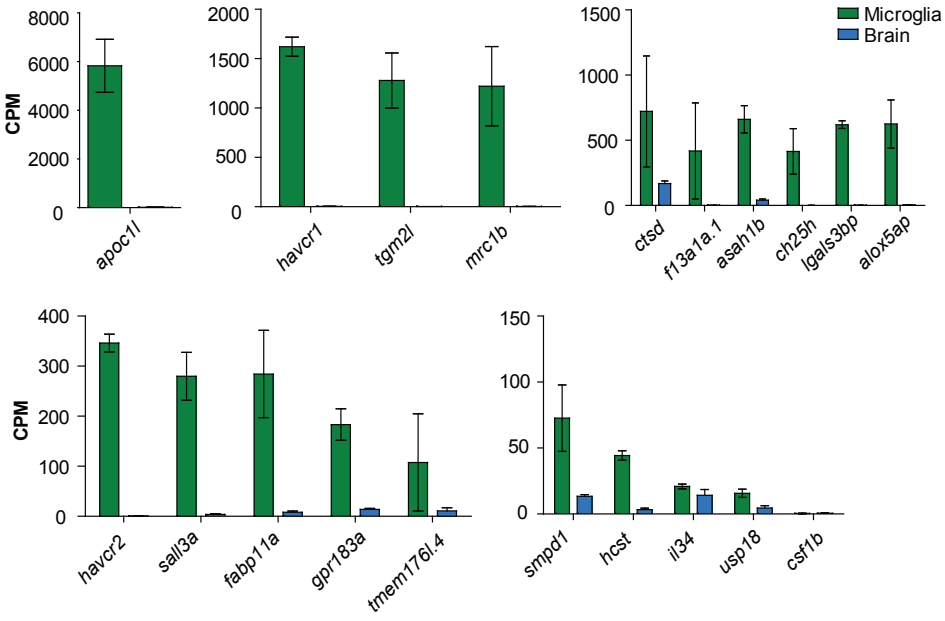

Fig S1. Expression of putative regulators of microglia development in the zebrafish brain. Bar graphs represent expression values of putative microglia regulators in microglia (green) and other brain cells (blue) observed in the microglia transcriptome[16].

Gene	gRNA sequence	Efficiency	R^2
alox5ap	GGATACGTACCCTACATTTC	32%	0.98
apoc1l	GGCCCAGGAGGAGCCCACAC	84%	0.90
asah1	AGCTGGAGGATTGCAGAAGT	52%	0.92
cstD	CGCGTCGGACGTGCAGAAAA	74%	0.90
csf1a	TGGGTGACAGAGTGCTTACA	91%	0.91
ch25h	GGTAGACTGTAATTGAGAAG	64%	0.89
csf1b	AGGACCGGGGATGTCCATCA	79%	0.79
f13a1a	GGTCAAACAAGATGTCGATG	30%	0.96
fabp11a	GGAGTCCACAATAGAGAGAG	-	-
havcr1	GGGAGCATATGATGGACTGA	85%	0.92
hcst	GGCTAGCGTACCAGTAGGTGG	49%	0.93
il34	CCATGGTCCAGTCCGAATGC	77%	0.8
mrc1b	GCGCACCACAGACGCTGGTC	83%	0.85
tgm2l	GGGTCTCTACAGCATGACTG	92%	0.92
gpr183	GACTCTGTACTCAGCCAACC	86%	0.94
lgals3bp	GGTCTACCATGATGGACAGT	84%	0.88
sall3a	GGAGTGGATGATTCAGACAG	79%	0.91
smpd1	CGACGGGGATGTAGAGACGG	83%	0.91
tmem176.4	GGGTCATCAATATTGCATTG	31%	0.94
usp18	TATGTCCAGCAGTTCAGTTG	11%	0.97

Table S1. gRNAs and their mutagenic efficiencies.

Chapter 5

Colony stimulating factor 1 receptor (*csf1r*) is dispensable for initial yolk sac macrophage development, but required for yolk sac- and early macrophage proliferation

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Abstract

Long-lived tissue macrophages exert essential functions during organogenesis, pathogenesis and tissue homeostasis, and disruption of their development can lead to disease. Some tissue macrophage populations, such as the microglia in the brain, are completely derived from yolk sac macrophages (YSMs) that seed tissues during early embryogenesis. The generation of early macrophage populations mostly depends on colony-stimulating factor 1 receptor (CSF1R), however, the exact role of CSF1R in tissue macrophage development is still unclear. Here we used zebrafish lacking functional Csf1r, to unravel how and when Csf1r is necessary for the development of early tissue macrophages from YSMs. We discovered that, although YSMs are present in *csf1r*-deficient zebrafish embryos, most of them fail to adopt a branched morphology and colonize the embryo. RNA sequencing on embryonic macrophages (YSMs and early tissue macrophages) revealed that developmental stage-dependent transcriptional changes were similar in *csf1r*-deficient and control zebrafish embryos. Additionally, further indicating that the early differentiation of macrophages is independent of *csf1r,* the vast majority of genes specific to macrophages, as opposed to other blood cells, were expressed at normal levels in *csf1r*-deficient macrophages. In contrast, Genes associated with cell division were expressed at lower levels in *Csf1r*-deficient macrophages at all examined time points. In line with this, we observed severely impaired proliferation in *csf1r*-deficient macrophages after 48 hpf when control macrophages would adopt a branched morphology and leave the yolk. In all, these data imply that, independently of *csf1r*, the initial yolk sac macrophages develop and differentiate, but fail to colonize the embryo and are unable to proliferate during tissue colonization. This indicates that the Csf1r is important for tissue colonization and expansion of embryonic tissue macrophages.

Introduction

Tissue resident macrophages exert important functions in organogenesis, immune defense and homeostasis[1]. Indeed, perturbations in tissue resident macrophages can have devastating consequences ranging from defects in tissue development to neurodegeneration[2-5]. Most tissue macrophage populations are initially derived from embryonic yolk sac macrophages (YSMs). These YSMs, during their colonization of the vertebrate embryo, undergo a stepwise differentiation into tissue macrophages, after which they acquire distinct gene expression profiles[6,7]. Throughout life however, some are partly or completely replaced by bone marrow-derived macrophages, whereas others remain entirely of yolk sac origin. For example, in the heart, a subset of macrophages is of embryonic origin, while another subset is bone-marrow derived[8]. In contrast, microglia, the brain's macrophages, are completely self-renewing and are not replaced by bone marrow-derived macrophages under homeostatic conditions[9-13].

Colony stimulating factor 1 receptor (CSF1R), is a key regulator of macrophage development. In vitro, it drives the proliferation and differentiation of macrophages, and promotes their survival[14]. In vivo, the absence of Csf1r in the mouse results in a complete lack of microglia, whereas other subsets of tissue resident macrophages are affected to a varying degree[15]. This indicates that Csf1r may primarily be important for the development and/or maintenance of long-lived yolk sac-derived macrophage populations[9,15-17]. Previously, Ginhoux and colleagues showed that, from E12.5 onwards, Csf1r-/- mice lack YSMs[9]. However, already at ~E7.5, the first YSMs are generated, after which, at E10.5, they colonize the fetal liver[9,18]. It is therefore unknown, whether Csf1r is required for the development of early tissue macrophage precursors.

Zebrafish are highly suitable to study immune cell development in vivo as they develop ex utero, are genetically tractable and, are transparent during early development[19-21]. Additionally, the majority of genes found in mammals, including *CSF1R,* are also present in the zebrafish (chapter 6)[22]. Here, we used *csf1ra* and *csf1rb*-deficient zebrafish to elucidate the role of *csf1r*-signaling in embryonic tissue macrophage development. We found that, in *csf1r*-deficient zebrafish, early YSMs are morphologically indistinguishable from control YSMs, and that YSM numbers and proliferative rates are similar in *csf1r*-deficient and in control embryos. Whereas control YSMs adopted a branched morphology and started to colonize the embryo, *csf1r*-deficient YSMs stopped dividing, kept a rounded morphology and mostly failed to migrate out of the yolk sac. At the transcriptional level, *csf1r*-deficient macrophages did not show indications of abnormal or impaired differentiation. In contrast, they showed reduced expression of genes involved in cell division and also showed decreased proliferation, even the few that managed to leave the yolk sac. In all, loss of *csf1r* mainly affects YSM tissue colonization and proliferation in the yolk sac and upon tissue colonization, and does not appear to affect macrophage differentiation status. Csf1r therefore appears to be primarily important for macrophage self-renewal capacity and embryonic tissue colonization by macrophages.

Results

Zebrafish YSMs are formed independently of csf1r

To determine whether YSMs are formed in the absence of *csf1r* we used zebrafish deficient for both *csf1r* homologs, *csf1ra* and *csf1rb,* that have almost no microglia (referred to as *csf1r*^{DM})(chapter 6). Previous studies showed that in the zebrafish, YSMs

are present on the yolk sac at 22 hour post fertilization (hpf)[19,23]. In vivo imaging of GFP-expressing macrophages (Tg(mpeg:GFP)) in control zebrafish embryos between 24 and 42 hpf, showed that at 24 hpf, ~15 GFP+ YSMs were present in the yolk sac, which increased to ~45 at 42 hpf[24]. In *csf1r*^{DM} embryos, the numbers of YSMs at 29 and 42 hpf did not significantly differ from those in controls, indicating that YSMs are formed independently of *csf1r* (Fig. 1A,B).

As YSMs were still formed independently of Csf1r, we next investigated whether *csf1r*^{DM} YSMs have a defect in tissue colonization. We analyzed YSM morphology and numbers at later developmental stages. At 42 hpf, some control YSMs had acquired a branched morphology, characterized by the presence of several protrusions, whereas the majority of *csf1r*^{DM} YSMs remained rounded (Fig. 1C,D). At 52 hpf, nearly all YSMs and pre-macrophages in control embryos showed multiple protrusions, whereas *csf1r*^{DM} YSMs mostly lacked protrusions and were present in lower numbers (Fig. 1E). Previously, it was shown that the morphological transition of YSMs correlated with their altered migratory behavior and colonization of embryonic tissues including the brain[22,25]. Indeed, half of the YSMs had left the yolk sac epithelium in controls at 52 hpf (Fig. 1E,F). In contrast, in *csf1r*^{DM} embryos only 15% of the macrophages were found outside of the yolk sac. In controls, migration trajectories of GFP+ early macrophages into the embryo, as shown by maximal projection images acquired over ~16 hours, were present throughout the entire embryo (Fig. G). In contrast, the trajectories of *csf1r*^{DM} GFP+ cells indicated that very little migration outside of the yolk sac had occurred (Fig. 1G). The few cells that did leave the yolk sac in *csf1r*^{DM} mutant embryos seemed to be trapped between the eyes and the brain (Supplementary Movie 1)(Fig. 1G). Thus, even though the generation of YSMs appears independent of *csf1r, csf1r* deficient YSMs show an altered, more rounded morphology and show severely impaired migration away from the yolk sac.

RNA sequencing of YSMs and early macrophages reveals *csf1r*-independent macrophage differentiation

To investigate which molecular changes could underlie the morphological and migratory defects of *csf1r*^{DM} mutant macrophages, we next performed RNA-sequencing on FACS sorted GFP+ macrophages at 28 and 50 hpf. The GFP+ macrophage population isolated at 28 hpf mainly consisted of YSMs, whereas those isolated at 50 hpf contained both YSMs and embryonic tissue macrophages. These time points were chosen because at 28 hpf *csf1r*^{DM} YSMs were morphologically indistinguishable from control YSMs, while at 50 hpf, the *csf1r*^{DM} macrophages were clearly different from those found in control embryos (Fig. 2A).

Principal component analysis (PCA) of the macrophage gene expression data sets showed clustering of triplicate samples based on developmental stage (component 1) and genotype (component 2) (Fig. 2B). This suggests that even though gene expression differed between control and *csf1r*^{DM} macrophages at both time points, the gene expression changes that occurred over time were comparable in *csf1r*^{DM} and control embryos (Fig. 2B,C). In control and in *csf1r*^{DM} macrophages, respectively, 1022 and 1695 genes were differentially expressed between 28 and 50 hpf (Fig. 2C). Gene set enrichment analysis (GSEA) revealed, both in control and in *csf1r*^{DM} mutant macrophages, the enrichment of gene sets associated with neuronal and cellular development for genes with increased expression (Fig. 2D). Conversely, downregulated

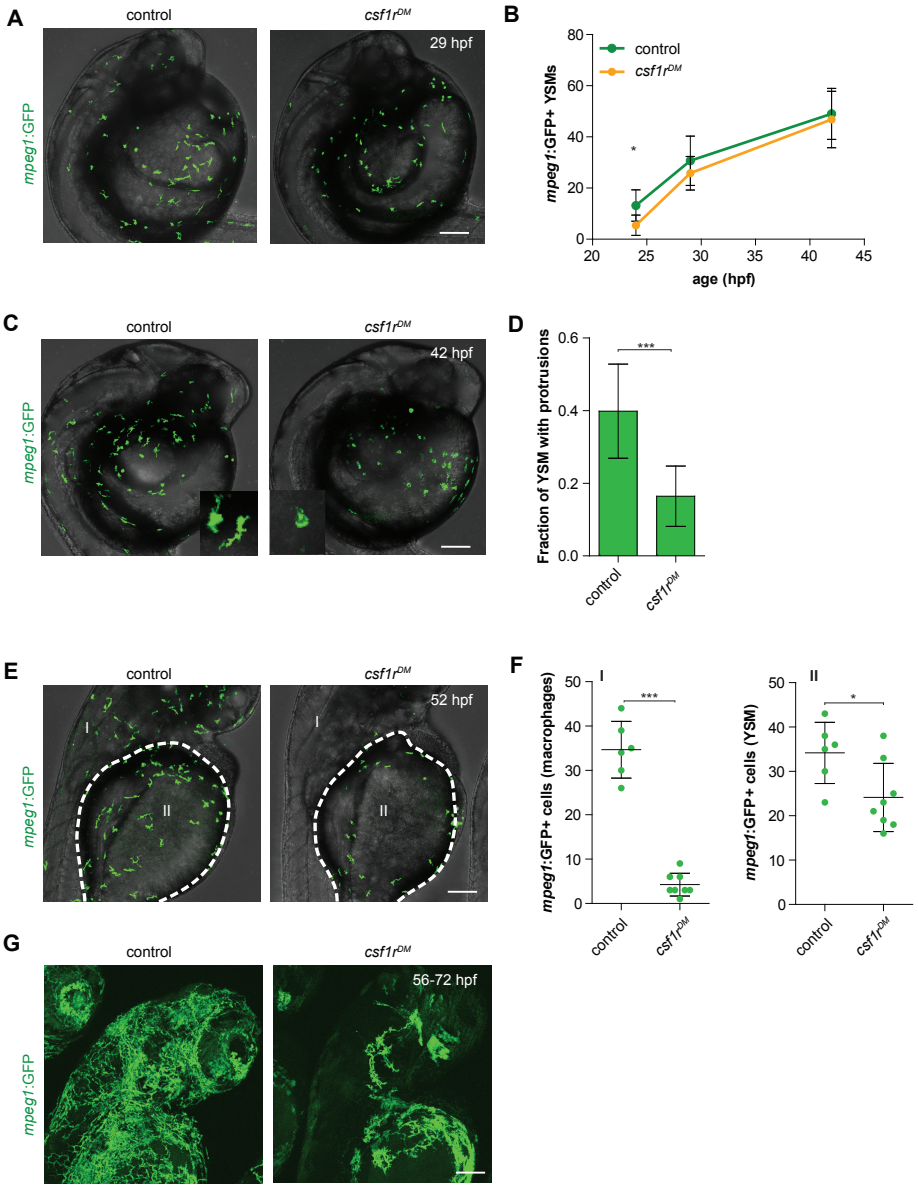

Fig. 1 GFP+ YSMs can be detected in control and *csf1r*^{DM} larvae
A Representative images of GFP+ myeloid progenitors located on the yolk sac (29 hpf). **B** Quantification of YSM numbers over time. **C** Representative images of GFP+ YSMs at 42 hpf used to quantify YSMs containing more than 1 protrusion in **D**. **E** Representative images of GFP+ positive YSMs at 52 hpf. The dotted line indicates the border between the embryonic tissue (I) and the yolk sac (II). **F** Quantification of *mpeg*-GFP+ macrophages that colonized the tissue (I) and YSMs located on the yolk sac (II). **G** Representative maximum projection of long term time lapse imaging of control and *csf1r*^{DM} larvae showing migratory trajectories of mpeg-GFP+ macrophages. Scale bars represent 100 μM. Error bars represent standard deviation. Statistical significance is calculated using ANOVA or student's T-tests *p < 0,05 **p < 0,01 ***p < 0,001.

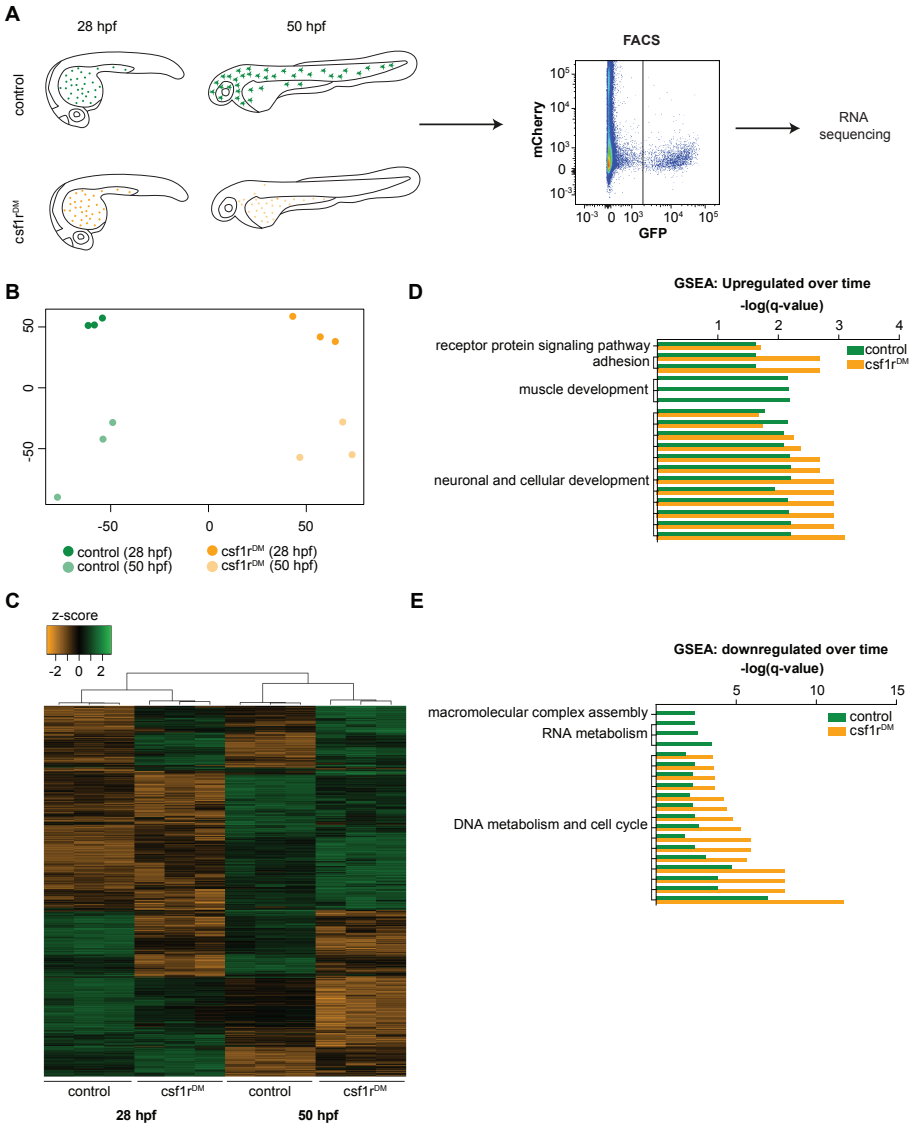

Fig. 2 RNA sequencing of YSMs at different developmental stages reveal many DE genes
A Schematic representation of the experimental set-up. GFP+ cells were isolated from both control and *csf1r*^{DM} larvae at 28 hpf and 50 hpf using FACS. These cells were used for RNA sequencing. **B** PCA analysis shows clustering of triplicates and segregation on genotype (component 1) and developmental stage (component 2). **C** Heat map showing all significantly differentially expressed genes (logFC > |1|; FDR < 0.01). **D** GO terms associated with the enriched genes upregulated over time in control and *csf1r*^{DM} macrophages. **E** Gene ontology (GO) terms associated with the enriched genes downregulated over time in control and *csf1r*^{DM} macrophages.

genes were enriched for cell cycle and DNA metabolism gene sets both in control and in *csf1r*^{DM} YSMs (Fig. 2E). Together, these PCA and GSEA analyses suggest that many of the specific changes in gene expression that accompany the transition from YSMs to early tissue macrophages occur independently of *csf1r.*

As CSF1R is needed for macrophage differentiation in vitro, the absence of CSF1R signaling in vivo, could result in impaired macrophage differentiation[14,26]. Therefore, we hypothesized that the rounded morphology of *csf1r*^{DM} macrophages, and their inability to colonize the embryo, could be due to a macrophage differentiation defect. However, either at 28 or at 50 hpf, we did not observe altered expression of genes typically highly expressed in macrophages, including chemokine and pathogen recognition receptors (*cxcr3, marco*, *mrc1*, *tlr1*), and myeloid transcription factors (*irf8*, *spi1a*, *cebpb*) in *csf1r*^{DM} macrophages (Fig. 3A,B). To assess macrophage differentiation in our *csf1r*^{DM} macrophages more systematically, we compared the gene expression profiles with a zebrafish macrophage-specific expression profile that was identified by single cell RNA sequencing[27]. We observed that, at both 28 and 50 hpf respectively, only ~5% of 2031 macrophage-specific genes were differentially expressed between control and *csf1r*^{DM} macrophages (Fig. 3C), suggesting that the induction of macrophage-specific gene expression occurs largely independent of *csf1r*.

Cell cycle related genes are downregulated in *csf1r*^{DM} macrophages

Even though the overall changes in gene expression between 28 and 50 hpf, were similar in control and in *csf1rDM* macrophages, there were extensive differences in gene expression between control and *csf1rDM* macrophages. At 28 and 50 hpf, we detected, 705 and 890 genes that were differentially expressed between *csf1r*^{DM} and controls, respectively (logFC > |1|; FDR < 0,01). GSEA revealed that, at 28 hpf, *csf1r*^{DM} macrophages showed lower expression of genes associated with DNA and RNA metabolism, and at 50 hpf, they showed lower expression of cell cycle-related genes (Fig. 4A,B). Gene ontology analysis revealed that, at both time points, the genes with higher expression in *csf1r*^{DM} macrophages, were mostly associated with blood coagulation, whereas the genes that, at both time points, were downregulated in *csf1r*^{DM} macrophages were associated with DNA replication (Fig. 4B). This suggests that embryonic *csf1r*^{DM} macrophages have a lower proliferative activity or capacity.

YSMs proliferate independently of *csf1r*, whereas microglia require *csf1r* to proliferate

As we observed lower expression of genes involved in cell cycle in *csf1r*^{DM} macrophages, we next assessed the proliferative rate of YSMs and brain macrophages, during the first days of development. Circulating monocytes are short-lived and are thought not to divide, whereas tissue macrophages, in particular the microglia in the brain, self-renew by proliferation[11,25,28-30]. Time lapse recordings of YSMs revealed that, between ~32 to 48 hpf, the proliferative rates were not significantly different in control and in *csf1r*^{DM} embryos. However, between ~56 and 72 hpf, control macrophages (YSMs and early tissue macrophages) still proliferated, whereas c*sf1r*^{DM} macrophages had completely stopped proliferating (Fig. 5A-C; Supplemental movie 2). This suggests that initially, the proliferation of YSMs is independent of *csf1r*, while later on, YSMs and early tissue macrophages require *csf1r* to proliferate and maintain their self-renewal capacity.

To determine whether *csf1r* is important for the proliferative expansion of early, yolk

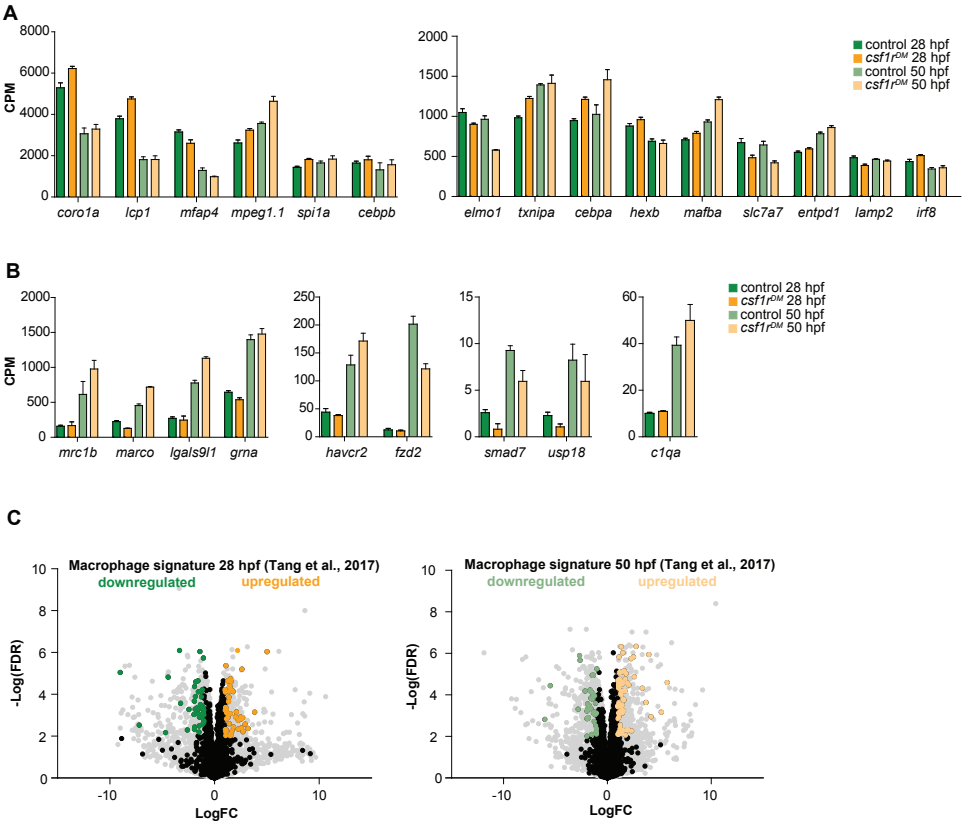

Fig. 3. Macrophage-specific gene expression is largely unaffected by loss of *csf1r*
A CPM values of 'macrophage signature' genes show high expression in all groups. **B** CPM values of 'macrophage signature' genes induced over time in control and *csf1r*^{DM} macrophages. **C** Volcano plot showing genes expression changes between control and *csf1r*^{DM} at 28 hpf and 50 hpf respectively. Light grey: all reads, Black: Macrophage genes[27]; Green: Macrophage genes significantly upregulated in control macrophages; Orange: Macrophage genes significantly upregulated in *csf1r*^{DM} macrophages (logFC > |1|; FDR < 0.01). Only 4 and 5% of the macrophage genes were significantly differentially expressed between control and *csf1r*^{DM} macrophages at 28 and 50 hpf respectively. Error bars represent standard deviation.

sac-derived, tissue macrophages, we determined, starting at 2 days post fertilization (dpf), the fraction of proliferative microglia at several consecutive days by Pcna immunostaining. In control embryos, when at 48 hpf the first macrophages arrive in the brain, very few early microglia were Pcna+, suggesting that they had low proliferative rates (Fig. 5C-F). Quantification of immunostained Lplastin+ microglia at 48, 72 and 96 hpf showed that, between 48 and 72 hpf, microglia numbers increased in control larvae (from ~5 to ~30 microglia). Previously, we showed that, in an allelic series of *csf1r* mutants, lowering the number of functional *csf1r* alleles correlate with lower microglia numbers, which could be due to a requirement for csf1r in early microglia proliferation (chapter 6). We found that, in 4-days-old control larvae, ~7 out of 35 microglia were Pcna+ and dividing, whereas, at 2, 3 and 4 dpf, no dividing microglia were found in most *csf1ra-/-, csf1ra-/-;b+/-* and *csf1r*^{DM} larvae (Fig. 5C-F). To independently verify this, we also performed EdU pulse labeling, which can be used to mark all proliferative events

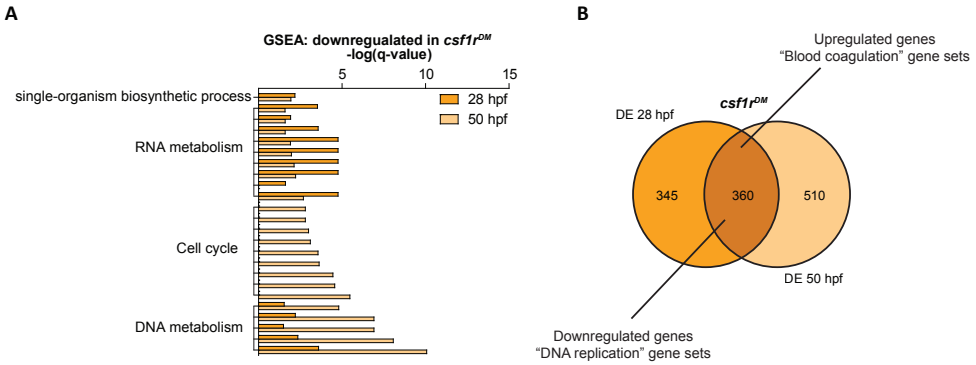

Fig. 4 Downregulated genes are associated with cell cycle-related GO terms.
A Bar graph showing the GO terms associated with enriched genes downregulated in *csf1r*^{DM} macrophages. **B** Venn diagram showing the amount of DE genes between control and *csf1r*^{DM} at 28 and 50 hpf and overlap between gene sets.

within the pulsed time window, and found that EdU+ microglia were completely absent in *csf1r*^{DM} larvae (Fig. 5G-I). This indicates that the proliferative expansion of microglia after brain colonization is *csf1r*-dependent, which indicates that in addition to a role in tissue colonization, *csf1r* is also required for local self-renewal of tissue macrophages upon colonization of target tissues.

Discussion

Here, we investigated the in vivo role of *csf1r* in the development of early tissue macrophages from YSMs in the vertebrate embryo. CSF1R is a well-studied regulator of macrophages: without CSF1R stimulation, macrophages do not proliferate nor differentiate in vitro[14]. In contrast, tissue macrophages in *Csf1r* knockout mice are differentially affected as these mice lack microglia and have diminished numbers of several subtypes of tissue macrophages[15]. However, it was still unknown how CSF1R precisely affects tissue macrophage development in vivo and when CSF1R is required during tissue macrophage development. We showed that YSMs are formed and that they, until 48 hpf, proliferate independently of *csf1r* in the zebrafish embryo. However, in contrast to control embryonic macrophages, most csf1rDM macrophages failed to adopt a branched morphology and colonize the embryo. RNA sequencing on isolated embryonic macrophages revealed no obvious *csf1r*-dependent macrophage differentiation defects. We did observe lower expression of genes associated with cell cycle and DNA replication in *csf1r*^{DM} embryos, pointing towards a *csf1r*-dependent proliferation defect. This was corroborated by the observation that, from 48 hpf onwards, *csf1r*^{DM} macrophages in the yolk sac and in the embryo failed to proliferate. Furthermore, the very few macrophages that manage to reach the brain were unable to proliferate and expand their population. In all, our data suggests that *csf1r* is largely dispensable for the acquisition of a macrophage cell fate in YSM, but instead, is required for YSMs to thrive and sustain a long-lived macrophage phenotype with the self-renewal capacity of tissue resident macrophages.

Even though CSF1R signaling appears to be necessary for macrophage differentiation in vitro, several studies have presented evidence that this is not the case

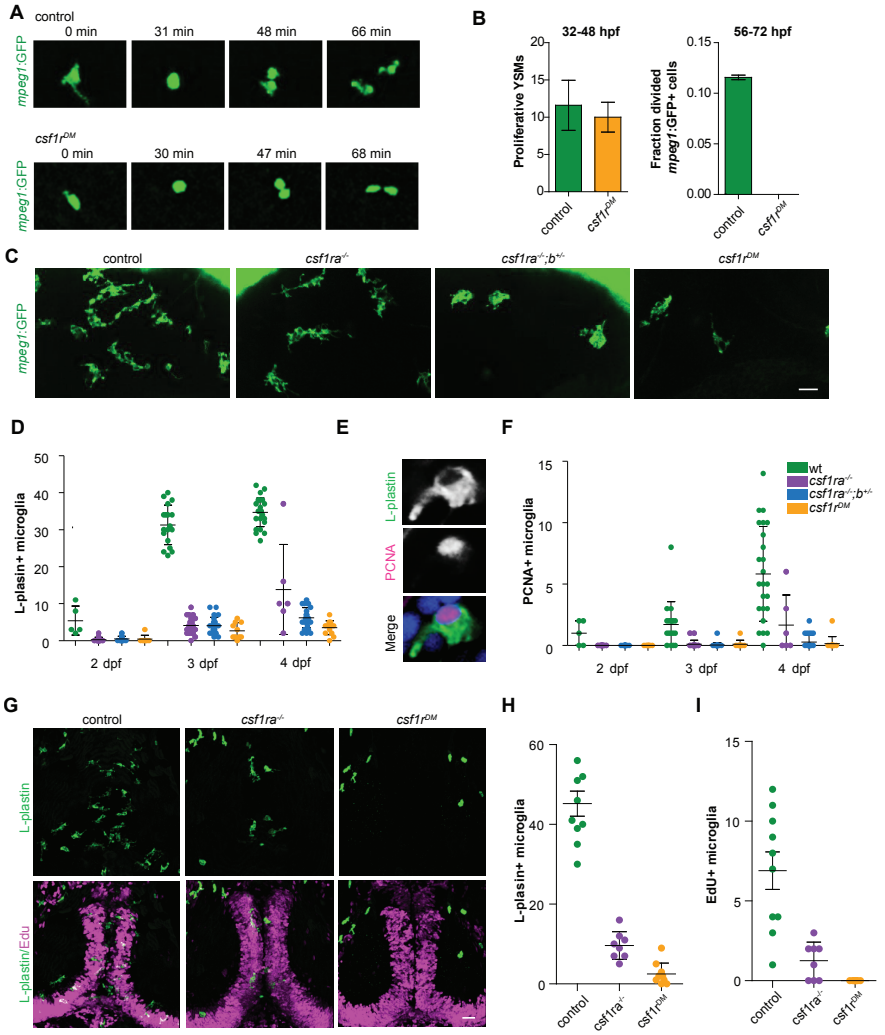

Fig. 5. Proliferation of YSMs is independent of csf1r, while microglia proliferation is absent in csf1r mutants

A Snap shots from dividing mpeg-GFP+ YSMs in control and csf1r^{DM} larvae (~36 hpf). **B** Quantification of proliferative YSMs during 16 hour time lapse imaging (~32 hpf - 48 hpf). **C** Representative images of L-plastin immunohistochemistry of microglia in different *csf1r* mutant larvae at 96 dpf. Scale bar represents 20 μM. **D** Quantification of L-plastin+ microglia in control and different csf1r mutants at 48, 72 and 96 hpf. **E** Representative images of a Pcna+/L-plastin+ microglia **F** Quantification Pcna+/L-plastin double positive microglia at 48, 72 and 96 hpf. **G** Representative images of L-plastin and Edu immunohistochemistry at 5 dpf. Scale bar represents 25 μM. **H** Quantification of L-plastin+ microglia in control, *csf1ra*$^{-/-}$ and *csf1r*^{DM} larvae at 5 dpf. **I** Quantification of L-plastin/EdU double positive microglia in control, *csf1ra*$^{-/-}$ and *csf1r*^{DM} larvae at 5 dpf. Error bars represent standard deviation.

in vivo. Both adult Csf1r$^{-/-}$ mice and *csf1r*^{DM} zebrafish almost completely lack microglia, but still have other tissue macrophage populations (data not shown)[15]. Furthermore, Csf1r-deficient bi-potential granulocyte-macrophage precursors (GMPs) have a normal lineage potential, which shows that macrophages can be formed independently of Csf1r[15,31,32]. Previously, in Csf1r-/- mice, the yolk sac macrophages were reported to be absent at E12.5[9]. However, in vivo imaging of mouse embryos indicated that, at E12, the majority of YSMs have migrated out of the yolk sac[25]. In zebrafish, *csf1r*^{DM} YSMs initially were morphologically and behaviorally indistinguishable from control YSMs, while at 2 dpf *csf1r*^{DM} YSM appeared to have severe defects. It remains to be tested whether also in Csf1r-/- mice, YSMs are formed initially, after which they would be progressively lost at later stages of development. In all, this shows that in vivo, at least in the zebrafish, yolk-sac macrophages can be formed independently of *csf1r*.

In Csf1r-deficient animals specific tissue macrophage populations, such as microglia, are absent, whereas other tissue macrophage populations persist[15]. It is possible that, due to loss of yolk-sac macrophages in csf1r-deficient animals, the loss of particular tissue macrophages, including the microglia, is explained by the extent to which a specific macrophage-subset remains of yolk-origin. The presence of specific tissue macrophage populations found in Csf1r-deficient animals may occur by bone marrow derived macrophages that differentiate into tissue-resident macrophages[11,29,33]. Genetic or chemical inhibition of CSF1R in adult mice results in depletion of specific tissue macrophages, including the microglia, within weeks, which indicates that Csf1r is important for macrophage survival in vivo[34,35]. In line with this, in *csf1r*^{DM} embryos, yolk sac-derived macrophages may show reduced survival, as at 52 hpf, the number of embryonic macrophages in *csf1r*^{DM} embryos appeared to be lower than those at 42 hpf. From 48 hpf onwards, embryonic *csf1r*^{DM} macrophages were also severely compromised in their proliferation, which together with a possibly reduced survival, may result in a quick depletion of yolk sac-derived macrophage precursors available for tissue colonization. Because of this, long-lived yolk sac-derived macrophage populations (e.g. microglia and Langerhans cells), with little contribution of bone-marrow derived macrophages, might be particularly sensitive to proliferative deficits due to *csf1r*-deficiency.

Csf1r^{DM} macrophages, in addition to showing a severe proliferation defect, also showed severely impaired migration out of the yolk sac. The few *csf1r*^{DM} YSMs that managed to escape the yolk sac appeared to mostly travel in the direction of the brain, but failed to enter the brain. Instead, they appeared to be stuck caudal to the eye. Others have shown that macrophages destined to become microglia enter the brain via the lateral periphery (scleral side of the eyes)[22,36]. This suggests that the few *csf1rDM* macrophages leaving the yolk sac, migrate towards the brain, but for some reason are unable to enter the brain. Although, it is unclear how microglia precursors are attracted to, and enter, the brain, it was recently shown that microglia precursors could travel towards the brain in response to apoptotic cells in the brain and retina[36-39]. We did not observe obvious differences in the expression of genes involved in chemotaxis or migration in *csf1r*^{DM} macrophages. Macrophages are attracted by chemokines or other cues via receptors causing their migration (chemotaxis). Analysis of gene expression levels of microglial genes possibly involved in this migration did not show any differences. Therefore, the migratory defect observed in these cells may be a direct consequence of the inability to respond to the Csf1r ligands Csf1 and Il34.

Here we provide insight into how the loss of *Csf1r* results in the absence of microglia observed in zebrafish, mouse and human (chapter 6)[15,17](personal communication Chang & Bennet). Apparently, in *csf1r*-mutants lacking microglia, yolk sac macrophages are present in the yolk sac, but fail to colonize the embryonic tissues and subsequently fail to expand in numbers by proliferation. Our data shows that *csf1r* is not a major regulator of macrophage development and differentiation per se, but rather, it is required for tissue colonization and expansion of yolk-sac derived macrophage populations.

Acknowledgements
We acknowledge Remco M Hoogenboezem for assistance in RNA sequencing.

Materials and Methods

Animals

C*sf1r* mutants were created as described in chapter 6. Tg(*mpeg1*:*egfp, Neuro*:Gal4, UAS:NTR-mCherry) were used as control animals[40]. Adult and larval fish were kept on a 14h/10h light-dark cycle at 28°C. Larvae were kept in HEPES-buffered E3 medium. Media was refreshed daily and at 24 hpf 0.003% 1-phenyl 2-thiourea (PTU) was added to prevent pigmentation. Animal experiments were approved by the Animal Experimentation Committee of the Erasmus MC.

Live imaging

Intravital imaging in zebrafish brains was largely performed as previously described[40]. Briefly, zebrafish larvae were mounted in 1.8% low melting point agarose containing 0.016% MS- 222 as sedative and anesthetic in HEPES-buffered E3. The imaging dish containing the embedded larva was filled with HEPES-buffered E3 containing 0.016% MS-222. Imaging was performed using a Leica SP5 intravital imaging setup with a 20x/1.0 NA water-dipping lens. Imaging of *mpeg1-gfp* was performed using the 488 nm laser. Analysis of imaging data was performed using imageJ (FIJI) and LAS AF software.

Immunofluorescence staining

Immunohistochemistry was performed as described[40,41]. Briefly, larvae were fixed in 4% PFA at 4°C overnight. Subsequently, they were dehydrated with a 25%, 50%, 75%, 100% MeOH series and stored at -20°C for at least 12 hours, and rehydrated in series followed by incubation in 150 mM Tris-HCl (pH=9.0) for 15 minutes at 70°C. Samples were then washed in PBS containing 0.04% Triton (PBST) and incubated in acetone for 20 minutes at -20°C. This was followed by washing steps in PBST and ddH_2O. Larvae were incubated for three hours in blocking buffer (10% goat serum, 1% Triton X-100 (Tx100), 1% BSA, 0.1% Tween-20 in PBS) at 4°C, followed by incubation in primary antibody buffer at 4°C for three days. Larvae were washed in 10% goat serum 1% Tx100 in PBS and PBS containing 1% TX100 for a few hours, followed by incubation in secondary antibody buffer at 4°C for two and a half days. Hereafter the secondary antibody was washed away using PBS. Primary antibody buffer: 1% goat serum, 0.8% Tx100, 1% BSA, 0.1% Tween-20 in PBS. Secondary antibody buffer: 0.8% goat serum, 1% BSA and PBS containing Hoechst. Primary antibodies: PCNA (1:250, Dako), L-plastin (1:500, gift from Yi Feng, University of Edinburgh). Secondary antibodies used were DyLight Alexa 488 (1:250) and DyLight Alexa 647 (1:250). Samples were imaged as described above.

EdU pulse-chase protocol

Larvae of 4 dpf were placed in a 24 wells plate in HEPES buffered (pH = 7.3) E3 containing 0.003% PTU and 0.5 mM EdU for 24 hours. Next, larvae were fixed in 4% PFA for 3 hours at room temperature, dehydrated with a 25%, 50%, 75%, 100% MeOH series and stored at -20°C for at least 12 hours. Rehydrated in series followed by a proteinase K (10μg/ml in PBS) incubation for an hour. Followed by 15 minute post fixation in 4% PFA. Larvae were further permeabilized in 1% DMSO in PBS-T. Thereafter 50μl Click-iT^{TM} (Invitrogen) reaction cocktail was added for 3 hours at room temperature protected from light. After washing steps larvae were subjected to immunolabelling using L-plastin (see section immunofluorescent labelling). Samples were imaged as described above.

Isolation of mpeg1-GFP+ cells from zebrafish larvae
At 28 hpf, 35 larvae were collected in 0.16% MS-222 solution to euthanize them before adding 5x Trypsin-EDTA (0.25% Trypsin, 0.1% EDTA in PBS). For *csf1r*^{DM} cells, at 50 hpf, 70 larvae were used as these mutants had fewer mpeg1-GFP positive cells. Microcentrifuge tubes containing zebrafish embryos were incubated on ice on a shaking platform to dissociate the cells. Next, the cell suspension was transferred to FACS tubes by running it over a 35 ìm cell strainer cap. PBS containing 10% fetal calf serum (FCS) was added over the strainer caps and the samples were centrifuges for 10 minutes 1000rpm at 4℃. The pellet was taken up in 300 μl PBS containing 10% FCS containing DAPI (1:1000). After analysis based on mpeg1-GFP expression cells were sorted by FACS, collected in Trizol, followed by RNA isolation (supplementary Fig. 2).

RNA sequencing
cDNA was synthesized and amplified using SMART-seq® V4 Ultra® Low Input RNA kit for Sequencing (Takara Bio USA, Inc.) following the manufacturer's protocol. Amplified cDNA was further processed according to TruSeq Sample Preparation v.2 Guide (Illumina) and paired end-sequenced (2$\times$75 bp) on the HiSeq 2500 (Illumina). Reads were mapped against the GRCz10 zebrafish genome. For differential gene expression analysis, GSEA and gene ontology we used the Bioconductor packages edgeR, Gage, and goseq, respectively [42-45].

Statistical analysis
For statistical analysis GraphPad was used to perform Student's T tests or ANOVAs. Results were regarded significant at $p < 0.05$.

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Chapter 6

In vivo, colony-stimulating factor 1 receptor (CSF1R) regulates microglia density and distribution, but not microglia differentiation

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Submitted

Abstract

Microglia are brain resident macrophages with trophic and phagocytic functions. Dominant loss-of-function mutations in a key microglia regulator, colony-stimulating factor 1 receptor (CSF1R), cause adult onset leukoencephalopathy with axonal spheroids (ALSP), a progressive white matter disorder. As it remains unclear precisely how CSF1R mutations affect microglia, we generated an allelic series of *csf1r* mutants in zebrafish to identify *csf1r*-dependent microglia changes. We found that *csf1r* mutations led to aberrant microglia density and distribution, and regional loss of microglia. Remaining microglia still had a microglia-specific gene expression signature, indicating that they had differentiated normally. Strikingly, we also observed lower microglia numbers and widespread microglia depletion in post mortem brain tissue of ALSP patients. Both in zebrafish and in human disease, local microglia loss also presented in regions without obvious pathology. Together, this implies that CSF1R mainly regulates microglia density and that early loss of microglia may contribute to ALSP pathogenesis.

Key words: Microglia, leukoencephalopathy, ALSP, colony-stimulating factor 1 receptor, transcriptomics, zebrafish, HDLS

Introduction

Microglia are specialized brain macrophages whose functions in the brain include phagocytosis and provision of trophic support[1-6]. Mutations in several genes that are highly expressed in microglia cause progressive white matter brain diseases[7-11]. For example, dominant loss-of-function mutations in colony-stimulating factor 1 receptor (CSF1R) cause adult-onset leukoencephalopathy with axonal spheroids and pigmented glia (ALSP), also known as hereditary diffuse leukoencephalopathy with axonal spheroids (HDLS)[12,13]. The almost exclusive expression of CSF1R in microglia suggests that ALSP pathogenesis involves microglia dysfunction. But where one study showed reduced microglia numbers in cortical layers 3 and 4 in postmortem end-stage ALSP brain sections, another showed increased microglia numbers during earlier ALSP disease stages[14,15]. The mechanism whereby heterozygous CSF1R mutations affect microglia, and consequently brain homeostasis, is still unknown. Insight into ALSP pathogenesis will therefore contribute to our understanding of microglia function in the vertebrate brain and of microglia involvement in other brain diseases.

Even though CSF1R signal transduction has been studied extensively in macrophages, it is not entirely clear how defective CSF1R signaling affects microglia *in vivo*. Activation by one of the two CSF1R ligands, colony-stimulating factor 1 (CSF-1 or M-CSF) or interleukin 34 (IL-34) leads to auto-phosphorylation of the tyrosine kinase receptor. *In vitro*, downstream activation of signal transduction pathways regulates the production, survival, differentiation and function of macrophages[16-21]. Genetic evidence for the consequences of CSF1R activation *in vivo* indicates that CSF1R primarily plays a homeostatic role in regulating the viability and proliferation of microglia[22,23]. Indeed, genetic deficiency of CSF1R signaling reduces protection against bacterial infection, mainly by limiting macrophage supply[17,24-27]. In contrast, by showing that Csf1r-/- macrophage precursors have the same lineage potential as those in wildtype, differentiating efficiently into macrophages, but failing to form colonies, a recent study concluded that Csf1r deficiency has little effect on myeloid differentiation *in vivo*[28].

Loss of Csf1r in mice leads to an almost complete absence of microglia, and also to severe developmental abnormalities and a shorter lifespan[21]. Csf1r-/- brains show widened cerebral ventricles, which is also observed in ALSP patients. Mice lacking microglia also show cerebrovascular defects and reduced numbers of oligodendrocyte lineage cells[21,29,30]. In addition, postnatal pharmacological inhibition of CSF1R in mice reduces the number of oligodendrocytes and oligodendrocyte precursor cells (OPCs) in a region-dependent manner[29]. The latter effect could predispose to myelination defects in later life.

To understand the effect of CSF1R haploinsufficiency on microglia we used the zebrafish as a model organism. Zebrafish are an upcoming genetic model organism to study brain diseases, including leukoencephalopathies[31,32]. They are highly suitable for *in vivo* imaging, as they develop externally and are transparent at early stages[32-34]. Previously, we identified the zebrafish microglia transcriptome, which shares high similarity with mouse and human microglia transcriptomes[35,36]. Zebrafish express two homologs of human CSF1R: *csf1ra* and *csf1rb*. We found that zebrafish *csf1ra* mutants show reduced microglia numbers only during development, thereby partially mimicking mouse mutants[37-39]. This suggests that the cellular functions of CSF1R are highly conserved between species, but that zebrafish *csf1rb* and *csf1ra* are likely redundant. In the present study we therefore created an allelic series of zebrafish *csf1r*

loss-of-function mutants in which we observed a local loss of microglia, a general reduction in microglia numbers, and an aberrant distribution of microglia. These microglial abnormalities were independent of microglia differentiation status. As we found that dysregulation of microglia density was as a primary consequence of *csf1r* haploinsufficiency, we next investigated whether CSF1R haploinsufficiency also affects microglia density in postmortem brain tissue of ALSP patients. This revealed widespread depletion of microglia and a general reduction in microglia density. In humans and zebrafish alike, changes in microglia density and distribution in the absence of obvious myelin pathology implied that loss of microglia may be an early event in ALSP pathogenesis.

Results

Zebrafish *csf1ra* and*csf1rb* together are functionally homologous to mammalian CSF1R

To study how CSF1R mutations affect microglia and the brain, we exploited the fact that zebrafish have two homologs for human CSF1R: *csf1ra* and *csf1rb.* Both of these are highly expressed in adult zebrafish microglia (Fig. 1A)[35]. Unlike Csf1r knock-out mice, which are almost completely devoid of microglia, zebrafish with homozygous loss-of-function mutations only in *csf1ra* (from here on: *csf1ra*$^{-/-}$), show reduced microglia numbers only during early development[40]. This suggests that *csf1rb* and *csf1ra* share a role in microglia development.

To test this, we introduced a premature stop codon in exon 3 of the *csf1rb* gene by TALEN-mediated genome editing; and assessed microglia numbers by neutral red staining (Fig. 1B, S1), which can be used to label microglia in zebrafish larvae *in vivo*. Whereas the microglia numbers in homozygous *csf1rb* mutants were a little smaller than in wild type , mutants deficient in both *csf1ra* and *csf1rb* (from here on: *csf1r*^{DM}), were almost completely devoid of microglia (Fig. 1C,D). The absence of microglia in *csf1r*^{DM} mutants was confirmed in larval and adult zebrafish by immunostaining for L-plastin (Fig. 1E, 2F,G). Adult *csf1r*^{DM} animals were viable, and, in-cross mating of *csf1r*^{DM}, adult animals produced viable homozygous mutant offspring (data not shown). However, after around three months of age, mutant animals occasionally showed seizure-like behavior, and their survival rate was lower than that of wild type animals (data not shown). Some *csf1r*^{DM} brains displayed signs of cerebral hemorrhaging that were consistent with the hemorrhages previously reported in Csf1r-/- mice[21]. These data show that zebrafish *csf1ra* and *csf1rb* both regulate the development of the microglia population, and both are thus functionally homologous to mammalian CSF1R.

Csf1r regulates microglia density and distribution independently of brain pathology

To investigate whether any pathological hallmarks of ALSP are also present in *csf1r* mutant zebrafish, we assessed tissue and white matter integrity in adult *csf1ra*$^{-/-}$, *csf1ra*$^{-/-}$;*b*$^{+/-}$ and *csf1r*^{DM} mutants. Hematoxilin and eosin labeling did not reveal signs of brain pathology (data not shown). Neither did immunolabeling for Claudin K (Cldnk)—which labels myelin tracts throughout the zebrafish brain—reveal major loss of myelin, even in *csf1r*^{DM} mutants (Fig. S2A)[41]. To determine whether *csf1r* mutants display more subtle myelin abnormalities, such as degeneration, hypomyelination, or hypermyelination, we therefore analyzed their white matter by electron microscopy (EM).

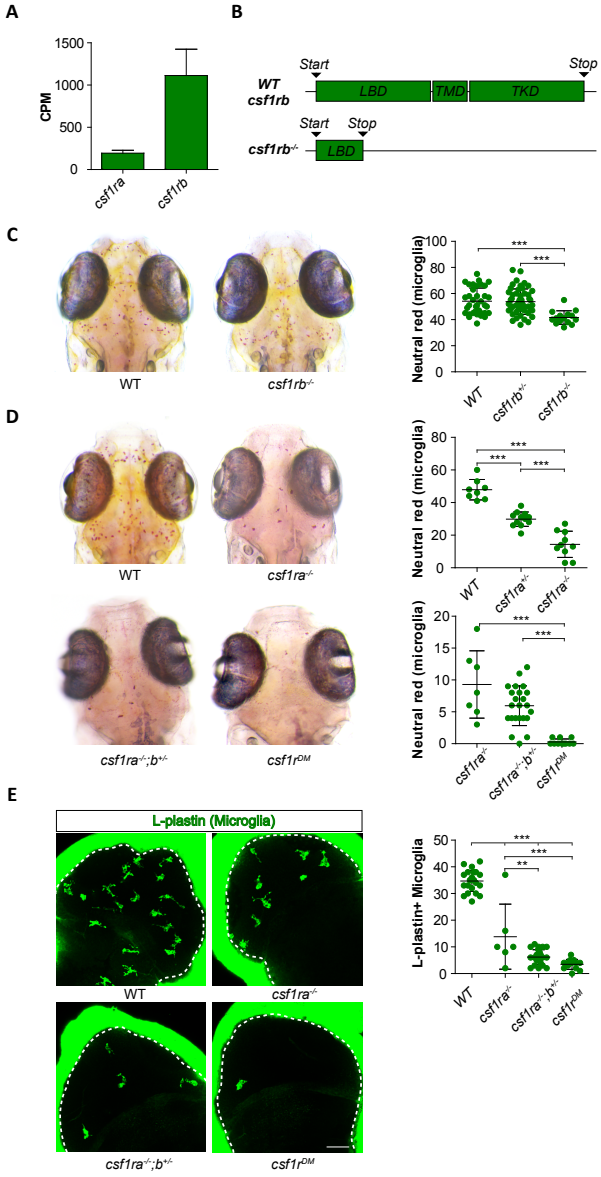

Fig. 1 Microglia numbers during development are *csf1r* dosage dependent.
A CPM expression values of *csf1ra* and *csf1rb* from our previous RNA sequencing study in adult zebrafish microglia[35]. **B** Schematic representation of the *csf1rb* mutation introduced with TALEN-mediated genome editing. **C,D** At 5 dpf WT, *csf1ra*$^{-/-}$, *csf1rb*$^{-/-}$, *csf1ra*$^{-/-}$;*b*$^{+/-}$, *csf1r*^{DM} and larvae were treated with neutral red for 2.5 h. Images were acquired with a stereomicroscope and microglia numbers were determined by counting the number of neutral red dots. **E** 4 dpf WT, *csf1r*$^{a-/-}$, *csf1r*$^{a-/-b+/-}$ *and csf1r*^{DM} were labeled with an antibody against L-plastin staining[88], and L-plastin-positive cells were quantified in the optic tecti. CPM = counts per million, LBD = Ligand-binding domain, TMD = Transmembrane domain, TKD = Tyrosine kinase domain, dpf = days post fertilization. Error bars represent the standard deviation. **p < 0.01, ***p < 0.001 (one-way ANOVA, Bonferroni multiple testing correction). Scale bar = 40 μm.

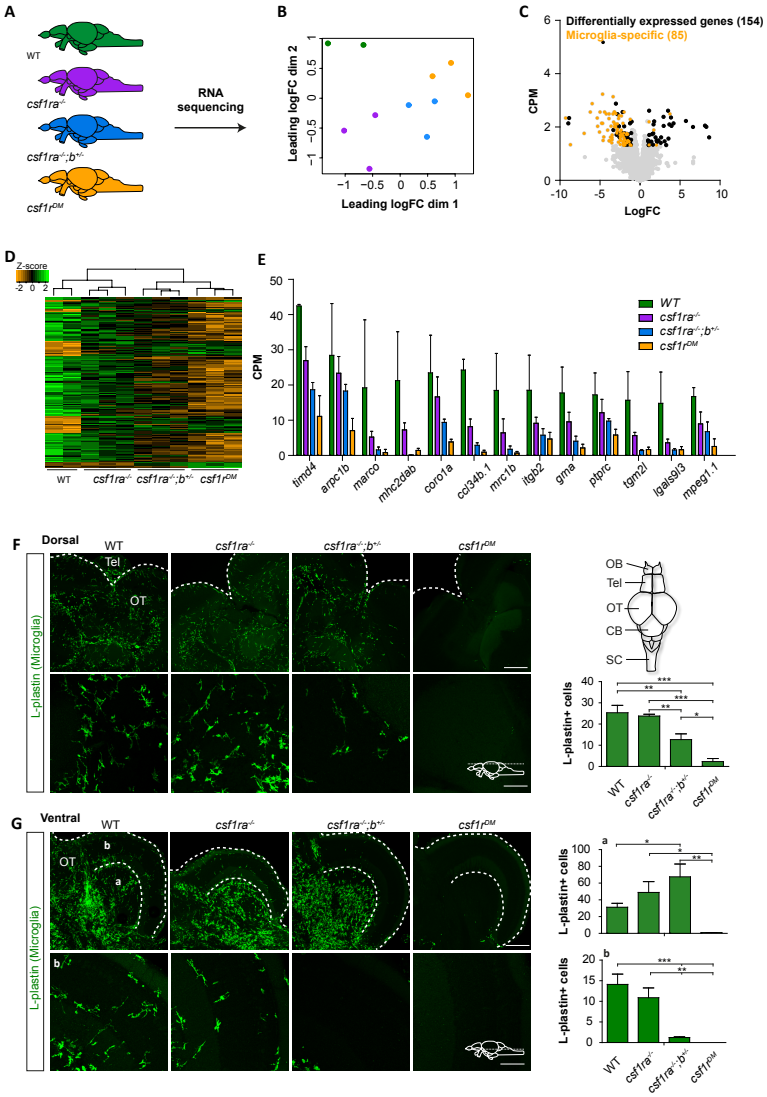

Fig. 2 Altered microglia distribution and numbers in the adult *csf1r* mutant brain.
A Schematic representation of RNA sequencing experiment. RNA was isolated from whole brains of 9-month-old WT, *csf1ra*$^{-/-}$, *csf1ra*$^{-/-}$;*b*$^{+/-}$ and *csf1r*^{DM} fish (3 brains per sample). **B** Multidimensional scaling plot. **C** Volcano plot with genes differentially expressed between *csf1r*^{DM} and WT fish. Yellow dots represent genes that are part of the zebrafish microglia transcriptome[35]. Black dots represent the other differentially expressed genes. Gray dots are all detected genes. **D** Heat map with genes differentially expressed between *csf1r*^{DM} and WT fish genes. **E** Expression values of differentially expressed microglia-specific genes. **F,G** Representative images of 5-month-old WT (n=3), *csf1ra*$^{-/-}$ (n=3), *csf1ra*$^{-/-}$;*b*$^{+/-}$ (n=3) and *csf1r*^{DM} (n=3) zebrafish brain sections stained with antibodies against L-plastin (microglia). Microglia were quantified in 3 areas (3.1 x 10^{-3} mm^3) per brain region per patient. Scale bar = 200 μm (low magnification **F**and **G**), 50 μm (high magnification **F** and **G**). Genes were differentially expressed with FDR < 0.05 and LogFC > |1|. FDR = false discovery rate, LogFC = Log fold change. Error bars represent standard deviation. *p < 0.05, **p < 0.01, ***p < 0.001 (one-way ANOVA, Bonferroni multiple testing correction).

We observed highly myelinated regions in the midbrain containing multilayered myelin sheets, which resembled those in mammals, but no apparent abnormalities in the multilayered myelin sheets in *csf1r* mutants (Fig. S2B). Immunolabeling for Sox10 also indicated normal numbers of oligodendrocyte lineage cells in *csf1r* mutants (Fig. S2C). Together, this indicates that *csf1r* deficiency in zebrafish does not result in overt myelin degeneration at this adult stage.

To establish whether loss of *csf1r* causes more subtle pathological changes, we performed RNA sequencing on adult *csf1r*-mutant zebrafish brains (Fig. 2A). Multidimensional scaling of gene expression data showed clustering of the samples based on the *csf1r* mutation status (WT, *csf1ra*$^{-/-}$, *csf1ra*$^{-/-}$;*b*$^{+/-}$, *csf1r*^{DM}), indicating *csf1r*-dependent changes in gene expression (Fig. 2B). Differential gene expression analysis between wild type and *csf1r*^{DM} mutant brains revealed 154 differentially expressed genes, 85 of which (e.g. *spi1b*, *irf8*, *csf1ra* and *csf1rb*) we had previously identified as part of the zebrafish microglial transcriptome (Fig. 2C,D,E)[35]. Hierarchical clustering of the samples on the basis of 154 differentially expressed genes revealed that *csf1ra*$^{-/-}$;*b*$^{+/-}$ mutants clustered with *csf1r*^{DM} mutants, whereas *csf1ra*$^{-/-}$ mutants clustered with wild type (Fig. 2D). This suggests that loss of *csf1r* leads mainly to reduced expression of microglia-specific genes, which indicates that loss of *csf1r* in zebrafish predominantly affects microglia. This indicates that *csf1r* deficiency and thus loss of microglia cause very few molecular changes and no obvious myelin-related pathology in adult zebrafish.

Previous studies indicate that the density of tissue macrophages, including the microglia, is affected by reduced CSF1R signaling[42-47]. To validate this in zebrafish, we used neutral red labeling and immunohistochemistry to assess microglia numbers in a series of *csf1r* mutant zebrafish larvae consisting of *csf1ra*$^{+/-}$, *csf1ra*$^{-/-}$, *csf1ra*$^{-/-}$;*b*$^{+/-}$ and *csf1r*^{DM} animals. In the larval stage, a gradual reduction in the number of *csf1r* alleles resulted in a corresponding decrease in microglia numbers (Fig. 1C,D,E). The greater reduction in microglia numbers in *csf1ra*$^{-/-}$ mutants than in *csf1rb*$^{-/-}$ mutants suggests that *csf1ra* is more important during early development. In adult zebrafish, however, microglia numbers in *csf1rb*$^{-/-}$ mutants were strongly reduced, whereas in *csf1ra*$^{-/-}$ mutants they were more comparable to those in wild type (Fig. 2F, S2D), suggesting differential requirements of *csf1ra* and *csf1rb* in microglia at different developmental stages. Surprisingly, in 5-month-old adult *csf1ra*$^{-/-}$;*b*$^{+/-}$ mutants, we observed that while microglia were absent in the dorsolateral side of the optic tectum, they appeared to accumulate in the underlying deep brain regions (Fig. 2G).

Csf1r-deficient microglia increase expression of genes involved in chemotaxis and migration

To assess in more detail how *csf1r* deficiency affects microglia independently of brain pathology, we performed RNA sequencing on FACS-sorted microglia from wild type, *csf1ra*$^{-/-}$ and *csf1ra*$^{-/-}$;*b*$^{+/-}$ mutant brains (Fig. 3A). Multidimensional scaling revealed clustering of the samples on the basis of *csf1r* mutation status, indicating *csf1r*-dependent changes in microglial gene expression (Fig. 3B). Because csf1ra$^{-/-}$;b$^{+/-}$ mutant microglial phenotypes show features of ALSP patient microglia, we compared microglia gene expression of these mutants with that of controls. We identified 1466 genes that were differentially expressed between *csf1ra*$^{-/-}$;*b*$^{+/-}$ mutant and wildtype microglia (Fig. 3C). Interestingly, the normalized expression values of 750 out of the

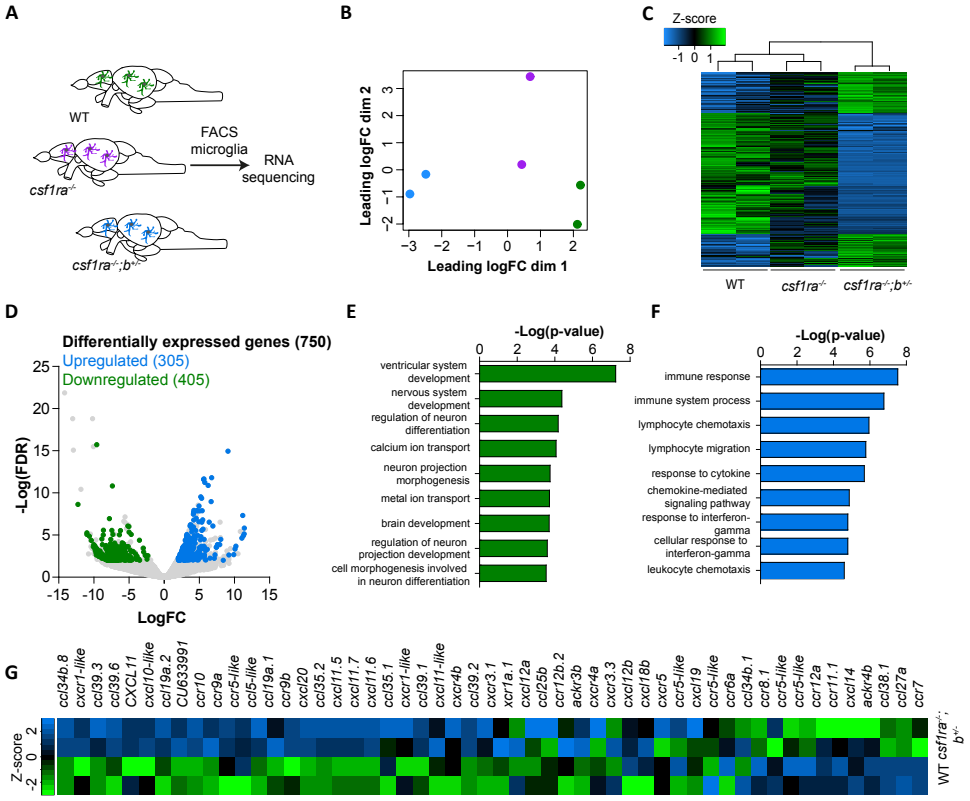

Fig. 3 RNA sequencing reveals increased expression of genes associated with chemotaxis in *csf1ra*$^{-/-}$*;b*$^{+/-}$ mutants.
A Schematic representation of RNA sequencing experiment. Microglia were FACS-sorted from WT (4 brains per sample, 2 samples); csf1ra$^{-/-}$ (4 brains per sample, 2 samples); and csf1ra$^{-/-}$;b$^{+/-}$ (4-5 brains per sample, 2 samples). **B** Multidimensional scaling plot. **C** Heat map of differentially expressed genes between *csf1ra*$^{-/-}$*;b*$^{+/-}$ and WT microglia. **D** Volcano plot of differentially expressed genes (*csf1ra*$^{-/-}$*;b*$^{+/-}$ vs WT), whose expression values in *csf1ra*$^{-/-}$ mutants laybetween those of WT and *csf1ra*$^{-/-}$*;b*$^{+/-}$ mutants. **E, F** Gene ontology analysis was performed on the genes that showed a *csf1r*-dependent increase in expression (**E**) and decrease in expression (**F**). **G** Heat map with expression z-scores for all chemokines and chemokine receptors that are expressed in zebrafish microglia. Genes were differentially expressed with FDR < 0.01 and LogFC > |2|.

1466 differentially expressed genes in *csf1ra*$^{-/-}$ mutant microglia lay in between those of *csf1ra*$^{-/-}$*;b*$^{+/-}$ and wild-type microglia (Fig. 3C,D). This indicates that many genes show *csf1r* dependent changes in expression, and therefore that changes in expression of these 750 genes are probably a primary consequence of *csf1r* deficiency. Gene ontology analysis on genes that showed *csf1r*-dependent changes in expression revealed that whereas downregulated genes were associated with brain and nervous system development, and with regulation of neuronal differentiation (Fig. 3E), upregulated genes were mainly associated with immune response, immune system process, and leukocyte chemotaxis (Fig. 3F). The differentially expressed genes in the gene ontology classes associated with the upregulated genes were mainly chemokines and chemokine receptors (e.g. *cxcl12a*, *ccl25b*, *ccl19a.1, cxcr4b*) (Fig. 3G). In fact, the expression of most chemokines and chemokine receptors in zebrafish microglia was

higher in *csf1ra*$^{-/-}$;*b*$^{+/-}$ mutants than in wild types (Fig. 3G), which may explain the aberrant microglia distribution in csf1ra-/-;b+/- mutants.

To test whether the expressional changes observed in *csf1ra*$^{-/-}$;*b*$^{+/-}$ microglia and brain indicated a general microglia differentiation defect, we investigated whether adult *csf1r*-mutants showed a loss of microglia-specific gene expression or a gain in gene expression associated with immature microglia or macrophages. Only 8 of the 300 most microglia-specific genes in zebrafish (many of which are also included in the mouse and human homeostatic microglia signature e.g. *slco2b1*, *pdgfba*, *scn4bb*) were significantly downregulated in *csf1ra*$^{-/-}$;*b*$^{+/-}$ microglia, suggesting that there is no loss of a homeostatic microglia signature (Fig. 4A,D,E)[35,36,48,49].

Next, we analyzed the expression of 378 zebrafish orthologs for genes that are strongly downregulated during microglia differentiation in the mouse brain to assess whether *csf1r* mutant microglia fail to downregulate genes specific to immature microglia [50]. Expression of only 10 of these 378 genes was increased in *csf1ra*$^{-/-}$;*b*$^{+/-}$ mutant microglia when compared to the expression in wild type microglia (Fig. 4B,F). We also found no evidence for increased expression of genes that discern microglia and macrophages (Fig. 4C)[51]. Additionally, *csf1ra*$^{-/-}$;*b*$^{+/-}$ microglia were still highly ramified and showed no signs of activation (Fig. 4G). This suggests that the *csf1r*-dependent changes in microglial gene expression are largely independent of differentiation status. Together, these data imply that the changes in the expression of genes involved in chemotaxis and cell migration in *csf1r* mutants are a specific consequence of *csf1r* deficiency and not of a global differentiation defect.

The damage-induced proliferative response of *csf1r* mutant microglia is delayed

Microglia respond quickly to damage by migration and proliferation, and Csf1r has been linked to this proliferative response of microglia[52,53]. Therefore, to assess whether proliferation defects could be linked to aberrant microglia localization, and possibly to microglia migration, we used our previously established neuronal ablation model in adult zebrafish. In this model, metronidazole (MTZ) treatment in zebrafish with brain-specific transgenic expression of nitroreductase (NTR) results in neuronal cell death[5,54]. We have shown previously that increasing the local demand for microglia by inducing neuronal cell death causes a strong local proliferative response by microglia[35]. To investigate whether microglia proliferation depends on *csf1r* dosage, we used PCNA as a cell-proliferation marker to assess microglia proliferation upon induction of neuronal ablation. One day after treatment, control NTR transgenic larvae showed the fraction of PCNA+ microglia had increased locally, with a corresponding increase in microglia numbers (Fig. 5A). In contrast, *csf1r* mutant microglia had not yet increased significantly in the fraction of PCNA+ microglia (Fig. 5A). Intriguingly, at 2 days post treatment, the fractions of PCNA+ microglia were similar in control and *csf1r* mutants (Fig. 5B). This showed that although *csf1ra*$^{-/-}$ and *csf1ra*$^{-/-}$;*b*$^{+/-}$ mutant microglia were able to mount a proliferative response, the proliferative response was delayed. At both 1 and 2 days post-treatment, however, the relative increase in microglia numbers, was much stronger in *csf1r* mutants than in controls. In fact, while the relative increase in control microglia numbers was proportional to the PCNA+ microglia fraction, the magnitude of the increase in the number of in *csf1r* mutant microglia was larger than the PCNA+ microglia fraction and therefore cannot be explained by proliferation alone. Thus, in *csf1r* mutants, microglia proliferation in response to neuronal death was delayed.

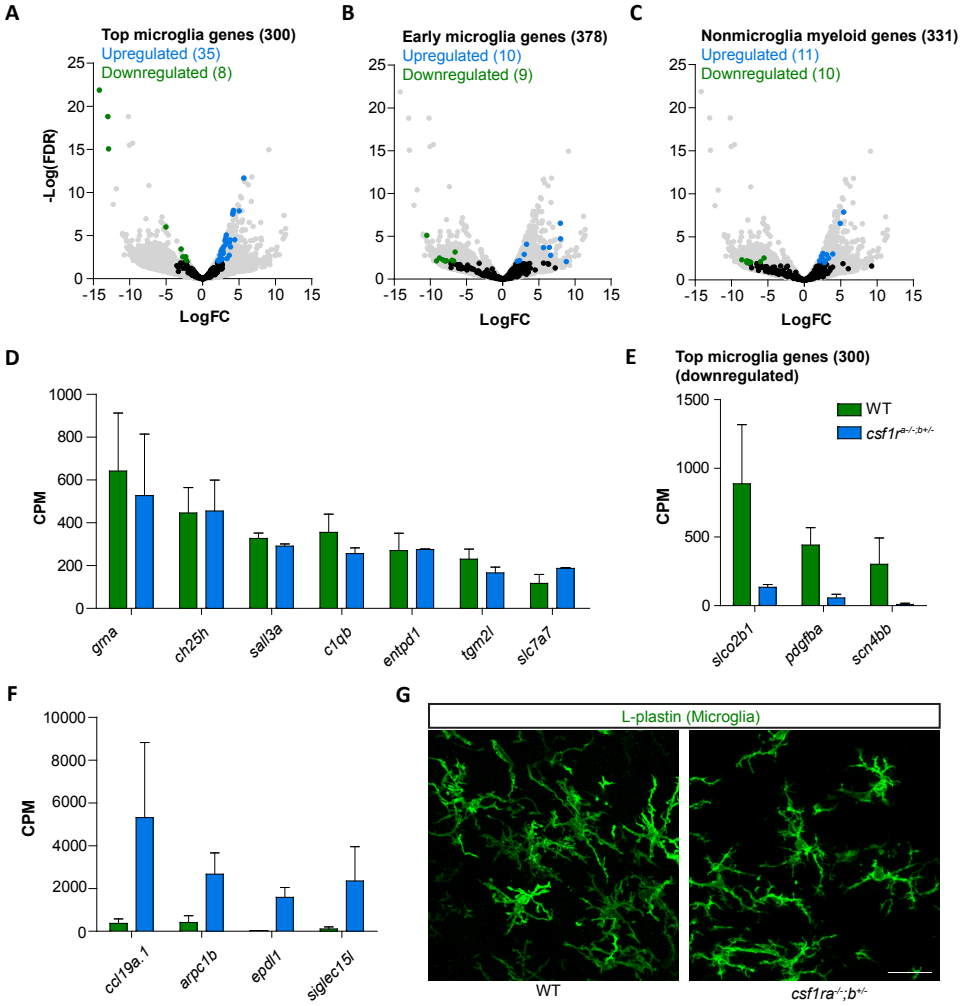

Fig. 4 Differential gene expression of *csf1r*-deficient microglia shows normal microglia differentiation. **A** Volcano plot showing expressional changes of the 300 most highly expressed microglia-specific genes in *csf1ra*$^{-/-}$*;b*$^{+/-}$ mutant microglia[35]. **B** Volcano plot showing the expressional changes in *csf1ra*$^{-/-}$*;b*$^{+/-}$ mutant microglia of normally downregulated genes during differentiation[50] and of genes normally expressed in other macrophages in the CNS[51]. **C,D** Expression values of zebrafish microglia-specific genes. **E** Expression values of downregulated microglia-specific genes. **F** Representative images of microglia (5-month-old fish) labeled with an antibody against L-plastin. CPM = Counts per Million. Scale bar = 20 μm.

Nevertheless, as numbers still increased it seems that potential initial proliferation deficiencies were compensated through microglia recruitment. Therefore, the aberrant distribution of microglia upon *csf1r* deficiency may be a compensatory mechanism intended to meet the brain's local demand for microglia.

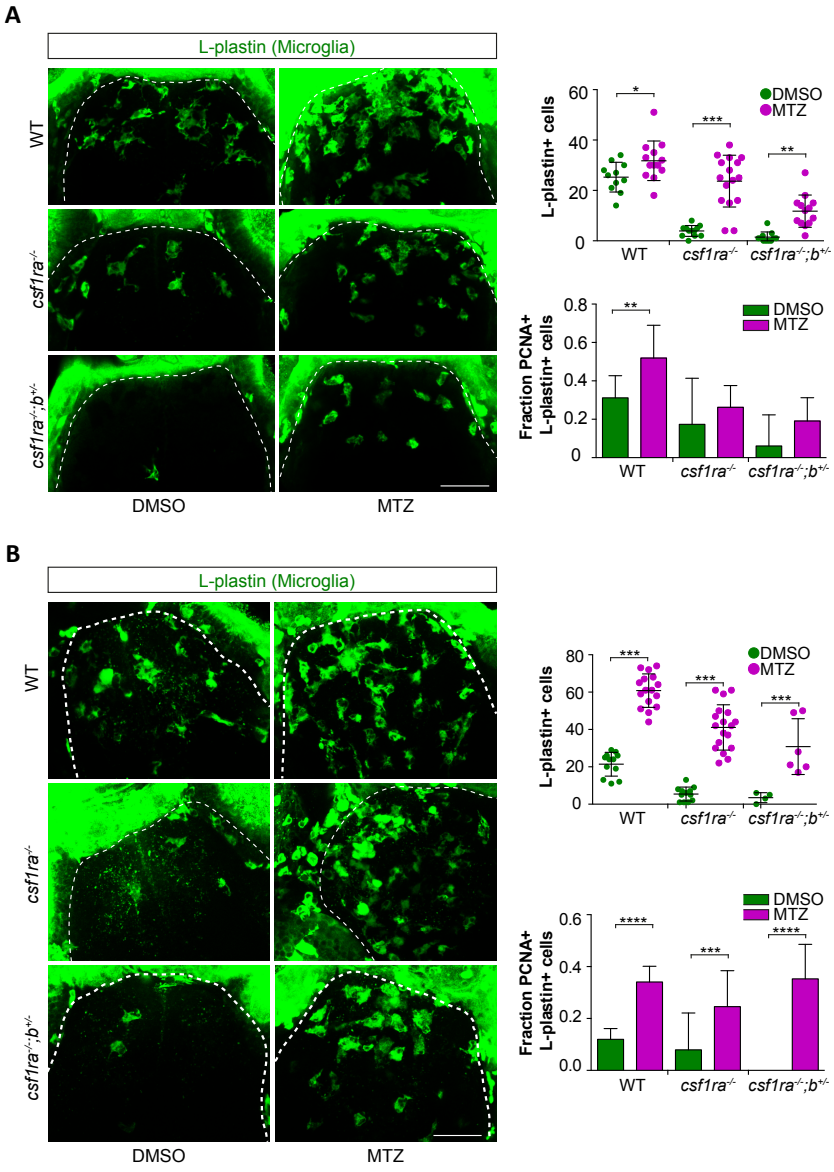

Fig. 5 Response to neuronal cell death of Csf1r mutant microglia dependents more on recruitment than on proliferation .

We used our previously described conditional neuronal ablation model[5,54], in which treatment with metronidazole (MTZ) leads to selective ablation of neurons with transgenic expression of nitroreductase (the *nsfB* gene encoding nitroreductase, NTR). WT, *csf1ra*$^{-/-}$ and *csf1ra*$^{-/-}$;*b*$^{+/-}$ larvae were treated with MTZ at 5 dpf for 16 hours and fixed for immunohistochemistry (whole mount) at 6 dpf (**A**) and 7dpf (**B**). Immunostaining was performed for dividing (PCNA+) microglia (L-plastin+), and the entire forebrain was imaged and quantified. Scale bar = 40 μm (**A, B**). Error bars represent the standard deviation. *p < 0.05, **p < 0.01, ***p < 0.001 (one-way ANOVA, Bonferroni multiple testing correction).

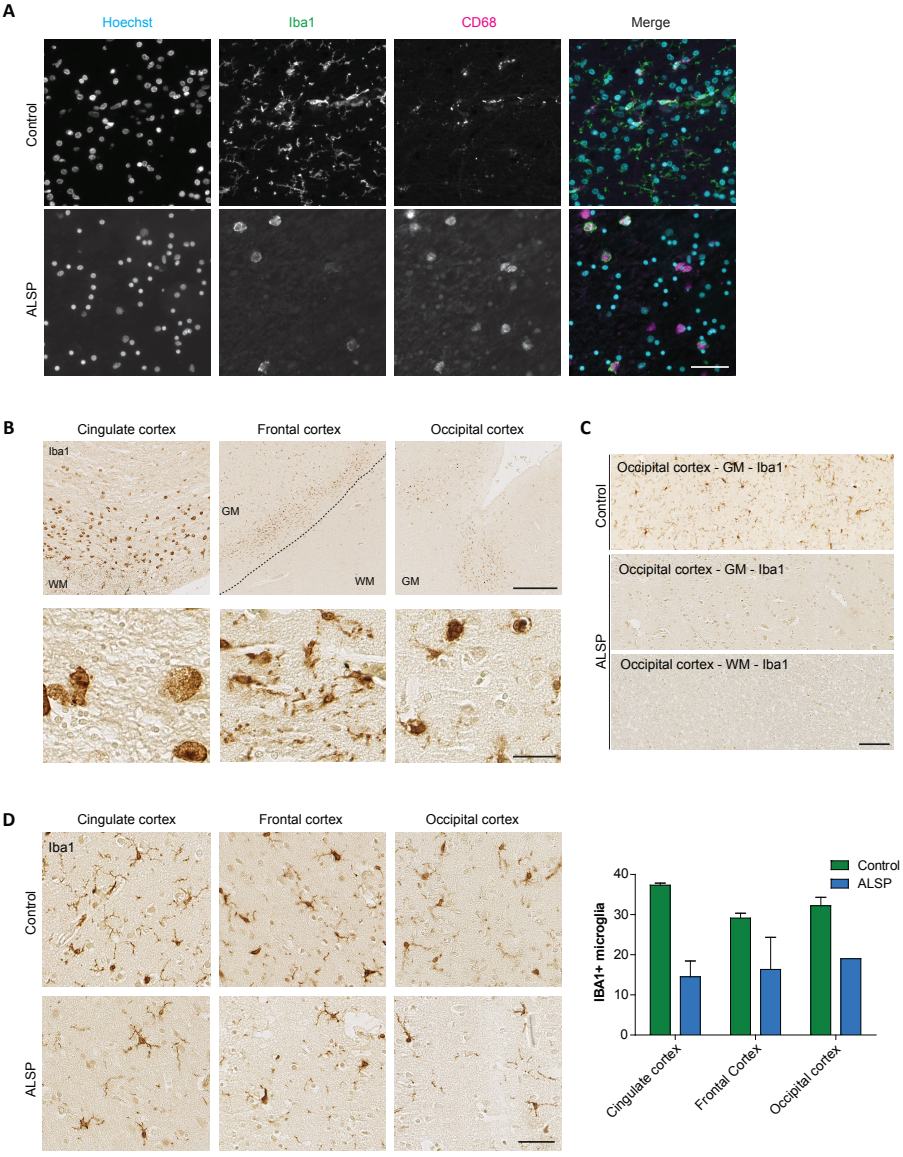

Fig. 6 ALSP patient brains show widespread microglia depletion. IBA1 and CD68 staining were used to characterize microglia in white matter and gray matter of two control donors and two ALSP patients. **A** IBA1 and CD68 antibody labeling of ALSP patient and control tissue. **B** Clusters of IBA1+ microglia are apparent at the borders between the gray matter and the white matter. **C** Severe depletion of IBA1+ microglia in the gray and white matter of the occipital cortex. **D** The ramified morphology of microglia in gray-matter areas of ALSP patients was similar to that in controls. Quantification of microglia numbers in gray matter areas of the cingulate cortex, frontal cortex, and occipital cortex which showed a homogeneous distribution of ramified microglia (3 gray matter areas per brain region per patient). **D** Scale bar = 50 μm **(A)**, 500 μm (**B**, low magnification), 30 μm (**B**, high magnification), 100 μm(**C**), 50 μm (**D**) . WM = white matter, GM = gray matter.

Severe microglia depletion in gray and white matter of postmortem ALSP patient brains

It has been reported that degenerated white matter in the brains of ALSP patients contains many CD68+ myeloid cells and reduced numbers of IBA1-positive microglia[14,15,55,56].We wanted to investigate whether altered microglia density and distribution, as we identified in the zebrafish would recur in the non-degenerated brain tissue of ALSP patients. By immunohistochemistry, we therefore analyzed microglia morphology, distribution and density in gray matter, normal-appearing white matter (NAWM) (occipital lobe); and degenerated white matter (middle frontal gyrus and cingulate gyrus) of two ALSP patients and age-matched controls (Fig. S3a). As in previous studies, we observed numerous HLA-DR+ and CD68+ cells in degenerated white matter, whereas IBA1+ cells were almost completely absent (Fig. 6A, S3B,C). Most IBA1+ microglia still present in the degenerated white matter appeared in clusters of ~10-100 cells. Interestingly, with the exception of sparse microglia clusters, microglia in the NAWM and gray matter were also severely depleted (Fig. 6B,C). Many of these IBA1+ microglia clusters were located at the border between the gray and the white matter (Fig. 6B). Whereas the few IBA1+ cells present in the white matter looked like foam cells, microglia in the gray matter and NAWM either looked activated or had a normal ramified morphology (Fig. 6B). In all the brain areas examined we also observed areas of gray matter in which IBA1+ microglia had a normal distribution and a ramified morphology. However, the density of these microglia was lower in ALSP patient brain sections than in controls (Fig 6D). This is reminiscent of the pathology observed in our zebrafish experiments, where we found regional differences in microglia density in unaffected brain tissue (Fig. 6B,D). The loss of IBA1+ microglia, the aberrant microglia distribution and altered morphology in microglia clusters—not only in the gray matter, but also in NAWM—indicates that microglial changes could precede white matter degeneration.

Discussion

Although mutations in genes that are particularly important for the microglia can cause severe brain disorders, it is still unclear whether pathogenesis involves a gain or loss of specific microglia activities. Here, we used the zebrafish to investigate the impact of a gradual reduction in functional *csf1r* alleles on microglia numbers, microglia differentiation status and their response to tissue damage. We found that *Csf1r* haploinsufficiency was correlated with a lower density of microglia, altered microglia distribution and local microglia depletion. Loss of three *csf1r* alleles did not severely impede the proliferative and phagocytic response of microglia to dying neurons, nor did it affect the homeostatic microglia signature. Instead, in response to increased phagocytic demand, *csf1r* mutant microglia increased their numbers locally through recruitment rather than proliferation. Accordingly, the expression of migration and chemotaxis genes in *csf1r* mutants was also increased. We also showed that, in the absence of extensive white matter degeneration, CSF1R haploinsufficiency results in widespread depletion and aberrant distribution of IBA1+ microglia in humans. These findings support the presence of a disease mechanism in which CSF1R haploinsufficiency reduces microglia density, causes microglial relocation, and results in depletion of functional microglia. This indicates that loss of microglia may be an early pathogenic event in ALSP patients.

CSF1R coding sequence and function are well conserved across species. CSF1R-deficient zebrafish, rodents and most likely humans lack microglia, are osteopetrotic and occasionally show cerebral hemorrhages (data not shown)[21,57]. Our data indicate that *csf1r* haploinsufficiency leads to a local loss of microglia, possibly through the maldistribution of microglia. This is similar to the aberrant distribution of microglia and widespread loss of microglia we observed in the NAWM and gray matter of post mortem ALSP patient brains. Interestingly, in the early stages of ALSP, microglia numbers are higher than in controls and microglia appear activated in specific brain regions. This is reminiscent of the increased microglia density we observed in deep brain regions of *csf1ra*$^{-/-}$;*b*$^{+/-}$ haploinsufficient zebrafish[14]. Similarly, microglia numbers are also higher in some brain regions in heterozygous Csf1r mutant mice than in control animals[58]. Between them, these observations indicate that microglia are lost over time.

Although microglia are efficient phagocytes that clear dead cells, dysfunctional synapses and myelin[6,59-61], the accumulation of myelin debris in microglia can compromise their phagocytic capacity and can also lead to microglial senescence[6,62,63]. Accumulation of myelin debris may therefore contribute to the progressive loss of functional IBA1+ microglia over the course of the disease. In fact, it was shown in a tuberculosis infection model that, due to reduced *csf1r* signaling, the loss of macrophages was driven by a failure to meet phagocytic demand[26]. Consistent with this idea, the morphology of microglia among clusters in ALSP patients ranged from ramified to completely round and foamy in appearance, due most likely to the accumulation of phagocytized myelin debris in microglia[62]. Simultaneously, it is possible that one functional CSF1R copy is not sufficient to sustain both normal microglia survival and proliferation, as microglia turn over in humans in adulthood[64,65]. Together, this indicates that CSF1R-dependent loss of microglia in ALSP patients may be progressive.

The absence of overt neuropathology or myelin pathology in *csf1r* mutant zebrafish may be related either to the fact that the central nervous system of the zebrafish is smaller or less complex than that of humans, or to the time needed for the pathology to develop in humans. The pathological hallmarks of ALSP are observed mainly in the neocortex, which is unique to mammals and has expanded immensely during evolution, particularly in primates and humans [66-68]. As the neocortex is rich in white matter, it may be more susceptible to pathology than the zebrafish brain, in which there is relatively little white matter[69]. Consistent with this, mutations that result in a relatively mild pathology in mice lead to severe leukodystrophy in humans[58,70]. Additionally, it takes about 30-40 years before ALSP becomes symptomatic, whereas mice and zebrafish live only a few years[13]. Even though the *csf1r* mutant zebrafish brain is relatively unaffected, the direct effects of *csf1r* mutations on microglia as described here were very similar to those in humans. Therefore we argue that the zebrafish is a good model organism for distinguishing between the direct effects of *csf1r* mutations on microglia and the indirect responses of microglia to brain pathology.

Although it is still unknown how long-term depletion of microglia in adulthood would affect brain homeostasis, and how it might cause pathology, white matter degeneration is a hallmark of several other brain disorders classified as microgliopathies. For example, mutations in microglia genes TREM2 and TYROBP both cause Nasu-Hakola disease (NHD), which is also characterized by white matter pathology. Even though the precise pathogenic mechanisms remain elusive, these disorders support the idea that microglia are critical to the maintenance of myelin in adulthood. In fact, it was recently

shown in the adult brain that lower microglia numbers lead to a reduction in the numbers of oligodendrocytes or NG2+/PDGFRA+ oligodendrocyte precursors (OPCs) in many brain regions, including the corpus callosum[29]. We anticipate that a progressive depletion of microglia occurs in ALSP, which could lead to a lower number of myelinating cells in adulthood, and could thereby contribute to ALSP pathogenesis. Additionally, during normal aging, undigested myelin accumulates in microglia and could reduce their phagocytic capacity. As failure to clear myelin debris is a major issue preventing re-myelination, having lower microglia numbers could thereby cause an additional problem in the myelinated white matter[6,71,72]. Both of these consequences of microglia defects could contribute to a pathogenic mechanism that leads to the progressive loss of myelin observed in ALSP.

Although microglia dysfunction directly affect myelinating cells directly, evidence also suggests the involvement of the cerebral vasculature in microgliopathies. For example, functional brain imaging analysis by SPECT showed evidence of hypoperfusion in the frontotemporal cortex of an ALSP patient, which was consistent with possible vascular abnormalities in ALSP[73]. Vascular defects due to microglia dysfunction were also observed in studies of NHD patients and of NHD mouse models[74]. It has been shown that cerebral hypoperfusion, in its turn, can lead to white-matter loss[75-77]. Mechanistically, this could involve defective pericytes, the vascular mural cells that regulate vessel constriction and blood flow, as mice without microglia have diminished PDGFRB+ pericyte numbers[78,79]. The finding that pericyte defects cause white matter dysfunction further supports this possible mechanism[80]. Taken together, this suggests that a contribution to the white matter degeneration observed in ALSP patients is made by vascular alterations and by the potential loss of oligodendrocyte lineage cells as a consequence of microglia dysfunction.

Like tissue macrophages, the microglia influence the development and repair of organs by secreting trophic factors including insulin-like growth factor 1 (IGF1), and by mediating signaling between cells[81-84]. A growth factor that is highly expressed in wild type microglia, and strongly downregulated in *csf1r* haploinsufficient microglia, is platelet-derived growth factor B (PDGFB), which directly affects oligodendrocyte lineage cells and pericytes[51,85]. PDGFB-mediated activation of PDGF receptors on pericytes and on OPCs induces migration and proliferation of pericytes and OPCs[86]. Local depletion of microglia could lead to a failure to provide such trophic factors, which could contribute to ALSP pathogenesis by affecting the capacity to form new oligodendrocytes or the pericyte coverage of the blood brain barrier.

In conclusion, the greatest effect of CSF1R haploinsufficiency seems to be a general reduction in microglia density in addition to large areas completely devoid of microglia. The partial or complete lack of microglia occurs in normal appearing gray and white matter, which suggests that loss of microglia may eventually result in ALSP pathology. Our gene expression data in an allelic series of *csf1r*-deficient microglia and brains therefore provide an opportunity to further delineate not only the function of *csf1r* in microglia, but also the consequences for the brain. Elucidating these is crucial to a more comprehensive understanding of the physiological functions of microglia and microglia-dependent disease mechanisms. Several studies have shown that pharmacological inhibition of CSF1R causes microglia depletion and, in mouse models, that it ameliorates neurodegenerative disease-like symptoms by depleting microglia or diminishing their proliferation and activation[53,87-89]. As microglia depletion may underlie

and contribute to the development and progression of ALSP, this raises the question of whether long-term inhibition of CSF1R in neurodegenerative diseases like Alzheimer's disease is a viable treatment option[88,89]. This warrants further studies to determine how the brain is affected by loss of microglia interactions and microglia-derived factors, and to devise ways of promoting the supply of functional microglia.

Acknowledgements/Funding

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Materials and methods

Animals

The following transgenic zebrafish lines were used: Wild type AB, Tg(mpeg1:EGFP) fish expressing GFP under the control of the mpeg1 promotor[90], Tg(Neuro-Gal4, UAS:nsfB-mCherry, mpeg1:EGFP) with neuronal specific nsfB expression encoding nitroreductase (NTR)[54], Csf1ra$^{j4e1/j4e1}$ with a V614M substitution in the first kinase domain[38]. Csf1ra mutants were also crossed in with the transgenic lines specified above. The *csf1rb* deletion mutant was created by TALEN-mediated genome editing[91]. The TALEN arms targeted exon 3 (Fig. S1) of the *csf1r*b gene, resulting in a 4bp deletion and premature stop codon. Animal experiments were approved by the Animal Experimentation Committee at Erasmus MC, Rotterdam.

Vital dye labeling

For all experiments in larvae, embryos were kept in E3 medium containing 0.003% 1-phenyl 2-thiourea (PTU) (Sigma-Aldrich) from 22hpf onwards. To assess microglia numbers, 5 or 6 dpf larvae were treated with 2.5 μg/ml neutral red in E3+PTU medium at 28℃ for two hours. After two hours, larvae were incubated in E3+PTU for at least 30 minutes before imaging. For imaging, larvae were anesthetized with 0.016% MS-222 (Sigma-Aldrich) and embedded in 1.8% low melting point (LMP) agarose (VWR BDH Prolabo) with the dorsal side facing upwards. Neutral red images were taken using a Leica M165FC stereo microscope.

Induction neuronal cell death

For neuronal ablation, neuro-NTR transgenic zebrafish were used as described previously[54]. Larvae were treated with 2 mM MTZ at 28℃ for 16 hours. MTZ was washed away with E3+PTU medium. Larvae were euthanized and fixed at different times after the start of treatment, at 1 day post treatment (20h) and 2 days post treatment (48h).

Immunofluorescence staining

Whole mount larvae This was usually done as described previously[92]. In brief, larvae were fixed in 4% PFA at 4℃ overnight, dehydrated to 100% MeOH, and kept at -20℃ for at least two days. They were then rehydrated to PBSTw (PBS + 0,1% Tween). Antigens were retrieved by incubating the larvae in 150 mM Tris-HCl pH=9.0 for 5 min, followed by incubation at 70℃ for 15 min. Afterwards, larvae were washed with PBSTw (2x 10 min) and dH_2O (2x 5 min) and incubated in aceton at -20℃ for 20 min. This was followed by several washing steps in PBSTw (6x 5 min); dH2O (2x 5 min); and PBSTw (2x 5 min). The larvae were then incubated in blocking buffer (10% goat serum, 1% Triton X-100, 1% BSA in PBSTw) at 4℃ for 3h. Primary-antibody labeling (1st antibody in 1% goat serum, 0.8% Triton X-100, 1% BSA in PBSTw) was done at 4℃ for three days. Larvae were washed in PBS-TS (10% goat serum, 1% Triton X-100 in PBS) (3x 1h); PBST (1% Triton X-100 in PBS) (2x 10 min); and PBS-TS (2x 1h). The larvae were then incubated with the secondary antibody and Hoechst at 4℃ for 2.5 days. Before imaging, the larvae were washed with PBS-TS (3x 1h); and PBSTw (2x 1h). Primary antibodies: PCNA (1:250, Dako); and L-plastin (1:500; gift from Yi Feng, University of Edinburgh). Secondary antibodies: DyLight alexa 488 (1:500); and DyLight alexa 647 (1:500). Hoechst was used for nuclear staining. Images were taken with a Leica SP5 confocal microscope using a 20x water dipping lens (NA = 1.0). Total microglia numbers

were quantified for the entire brain. The microglia response to neuronal death was quantified in the larval forebrain.

Adult brain sections Immunostaining on adult brain slices was performed as described previously[35]. In short, fish were euthanized in ice water, after which the skull (containing the brain) was fixed in 4% PFA at 4℃ overnight. Subsequently, the brains were carefully removed and dehydrated with a 25%, 50%, 75%, 100% MeOH series and kept at -20℃ for at least 12 hours. After rehydration, brains were embedded in 4% w/v low melting point agarose in PBS and cut into 80 μm horizontal sections using a Microm HM 650V vibratome (ThermoFisher Scientific). Immunostainings on free-floating sections were performed as described. The sections were incubated in blocking buffer (10 % goat serum, 0.5% Triton-X100 in PBS) at room temperature for 75 min, followed by incubation with the primary antibody in blocking buffer at 4℃ overnight. The sections were thoroughly washed with PBST (0.5% Triton X-100) before incubation with the secondary antibody and Hoechst at 4℃ overnight. The brain slices were then washed with PBST (5 x 30-60 min) and mounted on microscope slides using vectashield mounting medium H1000 (Vector Laboratories). Primary antibodies: L-plastin (1:1000; gift from Yi Feng, University of Edinburgh); ClaudinK (1:1000; gift from Thomas and Catherina Becker, Edinburgh); and Sox10 (1:500, Genetex). Secondary antibodies used were DyLight alexa 488 and DyLight alexa 647 (1:500, ThermoFisher Scientific, Waltham, US), and alexa 594 (1:250, Invitrogen, Waltham, US). Imaging was done with a Zeiss LSM700 confocal microscope using a 20x lens (NA = 0.75).

Microglia isolation

Microglia were isolated as described[35]. Briefly, *mpeg1*:GFP expressing zebrafish were euthanized in ice water. The heads were severed behind the gills, and the lower jaw, gills and eyes were removed. The brains (4-5 per sample) were taken out of the skull and cut using scalpels. This was followed by dissociation in 0.25% trypsin and 0.1% EDTA in PBS for 2 hours at 4℃, while resuspending regularly. Upon complete dissociation of the brain, trypsin was inactivated by adding 1/6 volume of a stop solution (6 mM $CaCl_2$ in PBS). The cell was collected in a 22% Percoll solution after being run through a 70 μm cell strainer, and ice-cold PBS was placed on top of the cell suspension and centrifuged at 1000 x g at 4℃ for 45 minutes. The remaining cell pellet was resuspended in suspension solution (high-glucose DMEM without phenol red, 0.8 mM $CaCl_2$). The cell suspension was transferred to FACS tubes with 35 μm cell strainer caps and FAC-sorted using a FACSaria III cell sorter (BD biosciences, New Jersey, US). Dapi was added to exclude dead cells.

RNAseq library synthesis and bioinformatic analysis

FAC-sorted cells were collected and lysed in RNase-free water containing 0.2 % v/v Triton X-100 and RNase inhibitor. PolyA+ RNA was reverse transcribed using an oligo(dT) primer. Template switching by reverse transcriptase was achieved by using an LNA containing TSO oligo. The reverse-transcribed cDNA was pre-amplified with primers for 18 cycles followed by clean-up. Tagmentation was performed on 500 pg of the pre-amplified cDNA with Tn5 followed by gap repair. The tagmented library was extended with Illumina adaptor sequences by PCR for 14 cycles and then purified. The resulting sequencing library was measured on Bioanalyzer and equimolar samples were loaded onto a flowcell and sequenced according to the Illumina TruSeq v3 protocol on the HiSeq2500 with a single-read 50 bp and dual 9 bp indices. The sequencing reads

were mapped against the GRCz10 zebrafish genome using the HiSat2 aligner[93]. To quantify the aligned and filtered data, the Bioconductor package Genomic Ranges was used[94]. Differential gene expression analysis was performed using the Bioconductor package edgeR[95]. To assess the differentiation status of *csf1r* mutant microglia, the differential gene expression list was compared with previously published microglia expression profiles obtained in zebrafish and mice[35,50,51]. We used the Bioconductor tool Biomart to find high-confidence zebrafish orthologs for the genes from studies performed in mice. For gene ontology analysis the Bioconductor package goseq was used[96].

Electron microscopy

Electron microscopy was performed largely as described previously[54]. Briefly, 4 to 5-month-old zebrafish were euthanized in ice water. The brains were carefully removed and incubated in Zamboni's fixative (4% PFA, 2% glutaraldehyde, 0.2% picric acid, 0.1M cacodylate, pH = 7.4) at 4℃ overnight. Brains were washed in cacodylate (3x5 min) and postfixed in a solution containing 1% osmium tetroxide (OsO_4) and 1,5% potassiumferrocyanide ($K_4Fe(CN)_6$) on ice for 2 hours. The brains were then washed in milliQ water (3x5 min) and dehydrated in series of 30%, 50%, 70% ethanol (10 min each), followed by 3 x 20 min incubation in absolute ethanol. Brains were rinsed in acetone, followed by incubation in a 1:1 EPON:acetone solution overnight. Next, the brains were incubated in pure EPON for 1 hour. New EPON was added followed by incubation in EPON for 2 hours. Samples were put at 200 mbar vacuum for 30 min at 37 ℃. Polymerization took place at 58℃ for 3 days. Ultra-thin sections were subsequently cut, collected on formvar-coated single-slot grids, and stained with a 1% aqueous uranyl acetate solution for 20 min and subsequently for 1 min with lead citrate. Photographs were obtained using a JEOL 1010 electron microscope.

Human brain tissue

Human brain tissue samples were obtained from the Netherlands Brain Bank (www.brainbank.nl). All patients and controls, or their next of kin, had given informed consent for autopsy and the use of brain tissue for research purposes. Relevant clinical information was retrieved from the medical records and is summarized in **Table S1**. We obtained paraffin-embedded tissue blocks of cingulate gyrus, frontal cortex and occipital lobe from 2 ALSP patients and 2 age-matched controls without neurological disease.

Luxol fast blue staining

Paraffin sections seven ìm in thickness were collected on glass slides (Superfrost Plus, VWR international, Leuven, Belgium) and dried at 37℃. Sections were deparaffinized and hydrated to 95% ethanol and incubated in Luxol fast blue solution (0.1% w/v luxol fast blue, Sigma-Aldrich, in 95% ethanol) overnight at 55℃. The sections were then quickly rinsed in 95% ethanol and incubated in lithium carbonate solution (0.05% w/v, Sigma-Aldrich, in distilled H_2O) for 30 s. Next, the samples were rinsed in 70% ethanol and subsequently in ddH2O. The sections were dehydrated to 100% ethanol and mounted with Entallan (Merck). Whole slides were digitalized using a NanoZoomer digital slide scanner (Hamamatsu), and the slides were analyzed using Hamamatsu software NDPview2.

Immunohistochemistry

Paraffin sections seven ìm in thickness were collected on Superfrost Plus glass slides (VWR international, Leuven, Belgium) and dried at 37℃. Tissue sections were characterized for the presence of microglia by staining for IBA1 as previously described [97]. In brief, tissue sections were deparaffinized and rehydrated to distilled water. Endogenous peroxidase was quenched in 0.3% H_2O_2 (Merck, Darmstadt, Germany) in phosphate-buffered saline (PBS), and antigen was retrieved by heating the slides in citrate buffer (10 mM; pH 6.0). The sections were then incubated with normal horse serum blocking buffer (TBS supplemented with 2% horse serum, 1% bovine serum albumin (Sigma-Aldrich, St. Louis, USA); 0.1% Triton X-100 and 0.05% Tween (Merck)) for 30 min at RT. Thereafter, sections were incubated with rabbit anti-human IBA1 antibodies (1:1000 TBS-BSA 1%; WAKO Chemicals, Richmond, USA) overnight at 4℃. After rinsing, the sections were incubated with biotinylated goat anti-rabbit antibodies (1:400 in TBS-BSA 1%; Vector Laboratories, Burtingame, USA) for 1h at RT; this was followed by incubation with the avidin-biotin complex (1:800 in TBS; Vector Laboratories) for 45' at RT, The sections were then developed with 3,3'-diaminobenzidine (DAB; 0.05 mg/ml, Sigma-Aldrich). After washing in distilled water, slides were dehydrated and embedded in Entallan (Merck). In between all steps, sections were extensively rinsed in TBS. Whole slides were digitalized using a NanoZoomer digital slide scanner (Hamamatsu) and the slides were analyzed using Hamamatsu software NDPview2. The numbers of $IBA1^+$ microglia were quantified in 3 areas of 0.5x0.3 mm per brain region per patient.

For the CD68-IBA1 immunofluorescent double-staining, tissue sections were deparaffinized and rehydrated to distilled water. Antigen retrieval was performed by heating the slides in citrate buffer (10 mM; pH 6.0). Sections were then incubated with NHS blocking buffer for 30 min at RT. Thereafter, sections were incubated with rabbit anti-human IBA1 (1:1000 in TBS-BSA 1%; WAKO Chemicals, Richmond, USA) and mouse anti-human CD68 (1:200 in TBS-BSA 1%; clone KP-1) antibodies overnight at 4℃. After rinsing, the sections were incubated with secondary anti-rabbit-Cy3 and anti-mouse Alexa-488 antibodies (both 1:800 in TBS-BSA 1%; Jackson Laboratories) and Hoechst (1:1000 in TBS-BSA 1%) for 1h at RT. Autofluorescence was blocked by incubating the slides in Sudan Black solution for 7 min at RT. After washing for 1 min in 70% ethanol and extensively in TBS, the slides were embedded in Mowiol. In between all steps, sections were extensively rinsed in TBS.

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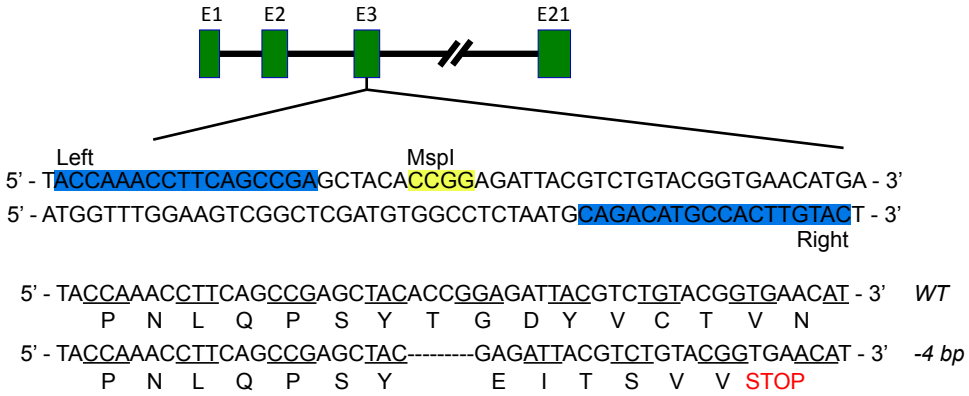
csf1rb



Genotyping
FW primer: CTTGCTGACAAATCCAGCAC
RV primer: AAACTAAATGCGGCCATACG

Fig S1
Schematic representation of *csf1rb* mutation. TALEN sequences are highlighted in blue. Restriction site for genotyping is highlighted in yellow. E = exon.

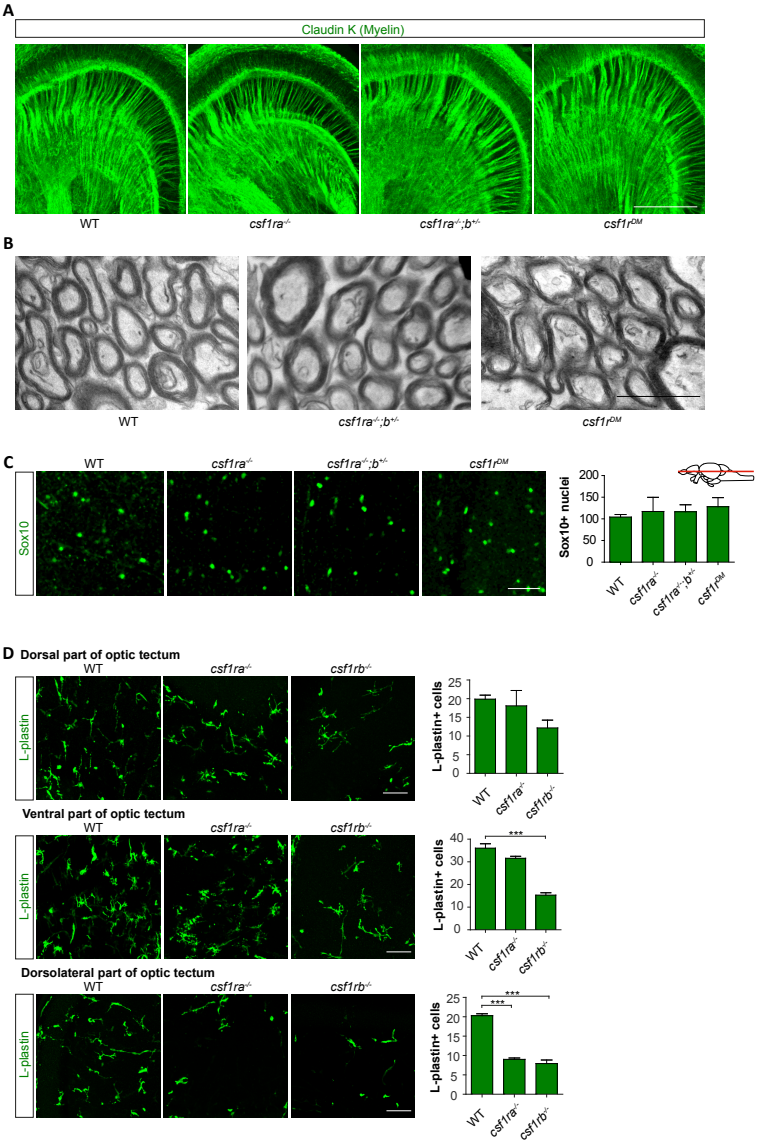


Fig S2

A Representative images of 5-month-old WT (n=3), *csf1ra*$^{-/-}$ (n=3), *csf1ra*$^{-/-}$;*b*$^{+/-}$ (n=3) and *csf1r*^{DM} (n=3) zebrafish brain sections stained with an antibody against ClaudinK (myelin). **B** Electron microscopy was performed on the brains of 5-month-old WT (n=3), *csf1ra*$^{-/-}$;*b*$^{+/-}$ (n=3) and *csf1r*^{DM} (n=3). Representative images of myelin in WT, *csf1ra*$^{-/-}$;*b*$^{+/-}$ and *csf1r*^{DM}. **C** Representative images of Sox10 (oligodendrocyte lineage cells) antibody labeling in deep brain regions of 5-month-old zebrafish. **D** Representative images of microglia in adult WT, *csf1ra*$^{-/-}$ and *csf1rb*$^{-/-}$ zebrafish. Quantification of microglia was done in 3 brain areas (3.1 x 10^{-3} mm^3) per region per brain. **B** Scale bars represent 200 μm (**A**), 1 μm (**B**), 40 μm (**C**), 50 μm (**D**).

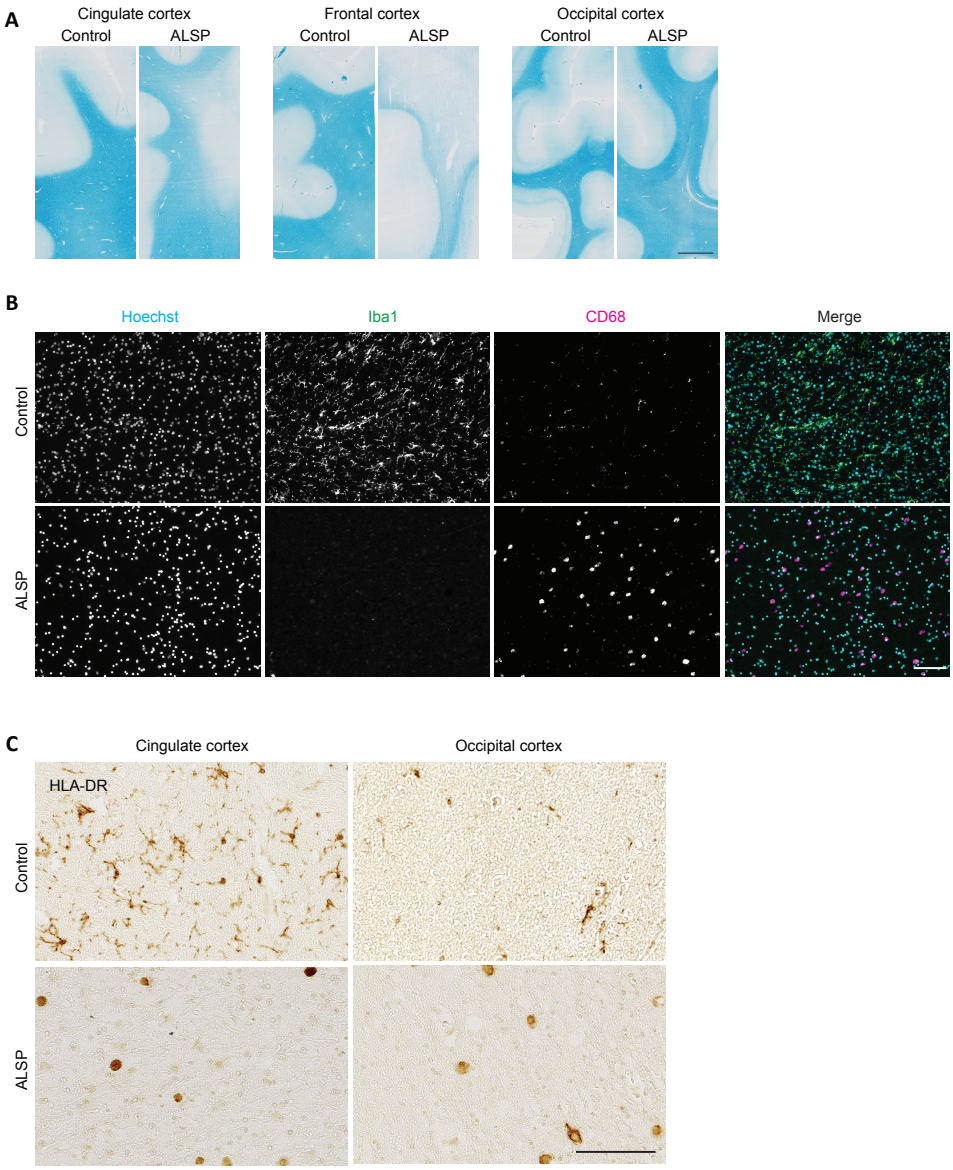


Fig S3
A Luxol fast blue staining in the cortex of the frontal and occipital lobe and the cingulate gyrus of ALSP patient and age-matched control. **B** Co-staining with IBA1 and CD68 in brain tissue of ALSP patient and age-matched control. **C** HLA-DR staining in the cortex of the occipital lobe and cingulate gyrus of ALSP patient and age-matched control. Scale bars represent 2 mm (**A**), 100 μm (**B,C**).

Patient	Age (y)	Gender	PM delay (h)	CSF1R Mutation	References
ALSP1	49	m	8:30	p.I794T	[98]
ALSP2	52	m	5:10	p.L630R	[98]
CTRL1	49	m	6:15	-	
CTRL2	53	m	14:25	-	

Table S1. Characteristics of the ALSP patients and controls

Chapter 7

Microglial activation by genetically targeted conditional neuronal ablation in the zebrafish

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Abstract

In neurodegenerative diseases activation of immune cells is thought to play a major role. Microglia are the main immune cells of the central nervous system. When encountering disease related stimuli microglia adopt an activated phenotype that typically includes a rounded morphology. The exact role of microglia or other potentially infiltrating myeloid cells in different brain diseases is not fully understood. In this chapter we present techniques in zebrafish to induce degeneration of neurons, to activate the microglia, and study activation phenotypes by immunohistochemistry and *in vivo* by fluorescence microscopic imaging.

Key words: zebrafish, microglia, macrophages, brain, phagocytosis, neurodegeneration, nitroreductase, live imaging, immunohistochemistry, aging, Alzheimer's disease

Introduction

The immune system plays a major role in many brain diseases, including diseases involving neuronal death. Microglia are brain-resident macrophages that are thought to be involved in the maintenance of brain homeostasis and the defense against pathogens and phagocytosis of dead cells and debris[1-4]. Upon encountering for example pathogens, tissue damage or protein aggregates, microglia adopt a rounded activated phenotype[2]. However, the exact mechanisms regulating microglia function and activation are still unclear. One reason for this lack of knowledge regarding microglia function is the relative inaccessibility of mammals and in particular the mammalian central nervous system (CNS) for *in vivo* studies.

Zebrafish are highly suitable for *in vivo* imaging studies, due to their rapid development and transparency during embryonic and larval stages[5]. Within 5 days after fertilization many organs, such as the heart, central nervous system, and cell types have developed, including innate immune cells and microglia. The zebrafish CNS is highly similar to the mammalian CNS with regard to development, anatomy and cell types. Zebrafish microglia show strong similarities with mammalian microglia in developmental ontogeny and function as they are highly effective in phagocytizing apoptotic neurons[6-9].

As in mammals, primitive yolk sac macrophages colonize the embryonic brain in zebrafish before their differentiation into microglia. Intriguingly, this process was already visualized in great detail almost a decade before this was elegantly shown by mouse lineage tracing studies[9,10].

Here, we present techniques to study microglial activation responses to neurodegeneration using the zebrafish as a model organism. We previously established transgenic brain-specific expression of the bacterial gene *nsfB* encoding the enzyme nitroreductase (NTR) as a specific, dose-dependent and reversible technique to induce neuronal cell death in zebrafish larvae[6,11]. NTR catalyzes the conversion of the prodrug metronidazole (MTZ) into a DNA-crosslinking agent that causes apoptosis in targeted cells. NTR-mediated neuronal ablation allows controlled onset and extent of neurodegeneration. This model helped us to identify a transient activation responses involving increased phagocytosis by microglia and as well as an increase in numbers which is followed by resolution involving apoptosis of microglia by *in vivo* imaging and immunohistochemistry[7]. Since MTZ effectively crosses the blood-brain barrier, this technique can also be applied to adult zebrafish (Oosterhof et al., unpublished data). In this chapter, we describe how to use NTR-mediated neurodegeneration and monitor microglia activation with high resolution *in vivo* and in fixed tissue in larval and adult zebrafish. The system is versatile as expression can be directed to virtually any cell type and therefore these protocols will also be applicable to other neuronal or glial cell types or populations of interest using different promoters.

Materials

Imaging microglial activation in response to neuronal ablation (larvae)

Induction of neuronal cell death

1. Zebrafish: tg(*NeuroG4*-mCherry, UAS-E1b:*nsfB*-mCherry, *Mpeg1*-GFP)[6,7,12-14].
2. Standard zebrafish supplies (28 - 28.5 ℃ incubator, 9 cm Petri dishes, breeding tanks, automated fish circulation system).
3. 60X E3 stock solution, dissolve 34.4 g NaCl, 1.52 g KCl, 5.8 g $CaCl_2$ and 9.8 g $MgSO_4$ in 2 L ddH_2O. Adjust the pH to
4. 2 with NaOH tablets and autoclave.
5. 1 M HEPES stock solution, dissolve 238.30 g HEPES in 1 L ddH_20. Adjust the pH to 7.3 with NaOH tablets and autoclave.
6. Buffered 1x E3 medium, Add 16.6 mL 60x E3 stock solution and 20 mL 1 M HEPES stock solution to 1 L ddH_2O.
7. 3%1-phenyl 2-thiourea (PTU) stock solution (1000x), dissolve 300 mg PTU in 10mL dimethylsulfoxide (DMSO).
8. 1M metronidazole (MTZ) stock solution, dissolve 1.71 g MTZ in 10 mL DMSO (**Note 1**).
9. 6-well or 12-well plate
10. Fluorescence dissection microscope (e.g. Leica M165V)

Live imaging and immunohistochemistry of microglial activation (Note 2)

1. 10x stock tricaine methanesulfonate solution (MS222; Sigma): add 0.1 g MS222 to 1 L buffered 1 x E3 medium and adjust the pH to 7.3 with NaOH tablets (**Note 3**). Final concentration = 0.16%.
2. 10x PBS stock solution: dissolve 80 g NaCl, 2 g KCl, 14.4 g Na_2HPO_4, 2.4 g KH_2PO_4 in 800 mL ddH_2O. Adjust pH to 7.4. Adjust volume to 1 L with additional distilled H_2O. Sterilize by autoclaving.
3. 1.8% low melting point (LMP) agarose (BDH Prolabo): dissolve 1.8 g of LMP agarose in 100 mL buffered 1x E3 medium. Heat in a microwave until it is completely dissolved (**Note 4**).
4. 4% Paraformaldehyde (PFA) in PBS
5. 1x PBS-T (0.4% triton X-100): add 2 mL Triton X-100 (Sigma) to 500mL 1x PBS
6. MeOH series in PBS (100%, 75%, 50%, 25%)
7. 10 mg/mL proteinase K stock solution (= 1000X solution)
8. Blocking solution: 1% BSA (0.01 g/mL), 1 % DMSO (10 μL/mL) in PBS-T
9. 1st antibody solution: 5% BSA (0.05 g/mL) in PBS-T
10. 2nd antibody solution: 2% BSA (0.02 g/mL) in PBS-T
11. Plastic Pasteur pipettes
12. Glass bottom imaging dishes (MatTek)
13. 1.5 mL microcentrifuge tubes
14. Tools to position larvae (e.g. gel loading tips)
15. Inverted confocal microscope (**Note 5**)

Imaging microglial activation in response to neuronal ablation (adult zebrafish)

Induction of neuronal cell death in the adult zebrafish brain

1. Adult zebrafish: tg(*NeuroG4*-mCherry, UAS:*nsfB*-mCherry, *mpeg1*-GFP)[6,7,12-14].
2. Standard zebrafish supplies (28 - 28.5 ℃ incubator, Petri dishes, breeding tanks, circulation system).
3. Dimethyl sulfoxide (DMSO)
4. Metronidazole (MTZ)

Isolation of fixed complete zebrafish brain

1. Ice water
2. Dissection microscope (e.g. Olympus SZX16, Leica M165V)
3. Scalpel
4. 2x forceps (Dumont #5)
5. Spring scissors - 8mm blades (F.S.T)
6. 1x PBS solution (pH 7.4)
7. MeOH series in PBS (100%, 75%, 50%, 25%)
8. 4% PFA in PBS
9. 1.5 mL microcentrifuge tubes

Sectioning of zebrafish brains for immunofluorescence staining

1. MeOH series in PBS (100%, 75%, 50%, 25%)
2. 4% Low-melting point agarose in PBS: Place the required amount of PBS in a 50 mL tube, add the required amount of low-melting point agarose and shake briefly until the solution is homogeneous. Place the tube in a beaker containing water and heat it in a microwave until all agarose is dissolved (**Note 6**).
3. 1x PBS solution
4. Plastic Pasteur pipette
5. Embedding base molds
6. Scalpel
7. Low-viscosity cyanoacrylate-based instant adhesive
8. 4% PFA in PBS
9. Vibratome (HM650V; Thermo Scientific)
10. 48-well plate

Immunofluorescence staining of zebrafish brain sections

1. Goat serum (Dako)
2. 1x PBS-T (0.5% triton X-100): add 2.5 mL Triton X-100 (Sigma) to 500 mL 1x PBS
3. Blocking buffer: Dissolve 1.5 g bovine serum albumin (BSA) in 50 mL PBST
4. Antibody solution 1: Dissolve 2.5 g BSA in 50 mL PBST
5. 1x PBS
6. Microscope slides
7. Vectashield mounting medium H1000
8. Glass cover slips
9. Parafilm

Methods

Imaging microglial activation in response to neuronal ablation (larvae)

Induction of neurodegeneration in zebrafish larvae

1. Day -1: Cross adult zebrafish tg(NeuroG4-mCherry; UAS:nsfB-mCherry; Mpeg1-GFP).
2. Day 0: Collect the eggs in buffered 1x E3 medium in a 9 cm Petri dish and raise the embryos in a 28-28.5℃ incubator.
3. At 1 day post fertilization (dpf) replace medium by buffered 1x E3 medium containing
4. 003 % PTU (1:1000 dilution from 3% PTU stock solution) (**Note 7)**
5. Screen embryos for bright mCherry signal on a fluorescence dissection microscope. Place the transgenic larvae in a new Petri dish. (**Note 8**).
6. At 3 dpf or later screen the larvae for *Mpeg1*-GFP expression (**Note 9)**.
7. Place the larvae in a 6-well or 12-well plate and replace the larval medium with buffered 1x E3 medium containing 0.003% PTU and 2 mM MTZ to induce neuronal cell ablation. Treatment with 2 mM MTZ for 16 h results in extensive neuronal cell ablation accompanied by microglia activation (**Note 10)**.
8. Wash larvae at least 3x with buffered 1x E3 medium containing 0.003% PTU (**Note 11**).
9. The larvae can be subjected to live imaging or fixation for later staining at the desired time point.

Live imaging zebrafish larvae

1. For live imaging anesthetize the larvae with 0.016% MS-222 in buffered E3 medium and place them in a glass bottom imaging dish (inverted confocal microscope) or in a 6 mm petri dish (upright confocal microscope) and place the dish under a dissection microscope.
2. Place 1.8% low melting point agarose on top of the larvae (**Note 12**).
3. For inverted confocal microscope: Quickly position the larvae upside down with their ‘nose’ facing the glass bottom before agarose solidifies (figure 1A)(**Note 13)**.
4. For upright confocal microscope: Position the larvae with their ‘nose’ touching the surface on top of the LMP agarose drop (figure 1B) (**Note 14**).
5. When the agarose has solidified fill the imaging dish with buffered 1x E3 medium containing 0.016% MS-222 to prevent drying of the sample (**Note 15)**.
6. Continue with imaging. Use appropriate laser wavelength (e.g. 488 nm to detect GFP and 543, 561 or 594 nm to detect mCherry). If possible, it is recommended to use the brightfield channel to include information about the imaging position (figure 2A) (**Note 16**).

Immunohistochemistry larvae

Prior to fixation anesthetize the larvae with 1x MS-222 and once they are anesthetized transfer them to a 1.5 mL micro centrifuge tube using a plastic Pasteur pipette or a cut 1 mL tip. It is recommended to perform all washing and incubation steps on a roller mixer unless stated otherwise (**Note 17**). NB: All steps are performed at room temperature (RT) in volumes of 1 mL unless stated otherwise.

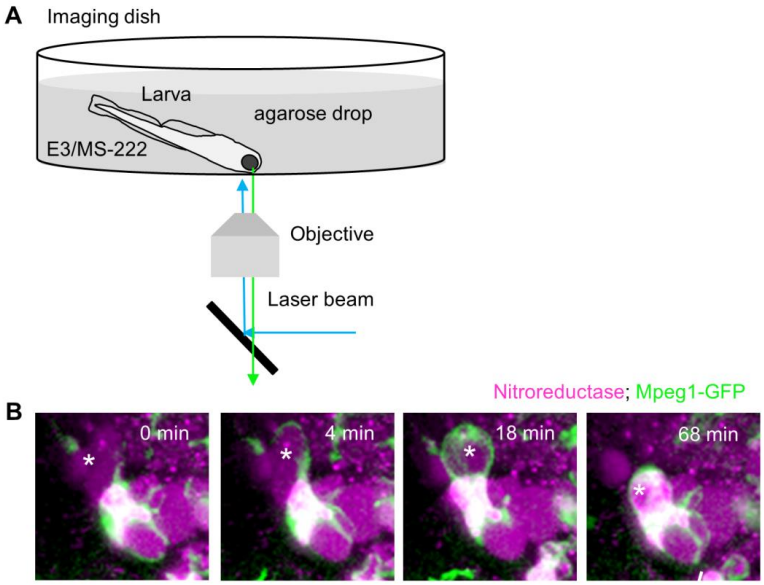

Fig 1. Live imaging in larval zebrafish. **A** Position of larva in imaging dish for imaging with an inverted confocal microscope. **B** *In vivo* stills showing a GFP-expressing microglia (in green) engulfing an apoptotic neuron (in magenta, marked with asterisks). Scale bar = 10 μm

1. Euthanize the larvae with an overdose of MS-222 and transfer the larvae to 1.5 mL microcentrifuge tubes (**Note 18**)
2. Briefly centrifuge the larvae in microcentrifuge tubes to collect them in the bottom of the tube and remove the medium.
3. Add 4% PFA and invert the microcentrifuge tubes five times to expose all the larvae to the PFA solution.
4. Incubate the larvae at 4℃ in 4% PFA for at least 16 h **(Note 19**).
5. Wash the larvae at least twice with PBS-T for 5 min.
6. Perform stepwise dehydration with a methanol (MeOH) series (25%, 50%, 75%, 100%) 5 min per step.
7. Store the larvae at -20℃ for at least 24 h. Larvae can be stored for a few months at -20℃ at this point (**Note 20**).
8. Perform stepwise rehydration with a MeOH series (75%, 50%, 25%, 0%), 5 min per step.
9. Remove the PBST and incubate the larvae in 10 μg/mL proteinase K in PBS-T for 40 min.
10. Remove the 10 μg/mL proteinase K solution and incubate the larvae in 4% PFA for 20 min.
11. Wash larvae at least twice with PBST for at least 5 min.
12. Remove the PBST and incubate the larvae in the blocking solution for 2 h.
13. Remove the blocking solution and incubate the larvae with 200 μL primary antibody solution containing 1:500 rabbit L-plastin (gift from Yi Feng, University of Edinburgh) at 4℃ on a shaker for at least 16 h (alternative incubation times may be required depending on the antibody) **(Note 21**).

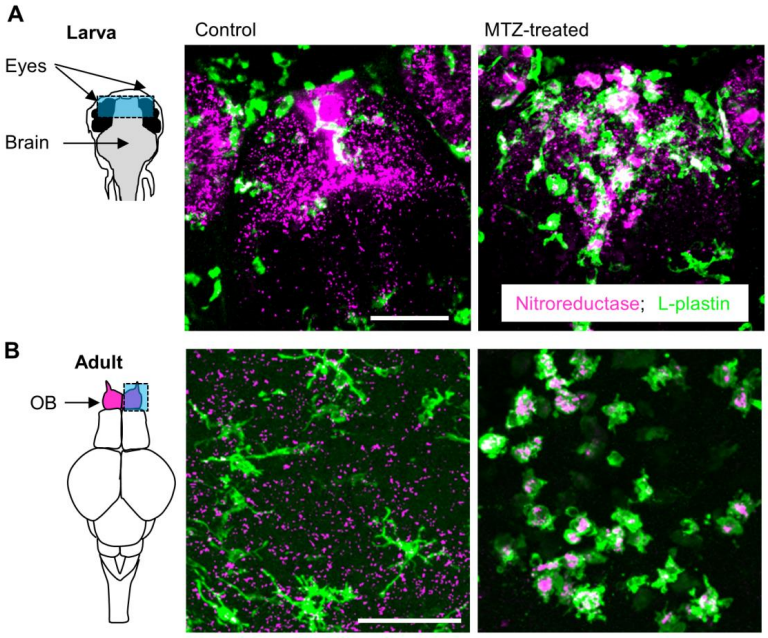

Fig 2. Immunostaining of zebrafish larvae and adult brains. A Activated microglia (in green L-plastin+) in 6 dpf larvae at 1 day post-treatment (dpt) with 2 mM MTZ for 16 h. Phagocytosed neurons are visible as bright mCherry-positive dots (in magenta). **B** Immunostaining in adult zebrafish brain olfactory bulbs after treatment with 5 mM MTZ for 48 h. Microglia are shown in green, apoptotic, engulfed neurons are shown in magenta. Scale bar = 50 μm.

14. Wash the larvae at least 10x with PBS-T for 30 min.
15. Remove the PBS-T and incubate the larvae in 200 μL secondary Alexa 488 labeled antibody solution (DyLight Alexa 488, 1:250) with 1:10.000 Hoechst on a shaker at 4℃ for at least 16 h (**Note 21**).
16. Wash the larvae at least six times with PBS-T for 10-15 min and continue for imaging (see mounting live imaging larvae)(**Note 13**).

Imaging microglial activation in response to neuronal ablation (adult zebrafish)

Metronidazole treatment adult fish

NB: During the entire experiment zebrafish can be fed according to the regular feeding regimen. Artemia or other live food is preferred since this causes less contamination of the water (**Note 22)**.

1. Dissolve 0.642 g MTZ in 3.5 mL DMSO to obtain a 1 M 1000 x stock solution and shake thoroughly (**Note 23**).
2. Fill a breeding tank with 750 mL water tapped from the aquarium water circulation system.
3. Transfer the dissolved MTZ to the breeding tank containing 750 mL fish water, resulting in a 5 mM MTZ solution (**Note 24**).
4. Transfer up to a maximum of 3 adult zebrafish to the breeding tank containing 5 mM MTZ (**Note 25**)

5. Place the breeding tank containing the fish in an incubator of 28 - 28.5 ℃ for 24 h (**Note 26**)
6. After the first 24 h of the experiment repeat step 30-34 once.

Isolation of fixed complete zebrafish brain

1. Euthanize the adult zebrafish on ice water.
2. Sever the head just behind the gills using a scalpel.
3. Place the head under a dissection microscope.
4. Remove the gills, jaws and remaining tissues until only the skull and eyes are left using forceps.
5. Place the skull containing the brain, eyes attached, in 4% PFA at 4℃ for at least 20 h (**Note 27**).
6. Wash the skulls containing the brains at least 3x with 1x PBS for at least 5 min at room temperature.
7. Place the skulls containing the brains with the ventral side facing upwards and carefully cut the optic nerves (white bands connecting the eyes to the head) with the scissors and remove the eyes.
8. Split the caudal half of the skull base in two equal parts with the break line in the rostral to caudal direction (**Note 28**).
9. Carefully remove the frontal part of the skull base (**Note 29**).
10. Remove the skull from the brain by pushing the caudal parts of the skull base apart.
11. Collect the brains in 1.5 mL micro centrifuge tubes (**Note 30**).
12. Perform stepwise dehydration with a methanol (MeOH) series (25%, 50%, 75%, 100%) 5 min per step at RT.
13. The brains can be stored at -20℃ for several weeks to months. For direct use, store them at -20℃ for at least 16 h.

Sectioning of zebrafish brains for immunofluorescence staining

1. Rehydrate the zebrafish brains in a methanol (MeOH) dilution series by subsequently incubating brains in 75% MeOH, 50% MeOH, 25% MeOH and 0% MeOH in 1x PBS at RT (**Note 31**).
2. Wash the brains once with 1x PBS to remove methanol traces.
3. Use the plastic Pasteur pipette to fill an embedding base mold with 4% LMP agarose (**Note 32**)
4. Place the zebrafish brain in the LMP agarose and position it in the desired orientation (**Note 33**)
5. Wait until the agarose gel has solidified (**Note 6**)
6. Remove the gel from the embedding base mold and cut it into a cube (using the scalpel) with at least 2 mm agarose on each side of the brain. Do not cut the agarose at the ventral side of the brain (**Note 34**).
7. Use the vibratome to cut 80 μm sections of the desired area.
8. For immunofluorescence staining transfer the brain sections to a 48-well plate containing 1x PBS (**Note 35**).

Immunofluorescence staining of zebrafish brain sections (Note 36)

1. Remove the PBS from the wells containing brain sections, add ~ 300 μL blocking buffer and incubate at room temperature on a shaker for 75 min.

2. Remove the blocking solution, add ~300 μL antibody solution 1 containing the primary L-plastin antibody (L-plastin, Rabbit, 1:1000, gift from Yi Feng, University of Edinburgh) and incubate at 4℃ on a shaker for at least 16 h.
3. Remove antibody solution and was at least 6x with PBST for at least 20 min.
4. Remove PBST, add ~300 μL blocking buffer containing the secondary antibody (e.g. Dylight α-Rabbit 488, 1:500) and incubate at 4℃ on a shaker for at least 16 h.
5. Remove antibody solution and wash at least 5x with 1x PBS for at least 20 min.
6. Transfer the brain sections to a microscope slide (**Note 37**)
7. Place small strips of Parafilm on either side of the brain sections and place 1-2 drops of Vectashield mounting medium on the brain sections. Cover with a cover glass and continue to imaging procedure (figure 2B)(**Note 38**).

Notes

1. Dissolved MTZ can be stored at -20℃ (preferably in aliquots).
2. Apoptotic cells can be labeled *in vivo* by combining imaging with secA5-YFP transgenic animals or in fixed tissue by TUNEL staining (Click-it Alexa Fluor 647 kit; Invitrogen) [15].
3. 10x MS-222 stock solution can be stored at -20℃. 10x MS-222 stock solution can be kept protected from light at 4℃ or room temperature for a few weeks.
4. In order to prevent setting of the gel, the 1.8% LMP agarose solution can be kept at 55℃.
5. We used a Zeiss LSM700, Zeiss LSM780 and Zeiss MPL for image acquisition. For imaging GFP a laser of 488 nm is necessary and for imaging of mCherry laser lines of 543, 555, 561 or 594 can be used. We used a 40x water lens with a numerical aperture (NA) of 1.2 for most of the image acquisition. In addition to a relatively high NA, this lens has a long working distance, which is ideal for making high quality images throughout the entire larval brain. It is possible to increase the quality of images by using lenses with a higher NA, however, these lenses usually have a shorter working distance, that do not allow imaging of the entire brain.
6. It is recommended to make the 1.8% LMP agarose solution when the brains have been rehydrated. In order to prevent premature setting of the gel, it is recommended to dissolve the agarose while the tube is placed in water and to keep it in the water while pipetting out the solution. Make sure there are no bubbles in the low melting point agarose transferred to the embedding base mold. This can make it more difficult to properly section tissue.
7. PTU prevents pigment formation [16]. As soon as the PTU is removed from the medium, pigment formation will occur. Therefore it is important to keep the PTU in the medium during the course of the experiment. An alternative to using PTU would be the use of fish without pigment formation (e.g. Casper[17]).
8. In case a line in which cell type-specific expression is accomplished by the use of the Gal4/UAS system in which the Gal4 driver as well as the NTR are labeled with mCherry, it is recommended to use the GFP filter for the selection of NTR/mCherry-positive larvae if possible. Only the larvae with the highest mCherry expression will show a red central nervous system while using the GFP filter. This reduces the number of NTR-negative larvae in the experiment that seem positive. Use a plastic Pasteur pipette or a 1 mL Gilson pipette with the tip cut off (0.5 cm) to transfer the

positive larvae to a new Petri dish. The brightest mCherry larvae are positive for both the neuronal gal4 driver and the UAS:NTR-mCherry construct.

9. From 2-3dpf onwards *Mpeg1*-GFP can be clearly observed. At 3 dpf the larvae have hatched and will be able to swim around. To sedate the larvae add some flaked ice to the Petri dishes and allow the ice to melt, while swirling the dish. Swirling will cause the sedated larvae to accumulate in the center of the dish.
10. Make sure the larvae are treated in the 28-28.5℃ incubator since lower temperatures may cause a weaker microglia response. The amount of induced cell death is dose and time dependent. Therefore one can choose to treat the larvae over a longer time period with a lower concentration or conversely to induce the same amount of cell death but over variable time periods (for more information see van Ham et al., 2012). Treatment with MTZ will work as soon as NTR is expressed.
11. The MTZ or DMSO containing solution is removed at the desired time point and the larvae are washed twice for 10 min with fresh HEPES buffered E3 containing
12. 003% PTU to remove the MTZ. We put 10-15 larvae per well in a 6-well plate.
13. When applying the 1.8% LMP agarose make sure the agarose has cooled to around 30℃ as higher temperatures may damage/kill larvae.
14. It is crucial to mount the larva as close as possible to the cover glass, to reduce the distance to the lens as high magnification objectives tend to have low working distance. Make sure the larva is positioned correctly before the agarose completely polymerizes. While the agarose is polymerizing keep the larva in place with a thin pipette tip or needle. We use Seque/Pro™ Capillary Pipet Tips from Roche.
15. For *in vivo* imaging using an upright microscope it is necessary to use a ceramic/dipping lens. To assure proper positioning of the larvae for imaging with an upright microscope it is also possible to mount them as described for the inverted microscope. Then the LMP agarose containing the larva can be taken out of the glass bottom dish and placed upside down in a droplet of LMP agarose in a 6 cm Petri dish. Make sure to use sufficient LMP agarose to prevent detachment of the agarose from the Petri dish, as this will cause the larva to move out of focus during the imaging (figure 1A).
16. Make sure to add the buffered 1x E3 medium with 0.016% MS-222 very carefully. The agarose detaches easily from the bottom of the dish. Addition of E3 medium is necessary to prevent shrinking of the agarose due to dehydration. Also the larva needs to stay anesthetized during the imaging.
17. When a water lens is used for long-term imaging, use Immersol instead of water as to avoid evaporation. It is crucial to put sufficient buffered 1x E3 medium in the imaging dish and to keep the lid on the dish to prevent evaporation and subsequent drying of the LMP agarose.
18. Put the 1.5 mL tubes in a 50 mL tube to place them on a roller mixer.
19. To assure equal antibody labeling in all larvae do not stain more than 20 larvae in a 1.5 mL tube.
20. Instead of overnight incubation at 4℃, 3 h at room temperature is also sufficient.
21. Fluorescence of mCherry is retained after immunohistochemistry, however when samples are stored for longer time periods (multiple months) mCherry fluorescence will fade.
22. In case incubation on a roller mixer is preferred, use at least 400 μL, preferably

more, antibody solution. Less solution may cause insufficient labeling due to larvae sticking to the walls of the tube diminisheing contact with the antibody solution.

23. Depending on the ablated brain area or cell type, fish may show impaired feeding behavior.
24. The solution should turn slightly yellow
25. After transferring the MTZ solution to the water, take care to mix the MTZ solution with water to obtain a completely transparent homogeneous solution. The mixing can be done by stirring the solution with a plastic Pasteur pipette.
26. We have performed these experiments in adult male and female fish of an age ranging from 3 - 9 months.
27. It is important to keep the fish at a temperature of at least 28°C. Lower temperatures may lead to a weaker microglia response to neuronal cell death.
28. PFA fixation within the skull facilitates the isolation of the brains and reduces the chance of damaging and losing brain tissue during dissection.
29. This is most easily done by pushing one arm of the forceps in between the brain and the caudal half of the skull base in the rostral to caudal direction. Subsequently squeezing of the forceps will result in a break line in the caudal part of the skull base. Then take out the forceps. Take care not to remove these parts of the skull base.
30. After the frontal part of the skull base has been removed the telencephalon and olfactory bulb should be exposed. This step is important, because otherwise the chance of losing (part of) the olfactory bulb is relatively high. Especially in the model described in this chapter, the effects are most pronounced in the olfactory bulb. Therefore it is important to preserve this part during the procedure.
31. It is possible to put multiple brains into a single microcentrifuge tube.
32. In order to assure optimal rehydration, the microcentrifuge tubes containing the brains can be put in a 50 mL tube during incubation and placed on a roller mixer.
33. For horizontal sections, position the brain with the dorsal side facing upward and the ventral side facing downward. For sectioning it is most convenient to place the brain as close to the surface as possible. The deeper the brain has sunk, the more agarose has to be cut before actual sectioning of the brain.
34. To speed up solidification of the gel, place the embedding base mold containing the brain on ice. Make sure to check regularly whether the brain is still in the desired position and correct when necessary. Sometimes during the solidification of the gel, the tissue sinks or tilts. This can be easily corrected as long as the gel has not completely solidified yet.
35. It is crucial that the ventral part is completely flat for optimal adhesion to the surface of the magnetic platform during the sectioning. Otherwise the agarose cube may detach. Also the position of the brain may change if the ventral part of the agarose tube is not properly cut.
36. An easy way to transfer the brain sections to a 48-well plate is to catch the sections on a microscope slide and then to use PBS to flush them into the desired well in the 48-well plate. It is possible to place multiple sections per well. 12-well plates can be used as well, however much more antibody will then be required.
37. All incubation and washing steps should be performed on a shaker.
38. One way to transfer the sections to the microscope slide is to first place a drop of PBS on the microscope slide and then scoop the sections out of the 48-well plate

using a 200 μL pipette tip. Then hold the tip in the drop of PBS. The brain section will detach. Then remove excess PBS.

39. These slides can be stored at 4℃ for several months.

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Chapter 8

General discussion

General discussion

Normal brain development and optimal brain performance are the result of extensive communication between different types of brain cells. Neurons and glial cells are the most prevalent brain cell types. For decades, glial cells were merely thought to be the glue holding the brain together, however it is now clear that glial cells have many essential brain-specific functions. For example, oligodendrocytes facilitate fast and efficient communication between neurons, whereas astrocytes provide metabolic support to meet the incredibly high energy demands of the brain[1]. Microglia, which unlike other glial cells are derived from primitive yolk sac macrophages (YSMs), form the brain's resident macrophage population that is important for the clearance of dead cells, debris and pathogens[2-4]. The immense cellular connectivity and the high degree of functional specialization of brain cells, make the brain extremely vulnerable to defects in any of those cell types. For example, genetic defects in any of the glial cell types can cause leukodystrophies, which are diseases characterized by white matter defects[5]. Also, aberrant functioning of multiple glial cell types is observed in most neurodegenerative disorders[3,5-8], which makes it difficult to discern the individual contributions of different glial cell types to the development and progression of brain disease. Especially for microglia this is challenging, because their functions and responses are highly sensitive to changes in the microenvironment, the type of pathogen- or damage-related trigger and their responses to damage are time-dependent[9-13]. Better understanding of microglia development and their roles in the developing and healthy adult brain in vivo could also indirectly provide valuable insights into the contribution of microglia to brain disease. Therefore, we here aimed to learn more about genetic regulation of microglia biology in the developing, healthy and diseased brain in vivo.

Zebrafish as a model organism in microglia research

Zebrafish larvae are highly suitable for in vivo imaging studies, because they are small, transparent, develop ex utero and are relatively easy to manipulate genetically. Forward genetic screens, as well as small molecule screens, have already led to identification of new gene functions relevant for human biology and new drugs with clinical application, respectively (reviewed in **chapter 2**)[14]. Of several main microglia regulators, such as colony stimulating factor 1 receptor (CSF1R), IRF8 and PU.1, the functions are well conserved between zebrafish, mice and humans[15-21]. For example, in all these species lack of functional CSF1R results in a complete absence of microglia (**chapter 6**)[17](Wang and Bennett, personal communication). When we started this research, only a handful of zebrafish microglia genes were known, and it was still unclear to what extent zebrafish microglia are similar to those in mammals. Therefore, we first characterized the zebrafish microglia transcriptome (**chapter 3**). This revealed that, for zebrafish homologs of mouse microglia signature genes, the majority were also specifically expressed in microglia (**chapter 3**)[22]. Recently, also the human microglia transcriptome was elucidated, revealing that human, mouse and zebrafish microglia gene expression signatures share remarkable overlap. For example, microglia signature genes, such as *P2RY12*, *CMKLR1* and *SLC7A7*, which are highly expressed in mice and in zebrafish microglia are also part of the human microglia transcriptome (Fig 1)[23,24]. This indicates that zebrafish microglia share a high degree of genetic similarity with human microglia and will be valuable for the discovery of new microglia regulators and functions relevant

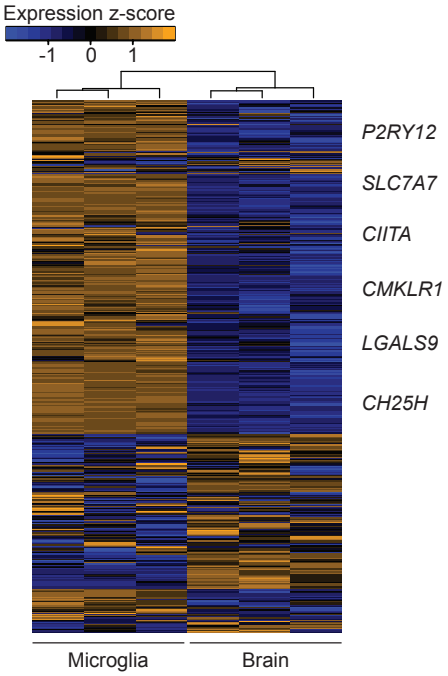

Fig 1. Zebrafish microglia express human microglia signature genes. Heatmap showing relative expression of zebrafish homologs of human microglia signature genes[22-24].

to human microglia biology.

The pathology observed in neurodegenerative diseases and in most mouse models that mimic them, are often accompanied by multiple pathological processes (e.g. protein aggregation) that can trigger immune responses in microglia. The immune responses in human disease and in these models generally include an increase in microglia numbers and secretion of inflammatory cytokines. Immune responses can be both cause and consequence of neuronal and glial dysfunction, which makes it difficult to identify the sequence of events leading to disease-related pathology[3,4,6]. Microglia, like all other macrophages, are particularly well equipped to sense and respond to molecular changes in their microenvironment[9-11,22,23]. Better understanding of microglia responses to individual disease-related triggers could therefore help in the prediction of events leading to the brain pathology observed in human brain disease. As neuronal cell death is a common denominator of neurodegenerative diseases, we used the zebrafish to assess how microglia respond to neuronal cell death specifically (**chapter 3**)[22]. Hereto, we used our previously established in vivo neuronal ablation model in which the microglia show a strong response to neuronal death specifically, which is followed by complete recovery of ablated brain tissue[13]. In **chapter 3** we showed that, despite a massive increase in proliferative and phagocytic activity, microglia did not show increased expression of inflammatory mediators or phagocytosis-related genes, some of which are associated with common neurodegenerative diseases[22,25,26]. This is consistent with the typical anti-inflammatory/immunologically silent response elicited to remove apoptotic cells[27]. Given that microglia clear dead cells and debris, and express

various genes needed for phagocytosis under homeostatic conditions, the absence of changes in these genes upon neuronal death suggests that microglia are already well-equipped to quickly meet increased phagocytic demands. Previously, we showed that the microglia response to neuronal death in the zebrafish resolves and that tissue recovers completely[13].

A widely held view in the neurodegenerative disease field is that chronic inflammation contributes to the progression of disease. As microglia appear to be prepared to deal effectively with increased phagocytic demand, death of neurons alone may be insufficient to elicit the more chronic inflammatory response found in neurodegenerative disease[28,29]. This chronic inflammation could be due to a failure to resolve an initially beneficial response to damage. Our data indicates that the death of neurons independent of other damage- or disease-related cues elicits a very different response than the excessive microglia-dependent inflammatory responses observed in human neurodegenerative disease[22]. Better understanding of the microglia responses to individual disease-associated triggers, such as protein aggregation, cellular injury and vascular damage, could help us to retrace the sequence of events leading to end-stage human neurodegenerative disease.

Unraveling microglia genetics in the zebrafish

Recent gene expression studies on mouse and human microglia, both in embryonic development and in the adult brain, have identified many genes that are potentially important for microglia biology with currently unknown function[23,24,30-32]. Identification of those genes that are important for microglia development and function, will contribute to better understanding of microglia biology in the healthy and in the diseased brain. In **chapter 4**, we developed a reverse genetic screening pipeline to facilitate the identification of genetic regulators of microglia development. Using this strategy, we identified zebrafish *il34* as a regulator of microglia development. In line with this, *Il34*-/- mice show reduced microglia numbers, which indicates that the function of IL34 is conserved between fish and mammals[33-35]. This also shows that our screening strategy is an effective method to discover regulators of microglia development. Current strategies to generate microglia in vitro, for example from human induced pluripotent stem cell (iPSC)-derived microglia, have yielded cells that share similarities with in vivo microglia, but lack the expression profile of mature microglia as identified in vivo[36-38]. Knowledge of the genes and molecular mechanisms driving microglia development and differentiation could also help to improve protocols to generate human induced pluripotent stem cell (iPSC)-derived microglia. The ability to generate microglia in vitro would be particularly interesting in the context of microgliopathies. In vitro microglia could be used to study the effects of patient-specific mutations on microglia functions or even to correct gene-function for cellular transplantation therapy.

Beyond genetic screening, our approach could also be used to identify small molecules affecting microglia. The small size of zebrafish embryos and the absorption of small molecules through the skin makes them highly suitable for high-throughput small molecule screens. Such screens have already led to the identification of diverse small molecules with novel biological modes of action and even several compounds with therapeutic benefit that are now used in the clinic or tested in clinical trials[39]. One example is the discovery of dorsomorphin, an inhibitor of bone morphogenetic protein (BMP) signaling[40]. Currently, a dorsomorphin derivative, LDN-193189, is being

developed as a treatment for fibrodysplasia ossificans progressiva (FOP), a disease involving overactive BMP receptor signaling. In this deadly disease, connective tissue transdifferentiate into bone[40,41].

Small molecule screens targeting microglia could help to discover mechanisms involved in microglia development and function, which might eventually lead to novel therapeutics as well. Importantly, depending on the disease, either replenishment or ablation of microglia could be the preferred strategy in the treatment of patients (see **chapter 6**). For example, inhibitors for CSF1R are already being tested to diminish microglia numbers in brain tumors, whereas transplantation of hematopoietic cells has been shown to reduce neurological deficits in some white matter disorders[42-45]. In such screens our software tool SpotNGlia would be particularly useful for automated image-based phenotyping, which typically is the most time-consuming/laborious component of such screens.

CSF1R as a regulator of microglia development and maintenance

CSF1R is a receptor tyrosine kinase that is required for the development of microglia. However, it is still unknown why CSF1R deficiency prevents microglia development, but not the development of most other tissue macrophage populations. Mostly based on extensive in vitro experimentation, it is thought that CSF1R is involved in the differentiation, proliferation and survival of macrophages, including microglia[46]. After microglia have colonized the brain, the generation of new microglia is thought to rely completely on self-renewal by local proliferation[2,47]. In **chapter 5 and 6** we showed evidence that *csf1r*-dependent proliferation and survival are important for the establishment and maintenance of microglia. We showed that, in zebrafish, complete or partial loss of *csf1r* compromises microglia density, which is most likely due to a defect in the generation of new microglia by proliferation and/or their decreased survival. In contrast, activating mutations in BRAF, a major component of MAP-kinase signaling downstream of CSF1R, in yolk sac macrophages, were recently shown to cause higher numbers of microglia and neurodegeneration[48]. Although we did not assess the role of *csf1r* in macrophage survival, *csf1r*-deficient YSM numbers declined after 42 hours post fertilization. Others have shown evidence that suppression of CSF1R in the mouse causes reduced survival of macrophages[46,49,50]. In fact, chemical inhibition and conditional genetic knockout of *Csf1r* in adult mice both cause over 90% lower microglia numbers within a week[49,50]. This all suggests that Csf1r regulates microglia density by controlling the rates of microglia generation and preventing their loss. Therefore, dysregulation of the balance between the generation of new microglia and loss of old microglia, for example through reductions in CSF1R might eventually result in a progressive loss of microglia. This might also explain the widespread loss of microglia we observed in post-mortem brain sections of deceased ALSP patients with only one functional copy of the gene.

Not all tissue-resident macrophage populations are affected equally in the absence of *Csf1r*, indicating that some subsets of tissue macrophages can be generated or maintained independently of *Csf1r*[51]. This is in line with our data showing that in zebrafish yolk sac macrophages show largely normal differentiation independent of *csf1r* (**chapter 5**). We also showed that reduced *csf1r* does not affect microglia differentiation status (**chapter 6**). Unlike microglia, most macrophage populations are completely or partly replaced by bone marrow-derived monocytes. For example, a subpopulation of

heart-resident macrophages is yolk sac-derived, whereas other cardiac macrophage subpopulations originate from the bone marrow[52]. We showed evidence that macrophages are formed, and can differentiate, independently of *csf1r*. Therefore, the monocyte origin of other tissue macrophages might explain the relatively normal densities of such macrophage populations in *Csf1r*-deficient animals[47,51]. This is further supported by evidence of others showing that CSF1R does not affect total monocyte counts in the blood in mice[53,54]. In all, contrary to the current idea that CSF1R is necessary for macrophage differentiation, our data strongly suggests that, in vivo, CSF1R only plays a minor role in acquisition of a macrophage phenotype.

CSF1R in brain disease

Mutations in several genes that are primarily expressed in microglia can cause devastating white matter disorders categorized as "microgliopathies"[55,56]. The mechanisms underlying these diseases are as yet unknown. Heterozygous loss-of-function mutations in CSF1R cause adult-onset leukoencephalopathy with axonal spheroids (ALSP), which is characterized by extensive degeneration of the white matter and presence of axonal spheroids[55]. Homozygous loss-of-function mutations in CSF1R cause structural brain defects and impaired myelinogenesis in infants (drs. Wang and Bennett, personal communication). In **chapter 6** we gained insight into mechanisms which could underlie the microgliopathy ALSP. In line with our findings in *csf1r* mutant zebrafish, we observed a significant reduction in microglia numbers in post mortem brain tissue of ALSP patients. Additionally, we noticed large millimeter-sized areas completely devoid of microglia in post mortem brain sections of ALSP patients (**chapter 6**). Given that in the brain CSF1R is almost exclusively expressed by microglia and appears to be crucial for the maintenance of microglia numbers, CSF1R-dependent brain pathology is most likely a direct or indirect consequence of microglia loss[17,57]. This concept is corroborated by the fact that an infant with *CSF1R* homozygous loss of function mutations also presented with main hallmarks of ALSP, such as brain calcification and widening of the ventricles, to a much more severe degree[58-60]. White matter pathology is a hallmark of ALSP and other microgliopathies, which indicates that microglia are crucial for white matter homeostasis in humans[55,56,58]. Furthermore, the reduction in myelin content as well as the absence of the corpus callosum in the infant with complete loss of *CSF1R*, suggests that in humans microglia are also involved in white matter development. This is supported by recent studies performed in mice showing direct roles for microglia in primary myelinogenesis[61]. Additionally, they are involved in the maintenance of numbers of myelin-forming cells, thereby affecting the production of myelin during embryogenesis and potentially throughout life[61-63]. Taken together with our findings, this supports the emerging concept that microglia serve critical roles in development and maintenance of the white matter (Fig 2). It is likely that direct interactions between membrane bound proteins or soluble factors derived from microglia would exert such an effect. Indeed, a recent study reports that a specific subset of microglia stimulates primary myelinogenesis through the production of insulin-like growth factor 1 (IGF1)[61]. It would be interesting to identify which other microglia factors explain their beneficial effects in brain development and homeostasis.

Based on the hypothesis that microglia are detrimental in brain diseases such as amyotrophic lateral sclerosis (ALS) and Alzheimer's disease, removal of microglia via inhibition of CSF1R is currently explored as a putative treatment option for these

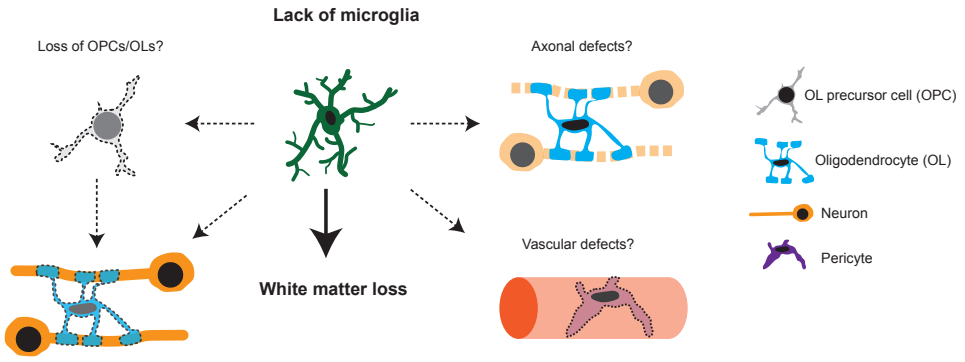

Fig 2. Different ways lack of microglia could lead to white matter loss. Most currently known microgliopathies are characterized by white matter degeneration. It is still unclear how long-term depletion of microglia in the adult brain would cause white matter degeneration. It is known that microglia numbers depletion in the adult mouse brain leads to reduced numbers of oligodendrocytes or NG2+/PDGFRA+ oligodendrocyte precursors (OPCs) in many brain regions. Although microglia dysfunction directly affect myelinating cells directly, evidence also suggests the involvement of the cerebral vasculature in microgliopathies. Mechanistically, this could involve defective pericytes, the vascular mural cells that regulate vessel constriction and blood flow, as mice without microglia have diminished PDGFRB+ pericyte numbers. Also, the expression of the growth factor PDGFb, which could activate PDGFRb on pericytes and PDGFRa on OPCs is strongly reduced in csf1r haploinsufficient microglia (see discussion chapter 6).

diseases[64-67]. Inhibition of CSF1R by GW2580 or PLX3397 results in effective, but temporary, >90% depletion of microglia in mice and improvement of cognitive or motor functions in mouse models for neurodegenerative disease[49,64,65]. However, our observations described in **chapter 6** and recent insights from genetic studies on human brain disease, indicate that there may potentially be severe adverse effects of microglia depletion as a strategy in human disease. Our data presented in **chapter 6** indicate that loss of microglia precedes and might even cause the white matter degeneration observed in ALSP patients. Defects in the white matter have been reported in neurodegenerative diseases such as Alzheimer's disease and ALS[68-70]. Therefore, removal of microglia through CSF1R inhibition in an already vulnerable degenerative brain might exacerbate disease instead. In fact, in a recent study by Yang and colleagues the removal of microglia in a mouse model for Parkinson's disease caused increased loss of dopaminergic neurons and exacerbated locomotor impairments[71].

Recent studies performed in healthy adult mice revealed that the ablation of microglia by administration of the CSF1R inhibitor PLX3397 for several weeks did not cause obvious adverse effects on the brain[49]. These findings however, do not exclude the chance of severe side effects of such a treatment in humans. The infant with homozygous mutations in *CSF1R* had severe structural brain defects, including agenesis of the corpus callosum, while in *Csf1r*-/- mice the corpus callosum was relatively unaffected[17]. Also, unlike *Csf1r* haploinsufficient mice, ALSP patients show severe white matter degeneration[72]. It is not uncommon that mutations that cause disease in humans, do not cause, or cause less severe, brain pathology in vertebrate model organisms. For example, the mouse model for metachromatic leukodystrophy, which is caused by an arylsufatase A deficiency, does not show the widespread demyelination that is observed in patients[73]. Also, to model for example human

Alzheimer's disease in mice, overexpression of sometimes multiple proteins containing different disease-related mutations is required to recapitulate main disease hallmarks[74]. Factors that might explain this relative absence of, or milder, brain pathology in neurodegenerative diseases models include the lower complexity of the brains of small model organism. Also, neurodegenerative diseases often take decades to develop in humans, whereas the maximum lifespan of both mouse and zebrafish in the lab typically is 2-3 years. Therefore, potentially very harmful side effects of microglia ablation-based treatment strategies could require a long(er) time to develop and could be easily missed in current animal models.

Future directions

The first microglia gene expression studies were performed on microglia isolated from whole brains[12,30-32]. However, like most other cell types, microglia show regional and stage-dependent functional heterogeneity[61,75-77]. Rapidly improving single-cell methods including single cell RNA sequencing have started to increase the resolution to identify microglia subsets with specific functions. A recent single-cell RNA sequencing study revealed that microglia at different stages of development show different gene expression patterns corresponding to the different stages of brain development[11]. In another recent study distinct microglia subpopulations important for primary myelinogenesis during brain development and for the clearance of amyloid plaques in a mouse model for Alzheimer's were identified[61,77]. Large scale single-cell techniques also enable us to detect intermediate cell states transitioning from one cellular phenotype to another, or rare cell-subclasses[76]. This technology could also be used to study the individual microglia responses to disease-associated triggers. In **chapter 5** we already showed that there are clear differences between the gene expression profiles of healthy and *csf1r*-deficient macrophages in zebrafish at different ages. Applying single-cell RNA sequencing to these specific stages could help us better understand which transcriptional changes underlie the transition from yolk sac macrophage to tissue-resident macrophage and how this is impaired in *csf1r*-deficient mutants. Additionally, such techniques could help identify changes in specific cell types and their subpopulations in microglia-deficient brains.

The identity and specialized functions of brain cells depend on the mutual interactions with cells and molecules in their microenvironment. The exact factors driving the acquisition of microglia identity and function in the brain are still largely unexplored. The epigenetic landscapes, comprising active and inactive genetic regulatory elements known as enhancers, of different tissue-resident macrophage populations, including microglia, are largely determined by the microenvironment[9-11,23]. However, the precise regulatory circuitry linking cues from the extracellular environment to the activity of enhancers, which regulate the expression of their target genes, remains to be identified. Better understanding of those enhancers and the transcription factors that bind to them, to drive the expression of specific groups of genes, will contribute to better understanding of the specific microenvironmental cues that drive microglia gene expression. Typically, based on DNA-sequence similarity, enhancers are thought to be poorly conserved between species. The location of enhancers relative to target genes, and their function, however, are thought to be conserved to a much higher degree. This is for example supported by a recent study, which showed that, between stickleback, zebrafish, mouse and humans, tissue specific enhancers, regulating gene expression

specific to intestinal sub-compartments, share high conservation of location and function[78]. Therefore, the zebrafish, beyond functional genetic screening, will likely be very useful for the identification of enhancer functions and concomitant transcription factors that are important for the acquisition and maintenance of cellular identity in vivo. It would be very interesting to determine whether the enhancers regulating gene expression in zebrafish microglia also share conserved locations across the genome with those in mouse and humans. Subsequently, as the importance and functions of individual enhancers are typically difficult to address in vivo, our genetic screening (**chapter 4**) could also be used to manipulate putative conserved enhancer regions to address their function.

Predicting the beneficial and detrimental consequences of treatment strategies aimed at microglia in human brain disease will benefit from fully understanding the interplay between microglia and other brain cell types will facilitate. Instead of focusing on the removal of microglia, promoting homeostatic functions by increasing microglia numbers could in some cases be the preferred strategy. Consistent with this idea, a few studies showed that injection of the CSF1R ligand CSF1, which is a stimulator of microglia proliferation, resulted in decreased cognitive decline and a reduction in disease-related protein aggregation in mouse models for Alzheimer's disease[79]. Another way to increase the numbers of functional microglia is by transplantation of hematopoietic stem cells (HSCs), which has been used successfully to stabilize the neurological deficits in patients diagnosed with leukodystrophy[44,45]. In fact, anecdotal evidence suggests that this could also benefit ALSP patients, as the disease process in an ALSP patient, who has been reported to have received a bone marrow transplant, was halted or significantly slowed[80]. Generally, better understanding of the role of microglia in the maintenance of brain homeostasis will provide valuable clues towards the restoration of brain homeostasis in brain diseases, even when microglia are not the main affected cell type.

In this thesis, we showed that zebrafish microglia share extensive similarity with mammalian microglia, based on gene expression, ontogeny and function, indicating that mechanistic insights and discoveries from zebrafish will be relevant to understand also mammalian microglia biology. We illustrate this by showing that one of the main regulators of microglia development and maintenance, *csf1r*, is functionally conserved between zebrafish, mice and even humans. Additionally, we showed evidence that, in the development of the human microgliopathy ALSP, a general reduction and microglia depletion, appears to be an early pathogenic event. Our findings therefore warrant further studies into the consequences of loss of microglia homeostatic activity. It is possible that loss of microglia homeostatic activity could contribute to other human brain diseases, such as Alzheimer's disease. In all, the studies presented in this thesis show that the zebrafish allows a holistic analysis of microglia, including their genetics, development and in vivo functions, to understand basic microglia biology, which is crucial to improve our understanding of the role of microglia in human brain disease.

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Appendix

Summary

Macrophages are immune cells with an enormous capacity for clearing dead cells, waste and pathogens. In addition to these functions, microglia, the brain's resident macrophages, are also important for the maintenance of neuronal connections and the secretion of trophic factors. Unlike most other tissue macrophages, microglia are completely derived from yolk sac-derived macrophages that colonize the developing brain during embryogenesis. Mutations in genes specifically expressed in microglia can cause rare, but severe white matter disorders by currently unknown mechanisms. Moreover, many gene variants associated with increased risk of developing diseases like Alzheimer's disease and multiple sclerosis appear to be most highly expressed in microglia compared to other brain cells. This suggests that some of their physiological roles are particularly important for the maintenance of brain homeostasis. However, despite decades of research the precise roles of microglia in the diseased- and in particular in the healthy brain in vivo are still unclear. Our aim was to better understand the role of microglia in the healthy and diseased brain using the zebrafish as a model organism.

In **chapter 2,** we described why the zebrafish is a suitable model organism to investigate the in vivo role of microglia, in the brain. Zebrafish are small tropical fresh water fish found in streams and ponds in the Himalayan region. They are highly suitable for in vivo studies, because of their small size, their transparency during embryonic development, and because it is relatively easy to genetically manipulate their genome. The majority of genes, cell types and tissues found in humans are also present, and functionally conserved in the zebrafish. The use of the zebrafish to study diverse biological or biomedical questions still continues to rise steeply and has already yielded tremendous insight into basic biological and disease-related mechanisms in vivo. Like mammalian microglia, zebrafish microglia have a ramified morphology, respond quickly to pathogens and efficiently clear dead cells and debris. However, when we started our studies it was still unknown whether zebrafish microglia are also genetically similar to mammalian microglia.

To investigate to what extent microglia identity is conserved between zebrafish and mammals, we purified microglia from zebrafish brains and performed RNA-sequencing to analyze their gene expression profiles (***chapter 3***). We observed that many microglia signature genes identified in mice and in humans, such as *CSF1R*, *TYROBP*, *CIITA*, *APOC1*, *P2RY12* and *SLC7A7*, are also specifically expressed in zebrafish microglia. We also found high expression of pathogen recognition receptors (e.g. Toll-like receptors), myeloid transcription factors (e.g. *spi1b*/*pu.1, irf8*) and other immune signaling components (e.g. *cd74*, complement factors, chemokine receptors). Direct comparison between zebrafish and mouse microglia gene expression profiles revealed that many of the mouse microglia signature genes are also specifically expressed in zebrafish microglia. This indicates that zebrafish could be used to gain insights into microglia genetics that are directly relevant for mammalian microglia biology.

We next used a similar strategy to characterize the transcriptional response of microglia in a pathological context. The pathology observed in neurodegenerative diseases is usually accompanied by immune responses. Due to the chronic and complex multifactorial nature of these diseases, it is difficult to discern first how microglia respond to individual disease-related cues specifically and second how these responses affect disease progression. As neuronal cell death is a common denominator of

neurodegenerative diseases, we used conditionally-induced neuronal cell death to investigate the acute response of microglia to the death of neurons specifically. We found that microglia don't show alterations in the expression of phagocytic genes and inflammatory mediators characteristic for neurodegenerative disease. In contrast, both at the transcriptional level and in vivo, they primarily appeared to respond by local proliferation. The subsequent increase in microglia numbers is most likely needed to meet the high phagocytic demand as a result of a local increase in dying neurons.

The high sensitivity of microglia to changes in their microenvironment has made it difficult to elucidate microglia functions in the homeostatic brain. Identification of functions associated with newly identified microglia signature genes could lead to the discovery of new regulators of microglia development and function. In ***chapter 4*** we present a scalable, semi-automated, reverse genetic screening pipeline to search for new genes with important roles in microglia development and function. We showed that CRISPR/Cas9-mediated gene disruption in zebrafish embryos effectively reproduces known mutant microglia phenotypes. Additionally, we developed a software tool, which we named SpotNGlia, to automatically quantify microglia numbers in zebrafish embryos. Using this strategy, we screened 20 selected genes for a possible involvement in microglia development. We identified zebrafish *il34*, the homolog of mammalian interleukin 34 (*Il34*), as a regulator of microglia development. In mammals IL34 is known to activate the CSF1 receptor (CSF1R), which is required for microglia development. This shows that our screening strategy in the zebrafish can be used to identify new genes that are important for mammalian microglia development.

In **chapter 5** and **6** we addressed the role of CSF1R (*csf1r* in zebrafish) in microglia development during embryogenesis (**chapter 5**) and its function in microglia in the adult brain (**chapter 6**). Even though CSF1R has been extensively studied for decades, its precise function in macrophages in vivo remains unclear. CSF1R functions, assigned primarily based on in vitro studies, include the survival, proliferation and differentiation of macrophages, including microglia. To study the role of *csf1r* in vivo, we generated an allelic series of *csf1r* mutant zebrafish. Similar to *Csf1r*-deficient mice, zebrafish lacking *csf1r* showed a complete loss of microglia and osteopetrosis, showing that CSF1R function is conserved between species. Intravital imaging in zebrafish embryos, revealed that *csf1r*-deficient yolk sac macrophages failed to colonize the brain. Furthermore, whereas we did not find evidence for a role of *csf1r* in the differentiation of microglia, both in developing and in adult microglia, *csf1r* appeared to regulate microglia numbers through proliferation. This insight may provide a potential mechanism for a human brain disorder, adult onset leukoencephalopathy with axonal spheroids (ALSP), which is characterized by severe white matter degeneration and caused by *CSF1R* haploinsufficiency. We observed, even in regions unaffected by extensive white matter degeneration, ~50% lower numbers of functional microglia in post mortem brain tissue of ALSP patients. In fact, millimeter-sized areas were completely devoid of microglia. This indicates that a gradual and/or a local loss of microglia, due to *CSF1R* haploinsufficiency, may be an important underlying cause of ALSP pathogenesis.

In all, the work presented in this thesis shows that microglia genetics and function, both in basic physiology and in the context of rare genetic human brain disease, can be effectively addressed in the zebrafish to gain new insight. Because of the significant contribution of microglia to the development and progression of brain disease, microglia are now recognized as a potential therapeutic target. As loss of microglia function may

be a contributing factor to disease, we first need to better understand the functions of microglia in the healthy brain. Accordingly, identification of additional microglia gene functions and understanding of the interactions between microglia and other brain cells in vivo will provide valuable new insights into microglia biology. Eventually, this will also help us understand when and how to manipulate microglia in disease.

Samenvatting

Macrofagen zijn immuuncellen met een enorme capaciteit voor het opruimen van bijvoorbeeld dode cellen en bacteriën. In alle weefsels zitten macrofagen die speciaal zijn aangepast voor specifieke functies binnen dat weefsel. Microglia zijn de macrofagen van de hersenen. Ze zijn betrokken bij de ontwikkeling en het goed functioneren van de hersenen. Verder lijken ze betrokken te zijn bij het ontstaan van hersenziekten en lijken ze het verloop van hersenziekten sterk te kunnen beïnvloeden. In tegenstelling tot de meeste hersencellen stammen microglia af van zogenaamde dooierzakmacrofagen, die tijdens de hele vroege embryonale ontwikkeling van de dooierzak naar de hersenen en andere organen migreren. Microglia zijn belangrijk voor het opruimen van dode cellen, ziekteverwekkers en afval, het onderhouden van neuronale verbindingen en het stimuleren van hersenontwikkeling door het uitscheiden van groeifactoren. Mutaties in genen die specifiek tot expressie komen in microglia kunnen zeldzame, ernstige neurodegeneratieve aandoeningen veroorzaken op vooralsnog onbekende wijze. Uit genetische studies in mensen blijkt dat genetische varianten die geassocieerd worden met een verhoogd risico op bijvoorbeeld de ziekte van Alzheimer of multiple sclerose, vaak in genen zitten die in de hersenen voornamelijk in microglia tot expressie komen. Ondanks tientallen jaren onderzoek is er nog veel onduidelijk over de functies van microglia in zowel het gezonde als in het zieke brein. Het doel van ons onderzoek was om meer inzicht in de genetische regulatie van microglia en de rol van microglia in het levende brein te krijgen. Hiervoor hebben we gebruik gemaakt van de bijzondere eigenschappen van de zebravis als modelorganisme, om microglia in levende hersenen te kunnen volgen en genetisch te kunnen manipuleren. Daarnaast hebben we post mortem humaan hersenweefsel gebruikt om te onderzoeken of onze bevindingen in de zebravis te vertalen zijn naar de mens.

In **hoofdstuk 2** leggen we uit waarom de zebravis een geschikt modelorganisme is om de werking van microglia, in het levende brein te kunnen onderzoeken. De zebravis is een kleine, tropische zoetwatervis die voorkomt in beekjes en vijvers in het Himalayagebied. Zebravissen zijn erg geschikt voor het visualiseren van processen in levende weefsels, omdat ze klein en transparant zijn tijdens de vroege ontwikkeling en omdat het relatief makkelijk is om ze genetisch te manipuleren. Homologe versies van de meeste genen, celtypes en weefsels in de mens zijn aanwezig en functioneel geconserveerd in de zebravis. Microglia in de zebravis hebben net als microglia in zoogdieren een sterk vertakte morfologie (vorm), reageren snel op weefselschade en kunnen snel en efficiënt dode cellen en afval opruimen. Echter, toen wij met ons onderzoek begonnen, was het nog onbekend in welke mate microglia van zebravissen en zoogdieren ook genetisch op elkaar lijken.

Om te onderzoeken in hoeverre de identiteit van microglia evolutionair geconserveerd is, en in hoeverre zebravis microglia lijken op die in de muis en in de mens, hebben we microglia uit zebravishersenen opgezuiverd en hun genexpressieprofiel in kaart gebracht met behulp van RNA sequencing (**hoofdstuk 3**). We hebben ontdekt dat veel van de microgliaspecifieke genen, zoals *CSF1R*, *TYROBP*, *CIITA*, *P2RY12* en SLC7A7, die geïdentificeerd zijn in de muis en in de mens ook specifiek in de microglia van zebravissen tot expressie komen. Daarnaast bleken microglia in de zebravis, net als die in zoogdieren, hoge expressie te hebben van receptoren die ziekteverwekkers herkennen, myeloïde transcriptiefactoren en vele andere moleculaire componenten die belangrijk zijn voor de cellen van het

afweersysteem. De identiteit van microglia lijkt dus in hoge mate evolutionair geconserveerd. Dit biedt ook perspectief wat betreft het ontrafelen van de functie van microgliagenen die bij ernstige hersenziektes betrokken zijn.

We hebben vervolgens een vergelijkbare strategie gebruikt om in kaart te brengen hoe microglia reageren op het afsterven van zenuwcellen (neuronen). In neurodegeneratieve ziektes is er vaak schade aan zenuwcellen, wat kan leiden tot activatie van het afweersysteem in de hersenen. Een hypothese binnen het Alzheimer onderzoek bijvoorbeeld is dat een te sterke, chronische activatie van het afweersysteem het ziekteverloop negatief beïnvloedt. Het is echter nog steeds onduidelijk welke precieze veranderingen in het degeneratieve brein chronische activatie van het afweersysteem veroorzaken en hoe dit vervolgens een negatieve invloed heeft op het ziekteverloop. Omdat het afsterven van zenuwcellen een belangrijk onderdeel is van alle neurodegeneratieve aandoeningen, hebben we gebruik gemaakt van transgene zebravissen waarin we specifiek neuronen kunnen laten degenereren. In dit model zagen we dat de acute dood van neuronen leidt tot een enorme toename van het aantal delende microglia, waarschijnlijk om snel zo veel mogelijk dode cellen op te kunnen ruimen. Verrassend genoeg vonden we geen toename in de expressie van genen die betrokken zijn bij de activatie van het afweersysteem die veelal gevonden wordt in neurodegeneratieve ziektes. Dit laat dus ook zien dat neuronale celdood op zichzelf niet per se leidt tot de ontstekingsreactie die wordt geassocieerd met neurodegeneratieve ziektes.

In het verleden was onderzoek naar microglia vooral gefocust op hun rol in ontstekingsreacties in de hersenen, waardoor pas recent ontdekt is dat microglia ook essentiële functies in het gezonde brein hebben. Als we erachter kunnen komen wat de functie is van de genen die microglia onderscheiden van andere cellen, dan zou dit bij kunnen dragen aan beter begrip van de functies van microglia in het gezonde brein. In **hoofdstuk 4** presenteren we een nieuwe strategie om nieuwe genen die belangrijk zijn voor de ontwikkeling en functie van microglia te kunnen ontdekken met behulp van genoomediting en automatische beeldanalyse van zebravislarven. Hierin laten we zien dat het mogelijk is om met behulp van CRISPR/Cas9 technologie genen te modificeren en bekende genetische afwijkingen in microglia te reproduceren. Om microglia-aantallen in de hersenen te kunnen kwantificeren hebben we software ontwikkeld, genaamd SpotNGlia, waarmee microglia automatisch gekwantificeerd kunnen worden. Met deze strategie hebben we 20 verschillende genen getest voor een mogelijke betrokkenheid bij de ontwikkeling van microglia. Hierbij hebben we het zebravisgen *il34*, een homoloog van interleukine 34 (Il34) in zoogdieren, geïdentificeerd als een belangrijke regulator van microgliaontwikkeling. In zoogdieren (mens en muis) activeert IL34 de colony stimulating factor 1 receptor (CSF1R), die noodzakelijk is voor de aanmaak van microglia. Hiermee tonen we aan dat onze strategie om nieuwe genen te vinden die belangrijk zijn voor microglia ontwikkeling in de zebravis goed werkt.

In **hoofdstuk 5** en **6** hebben we onderzocht wat de precieze functie van het *CSF1R* gen (*csf1r* in zebravissen) in microglia is tijdens de vroege embryonale ontwikkeling (**hoofdstuk 5**) en in het volwassen brein (**hoofdstuk 6)**. Hoewel er al tientallen jaren onderzoek naar CSF1R gedaan is, is het nog steeds onduidelijk wat de precieze functie is in macrofagen in vivo. Functies die met name op basis van in vitro (weefselkweek) studies toegewezen worden aan CSF1R zijn het positief beïnvloeden van de overleving, proliferatie en differentiatie van macrofagen, waaronder microglia. Om dit nader te

onderzoeken hebben we een serie zebravismutanten gemaakt met mutaties in *csf1r*, waaronder een mutant waarin functioneel *csf1r* ontbreekt. Met behulp van microscopie in levende zebravisembryo's hebben we laten zien dat *csf1r* nodig is voor de migratie van dooierzakmacrofagen naar embryonale weefsels, waaronder de hersenen. Verder hebben we gevonden we dat *csf1r* de aantallen microglia lijkt te reguleren in zowel het ontwikkelende als in het volwassen brein.

De regulatie van microglia-aantallen door CSF1R is met name interessant in de context van leukoencephalopathie met axonale sferoïden bij volwassenen (ALSP), een erfelijke ziekte die wordt veroorzaakt door mutaties in het CSF1R gen. De neuropathologie van ALSP wordt met name gekenmerkt door verlies van witte stof. De witte stof bevat vooral verbindingen tussen verschillende hersengebieden en ongeveer 50% van onze hersenen bestaat uit witte stof. In post mortem hersenweefsel van patiënten zagen we dat de aantallen microglia met ongeveer 50% afgenomen waren. Verder viel op dat er in grote oppervlakken van het hersenweefsel geen microglia meer aanwezig waren, zelfs daar waar er nog geen waarneembaar verlies van witte stof was. Dit impliceert dat een lokaal verlies van microglia als gevolg van verminderd CSF1R vroeg in het ziekteproces al aanwezig is en mogelijk een onderliggende oorzaak is voor de ontwikkeling van ALSP.

Dit proefschrift laat zien dat de zebravis een goed model organisme is om zowel fundamenteel wetenschappelijke als ook ziektegerelateerde vraagstukken met betrekking tot microglia genetica en functie te onderzoeken. Doordat microglia betrokken zijn bij de ontwikkeling van hersenziektes, en ze het verloop van zulke ziektes kunnen beïnvloeden, zijn microglia een potentieel doelwit voor nieuwe therapieën voor hersenaandoeningen. Depletie van microglia wordt bijvoorbeeld momenteel door verschillende onderzoeksgroepen onderzocht als mogelijke behandeling voor de ziekte van Alzheimer. Voor het voorspellen en minimaliseren van negatieve bijwerkingen van depletie van microglia is het echter noodzakelijk om de functies van microglia in het gezonde brein beter te begrijpen. Hiervoor zou het identificeren van nieuwe functies van microgliaspecifieke genen en interacties tussen microglia en andere hersencellen in vivo van grote waarde kunnen zijn. Dit soort onderzoek zal onze kennis over microglia doen toenemen, waardoor we beter leren begrijpen hoe manipulatie van microglia ingezet kan worden voor de behandeling van ziekte.

Gearfetting

Makrofagen binne ymmúnsellen mei in enoarme kapasiteit foar it opromjen fan bygelyks deade sellen en baktearjes. Yn alle weefsels sitte makrofagen dy't spesjaal oanpasse binne foar spesifike funksjes yn dat weefsel. Mikroglia binne de makrofagen fan de harsens. Sy binne behelle by de ûntwikkeling en it goed funksjonearjen fan de harsens. Fierder lykje se behelle te wêzen by it ûntstean fan harsensykten en lykje sy it ferrin fan harsensykten sterk beynfloedzje te kinnen. Yn tsjinstelling ta de measte harsensellen stamme mikroglia ôf fan saneamde djerrepûdemakrofagen dy't yn de tige iere embryonale ûntwikkeling fan de djerrepûde nei de harsens en oare organen migrearje. Mikroglia binne wichtich foar it opromjen fan deade sellen, sykteferwekkers en ôffal, it ûnderhâlden fan neuronale ferbinings en it stimulearjen fan harsenûntwikkeling troch it útskieden fan groeifaktoaren. Mutaasjes yn de genen dy't spesifyk ta ekspresje komme yn mikroglia kinne seldsume, slimme neurodegenerative oantaastings feroarsaakje op in foarearst ûnbekende wize. Ut genetyske stúdzjes by minsken blykt dat genetyske farianten dy't assosjearre wurde mei in ferhege risiko op bygelyks de sykte fan Alzheimer of multiple sclerose, faak yn genen sitte dy't yn de harsens benammen yn mikroglia ta ekspresje komme. Nettsjinsteande tsientallen jierren ûndersyk is der noch in soad ûndúdlik oer de funksjes fan mikroglia yn it sûne likegoed as it sike brein. It doel fan ús ûndersyk wie om mear ynsjoch yn de genetyske regulaasje fan mikroglia en de rol fan mikroglia yn it libbene brein te krijen. Dêrfoar hawwe wy gebrûk makke fan de bysûndere eigenskippen fan de sebrafisk as modelorganisme, om mikroglia yn libbene harsens folgje en genetysk manipulearje te kinnen. Dêrneist hawwe wy post mortem humaan harsenweefsel brûkt om te ûndersykjen of ús befinings yn de sebrafisk oer te setten binne nei de minsk.

Yn **haadstik 2** lizze wy út wêrom't de sebrafisk in geskikt modelorganisme is om de wurking fan mikroglia yn it libbene brein ûndersykje te kinnen. De sebrafisk is in lytse tropyske swietwetterfisk dy't foarkomt yn streamkes en fivers yn it Himalayagebiet. Sebrafisken binne tige geskikt foar it fisualisearjen fan prosessen yn libben weefsel, om't se lyts en transparant binne yn de iere ûntwikkeling en it relatyf maklik is om se genetysk te manipulearjen. Homologe ferzjes fan de measte genen, seltypen en weefsels dy't foarkomme yn minsken binne oanwêzich en funksjoneel konservearre yn de sebrafisk. Mikroglia yn de sebrafisk hawwe krekt as mikroglia yn sûchbisten in tige fersprantele morfology (foarm), reagearje fluch op weefselskea en binne by steat fluch en effisjint deade sellen en ôffal op te romjen. Lykwols, op it stuit dat wy mei ús ûndersyk úteinsetten wie it noch net bekend hoe bot mikroglia fan sebrafisken en sûchbisten ek genetysk opinoar lykje.

Om te ûndersykjen foar hoefier't de identiteit fan mikroglia evolúsjonêr konservearre is, en hoe bot mikroglia yn sebrafisen lykje op dy yn de mûs en de minsk, hawwe wy mikroglia út sebrafiskharsens opsuvere en dêrfan it genekspresjeprofyl yn kaart brocht mei RNA sequencing (**haadstik 3**). Wy hawwe fûn dat in soad mikrogliaspesifike genen, lykas *CSF1R*, *TYROBP*, *CIITA*, *P2PY12* en *SLC7A7*, dy't identifisearre binne yn de mûs en de minsk ek spesifyk ta ekspresje komme yn mikroglia fan sebrafisken. Ek die bliken dat mikroglia yn de sebrafisk, krekt lykas dy yn sûchbisten, hege ekspresje fan reseptoaren dy't sykteferwekkers werkenne, myeloïde transkripsjefaktoaren en in soad oare molekulêre komponinten dy't wichtich binne foar de sellen fan it ôfwarsysteem hawwe. De identiteit fan mikroglia liket dus sterk evolusjonêr konservearre. Dat biedt ek perspektyf oangeande it ûntraffeljen fan de funksje fan mikrogliagenen dy't by slimme

harsensykten belutsen binne.

Dêrnei hawwe wy in ferlykbere strategy brûkt om yn kaart te bringen hoe mikroglia reagearje op it ôfstjerren fan senuwsellen (neuronen). Yn neurodegenerative sykten is der faak skea oan senuwsellen, dat liede kin ta aktivaasje fan it ôfwarsysteem yn de harsens. In hypotese binnen it Alzheimer ûndersyk bygelyks is dat in te sterke, gronyske aktivaasje fan it ôfwarsysteem it sykteferrin negatyf beynfloedet. It is lykwols noch hieltyd ûndúdlik hokker krekte feroarings yn it degenerative brein gronyske aktivaasje fan it ôfwarsysteem feroarsaakje en hoe't dit ferfolgens in negative ynfloed hat op it sykteferrin. Om't it ôfstjerren fan senuwsellen in wichtige oerienkomst is fan alle neurodegenerative oandwanings, hawwe wy gebrûk makke fan transgene sebrafisken wêryn't wy spesifyk neuronen degenerearje litte kinne. Yn dat model seagen wy dat de akute dea fan neuronen liedt ta in enoarme tanimming fan it tal dielende mikroglia, nei alle gedachten om fluch sa folle mooglik deade sellen opromje te kinnen. Ta ús fernuvering fûnen wy gjin tanimming yn de ekspresje fan genen dy't behelle binne by de aktivaasje fan it ôfwarsysteem dy't almeast yn neurodegenerative sykten fûn wurde. Dat lit ek sjen dat de neurale seldea op himsels net perfoarst liedt ta de ûntstekkingsreaksje dy't assosjearre wurdt mei neurodegenerative sykten.

Yn it ferline waard by ûndersyk nei mikroglia benammen har rol by ûntstekkingsreaksjes yn de harsens foar de lins naam, wêrtroch noch mar krektlyn ûntdutsen is dat mikroglia ek essinsjele funksjes yn it sûne brein hawwe. At wy derefter komme kinne wat de funksje is fan de genen dy't mikroglia ûnderskiede fan oare sellen, dan soe dat bydrage kinne oan better begryp fan de funksjes fan mikroglia yn it sûne brein.

Yn **haadstik 4** presintearje wy in nije strategy om nije genen dy't wichtich binne foar de ûntwikkeling en funksje fan mikroglia ûntdekke te kinnen mei behelp fan genoomediting en automatyske byldanalyze fan sebrafisklarven. Dêryn litte wy sjen dat it mooglik is om mei help fan CRISPR/Cas9 technology genen te modifisearjen en bekende genetyske ôfwikings yn mikroglia te reprodusearjen. Om it tal mikroglia yn de harsens kwantifisearje te kinnen hawwe wy software, mei de namme SpotNGlia, ûntwikkele om mikroglia automatysk kwantifisearje te kinnen. Mei dy strategy hawwe wy 20 ferskillende genen test foar har mooglike belutsenens by de ûntwikkeling fan mikroglia. Dêrby hawwe wy it sebrafiskgen *il34*, in homolooch fan ynterleukine 34 (Il34) yn sûchbisten, identifisearre as in wichtige regulator fan mikroglia-ûntwikkeling. Yn sûchbisten (minsk en mûs) aktivearret IL34 de colony stimulating factor 1 receptor (CSF1R), dy't needsaaklik is foar it oanmeitsjen fan mikroglia. Dêrmei toane wy oan dat ús strategy om nije genen te finen dy't wichtich binne foar de ûntwikkeling fan mikroglia yn de sebrafisk goed wurket.

Yn **haadstik 5** en **6** hawwe wy ûndersocht wat de krekte funksje fan it CSF1R gen (*csf1r* yn sebrafisken) yn mikroglia is yn de iere embryonale ûntwikkeling (**haadstik 5**) en yn it folwoeksen brein (**haadstik 6**). Hoewol't CSF1R al tsientallen jierren ûndersocht wurdt, is it noch hieltyd net dúdlik wat de krekte funksje is yn makrofagen in vivo. Funksjes dy't benammen op basis fan in vitro (weefelkweek) stúdzjes tawiisd wurde oan CSF1R binne it posityf beynfloedzje fan de oerlevering, proliferaasje en differinsjaasje fan makrofagen, wêrûnder mikroglia. Om dat neier te ûndersykjen hawwe wy in searje sebrafiskmutanten makke mei mutaasjes yn *csf1r*, wêrûnder in mutant dêr't funksjoneel *csf1r* yn ûntbrekt. Mei behelp fan mikroskopy yn libbene sebrafiskembryo's hawwe wy sjen litten dat *csf1r* nedich is foar de migraasje fan djerrepûdemakrofagen nei

embryonale weefsels, wêrûnder de harsens. Fierder hawwe wy fûn dat *csf1r* de oantallen mikroglia liket te regulearjen yn it ûntwikkeljende likegoed as it folwoeksen brein.

De regulaasje fan it tal mikroglia troch CSF1R is benammen ynteressant yn de kontekst fan adult onset leukoencephalopathy with axonal spheroids (ALSP), in erflike sykte dy't feroarsake wurdt troch mutaasjes yn it CSF1R gen. De neuropatology fan ALSP wurdt benammen karakterisearre troch it ferlies fan wite stof. De wite stof befettet benammen ferbinings tusken ferskillende harsengebieten en sawat 50% fan ús harsens bestiet út wite stof. Yn post mortem harsenweefsel fan pasjinten seagen wy dat it tal mikroglia mei likernôch 50% tebek rûn. Fierder foel op dat der yn grutte stikken fan it harsenweefsel gjin mikroglia mear oanwêzich wienen, sels op it plak dêr't noch gjin waarnimber ferlies fan wite stof wie. Dat ymplisearret dat in lokaal ferlies fan mikroglia as gefolch fan fermindere CSF1R ier yn it sykteproses oanwêzich is en mooglik in ûnderlizzende oarsaak is fan de ûntwikkeling fan ALSP.

Dit proefskrift lit sjen dat de sebrafisk in goed modelorganisme is om fûneminteel wittenskiplike likegoed as ek sykterelatearre fraachstikken oangeande mikrogliagenetika en funksje te ûndersykjen. Om reden fan de belutsenens fan mikroglia by de ûntwikkeling fan harsensykten, en om't se it ferrin fan sokke sykten beynfloedzje kinne, foarmje mikroglia in mooglik doel foar de ûntwikkeling fan nije terapyen foar harsenoandwanings. Depleesje fan mikroglia wurdt op it stuit troch ferskillende ûndersyksgroepen ûndersocht as mooglike behanneling foar bygelyks de sykte fan Alzheimer. Foar it foarsizzen en minimalisearjen fan negative bywurkingen fan depleesje fan mikroglia is it lykwols needsaaklik om de funksjes fan mikroglia yn it sûne brein better te begripen. Dêrfoar soe it identifisearjen fan nije funksjes fan mikrogliaspesifike genen en ynteraksjes tusken mikroglia en oare harsensellen in vivo fan grutte wearde wêze kinne. Datsoarte fan ûndersyk sil ús kennis oer mikroglia fergrutsje, wat ús ek stypje sil by it begryp fan hoe't manipulaasje fan mikroglia ynset wurde kin foar de behanneling fan sykte.

Curriculum Vitae

Nynke Oosterhof was born in Drachten on February 9th, 1990. After she graduated from high school in 2008 (gymnasium, Drachtster Lyceum te Drachten), she started her bachelor degree in biology at the University of Groningen. Her bachelor degree was almost entirely focused on behavioral and neurosciences. In the last year of her bachelor she did two small research projects. One of these projects was performed in the lab of Prof. Dr. U. Eisel and was focused the beneficial role of the tumor necrosis factor 2 receptor (TNFR2). In the other research project she studied the role of syntaxin-4, galactosylceramide and sulfatide on MBP mRNA transport in oligodendrocytes. After she got her bachelor degree in 2011, she was admitted to the research master Behavioural and cognitive neurosciences at the University of Groningen. During her first research internship in the lab of Prof. E. Boddeke at the University of Groningen, she studied the role of microglia in a mouse model for multiple sclerosis. The year after she went to the lab of Prof. R.A. Quinlan at Durham University in the United Kingdom. There, she studied the role of aB-crystallin in mitochondrial movement. After graduating with honors in 2013, she started working on the role of microglia in the healthy and diseased brain in the lab of Dr. T.J. van Ham. The results of this work are described in this PhD thesis titled *"Microglia in the health and disease: Learning from the zebrafish"*. This thesis will be defended just before the summer of 2018.

List of publications

Oosterhof N, Dekens DW, Lawerman TF, van Dijk M. Yet another role for SIRT1: reduction of alpha-synuclein aggregation in stressed neurons. J Neurosci. 2012;32(19):6413-4.

van Ham TJ, Brady CA, Kalicharan RD, **Oosterhof N**, Kuipers J, Veenstra-Algra A, et al. Intravital correlated microscopy reveals differential macrophage and microglial dynamics during resolution of neuroinflammation. Dis Model Mech. 2014;7(7):857-69.

Oosterhof N, Boddeke E, van Ham TJ. Immune cell dynamics in the CNS: Learning from the zebrafish. Glia. 2015;63(5):719-35.

Oosterhof N, Holtman IR, Kuil LE, van der Linde HC, Boddeke EW, Eggen BJ, et al. Identification of a conserved and acute neurodegeneration-specific microglial transcriptome in the zebrafish. Glia. 2017;65(1):138-49.

Oosterhof N, Kuil LE, van Ham TJ. Microglial Activation by Genetically Targeted Conditional Neuronal Ablation in the Zebrafish. Methods Mol Biol. 2017;1559:377-90.

PhD Portfolio

Courses	Year	ECTS
BCN orientation course, Groningen	2013	3
Proefdierkunde, Groningen	2013	4
Microbiological safety, Groningen	2013	1
BCN Masterclass, Groningen	2013	0,5
The power of RNA-seq, Wageningen	2013	0,9
Cellular Imaging, Groningen	2014	2
BCN retreat, Groningen	2014	1
Microarray/RNAseq data analysis using R/BioC and webtools	2014	2
Advanced immunology	2015	1
Microscopic image Analysis: From theory to practice	2016	0,8
Research integrity	2017	0,3
Python programming	2017	1
Biomedical writing in english	2017	2
Workshops		
MGC PhD Workshop, Münster	2014	1
MGC PhD Workshop, Maastricht	2015	1
MGC PhD Workshop, Dortmund	2016	2
(Inter)national conferences		
GliaNed meeting, Utrecht	2014	0,5
European zebrafish meeting, Oslo	2015	2
Dutch neuroscience meeting, Lunteren	2015	1
GliaNed meeting, Groningen	2015	0,5
100 years of Phagocytes meeting, Sicily	2016	2
Dutch neuroscience meeting, Lunteren	2016	1
GliaNed meeting, Rijswijk	2017	0,5
Microglia symposium, Rijswijk	2017	0,5
Microglia meeting, Groningen	2017	1
European glia meeting, Edinburgh	2017	2
GliaNed meeting, Amsterdam	2018	0,5
Teaching		
Supervision Bachelor students Groningen	2014	1
Supervision Bachelor thesis Groningen	2014	1
Supervision Master student Molecular medicine	2014-2015	2
Supervision Master student from Leiden University	2015-2016	2
Lecture minor Genetica in de maatschappij	2016	1
Supervision Master student Molecular medicine	2016-2017	2
Total		**44**

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