Chapter 4
Lessons from In Vivo Imaging

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Abstract The first line of defense against injury or disease in the central nervous system (CNS) is through microglia. In the adult brain, microglia were long believed to stay in a dormant/resting state, activated only in the event of an insult to the brain. This view changed dramatically with the development of modern imaging techniques that allowed the study of microglial behavior in the intact brain over time to reveal the dynamic nature of their responses. In vivo imaging studies using two-photon microscopy revealed a previously unknown function for microglia: they continuously screen the intact brain parenchyma with their fine processes on a timescale of minutes. By doing so, they contact neuronal cell bodies, axons, dendrites, and dendritic spines and are believed to play a central role in sculpting neuronal networks during development, adulthood, and the normal aging process. Following acute trauma, or in neurodegenerative or neuroinflammatory diseases, microglial responses range from protective to harmful, underscoring the need to better understand their diverse roles in different pathological conditions. In this chapter we will introduce two-photon microscopy and compare the in vivo and in vitro imaging approaches for studying microglia. We will also discuss relevant mouse models available for in vivo imaging studies of microglia and review how such studies are constantly reshaping our understanding of the multifaceted role of microglia in the healthy and diseased CNS.

Keywords Microglia • In vivo imaging • Imaging technologies and methods • Mouse models • Two-photon microscopy • In vitro experiments • Alzheimer's disease • Multiple sclerosis

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Bullet Points

- This chapter highlights how in vivo imaging has revolutionized our understanding of microglial biology in the healthy and diseased central nervous system.
- We provide a brief introduction to available imaging technologies, methods, and mouse models used to study microglia in vivo, with a focus on two-photon microscopy.
- We compare findings from in vitro and in vivo imaging experiments and discuss how different experimental conditions have generated distinct and often conflicting conclusions regarding microglial functions.

4.1 Introduction

Microglia are the resident immune cells of the brain and spinal cord. They were first identified by Nissl and Robertson at the end of the nineteenth century. In 1932, Pio del Rio Hortega called them "microglia" for the first time and introduced some basic characteristics of the cells that are still valid today ((Kaur et al. 2001; Kettenmann et al. 2011), and for more information about the discovery of microglia, please see Chap. 2). For many decades after the identification and first description of microglia, their importance for central nervous system (CNS) physiology was still largely underappreciated. Moreover, their developmental origin remained an area of active debate until recently, almost 90 years after their discovery. We now know that microglia colonize the brain from the yolk-sac at the early stages of embryonic development and, unlike all other glial cells in the brain, are of mesodermal origin (Chan et al. 2007; Ginhoux et al. 2010; Kierdorf et al. 2013). Once they enter the CNS, microglia populate every part of the brain and spinal cord, and they remain present throughout life. In addition, their developmental links with myeloid progenitors and the monocytic lineage, as well as their ability to perform most of the typical immune cell functions (e.g., phagocytosis of dead cells, secretion of pro-inflammatory cytokines and chemokines, and antigen presentation), has established their classification as the resident immune cells of the CNS (see Chap. 5 for more information).

The classic immunological functions of microglia have been thoroughly described in several disease, injury, and infection paradigms (Kreutzberg 1996; Ransohoff and Brown 2012; Aguzzi et al. 2013). Interestingly, microglia have been directly associated with a robust morphological transformation, defined as their "activated state". For many decades, however, microglia were essentially assumed to be inactive in the absence of a challenge, with minimal or no involvement in physiological brain functions. The role of microglia in physiological conditions was significantly re-evaluated with the development of in vivo imaging approaches, which allowed the study of microglia in the intact brain for the first time. In fact, in vivo imaging studies with subcellular resolution became possible with the advent of two-photon microscopy (Denk et al. 1990), a major technological advance that

proved instrumental for revealing previously unknown functions for microglia in both the physiological and pathological CNS. While other in vivo imaging modalities like positron emission tomography (PET) and whole animal luminescent or fluorescent imaging allow monitoring of large areas of microglial activation throughout the brain, the spatial resolution of these techniques remains relatively low. In contrast, imaging individual microglial processes with two-photon microscopy uncovered the constant physical surveillance function of microglia, underscored their significance for the architecture and function of the brain, and thereby opened new avenues of exploration into the cellular neuroscience behind these dynamic behaviors. These new endeavors generated the need for new animal models for endogenous labeling of microglia with fluorescent proteins, as well as new in vivo imaging methods to allow access to previously unexplored areas of the CNS, such as the spinal cord. Microglia are also involved in pathological processes associated with essentially every CNS disease. Investigators are now taking advantage of these newly developed imaging resources and approaches to perform longitudinal in vivo imaging studies in various animal disease models, unveiling additional roles and distinct functions for microglia in neurodegenerative and neuroinflammatory diseases. In this chapter we will first make a brief and simplified introduction to available technologies, imaging methods, and mouse models that have made the in vivo imaging studies of microglia possible, with a particular focus on two-photon microscopy. We will then highlight some of the early attempts to image microglia in culture or in slice preparations and briefly discuss their advantages and limitations. Finally, we will discuss some of the key findings that were uncovered by in vivo imaging studies of microglia, underscoring how these findings challenged longstanding preconceptions about them and helped redefine our understanding of their role in both the physiological and pathological CNS.

4.2 In Vivo Imaging of Microglia: Technologies, Methods, and Mouse Models

Several in vivo imaging techniques such as magnetic resonance imaging (MRI), ultrasound imaging, PET, and whole animal luminescence or fluorescence microscopy allow the observation of biological phenomena in living organisms. Among these modalities, in vivo imaging specifically of microglial cells in the mammalian CNS has been performed with luminescence imaging, PET, and multi-photon or two-photon laser scanning microscopy. For example, luminescence imaging has allowed to study microglial activation in a model of transient ischemia using a transgenic mouse line with expression of both luciferase and green fluorescent protein (GFP) under the promoter of the toll-like receptor 2 (TLR2) (Lalancette-Hebert et al. 2009). This receptor, though not specific for microglia, is upregulated in these cells following certain brain insults. Bioluminescence imaging using a charged coupled device (CCD) camera showed TLR-2 upregulation indicative of microglial activation that was interestingly evident in the olfactory bulb a few hours before it

was detected over the site of infarction (Lalancette-Hebert et al. 2009). Microglial activation in different brain regions can also be detected using PET imaging and the radioligand [11C]PK11195 (Venneti et al. 2013). [11C]PK11195 binds to the translocator protein (TSPO) which has been shown to be upregulated in activated microglia upon brain injury and neurodegenerative conditions. However, this ligand is not specific for microglia, as it detects to a smaller degree also activated astrocytes. Moreover, [11C]PK11195 cannot be used to image microglia under physiological conditions (Venneti et al. 2013). New tracers are therefore needed to specifically image microglia in physiological and pathological conditions, using non-invasive modalities like PET and MRI imaging. Developing better microglia-specific tracers for these imaging modalities would be an invaluable resource for in vivo studies of microglial responses across large brain regions, not only in animal models but also in human patients. On the other hand, though PET and MRI are the most widely used clinically applicable imaging modalities, they can still only accomplish a relatively limited spatial resolution.

Among all available in vivo imaging modalities, two-photon microscopy can accomplish the highest spatial resolution. It is therefore typically used to track individual cells or even cellular compartments such as dendritic spines and microglial processes, as they partake in complex biological processes in real time, within their natural microenvironment, in both health and disease. This is accomplished by the use of specialized lasers that emit low-energy infrared light that penetrates below the surface of living organs, such as the brain, with minimal photobleaching and photodamage (Helmchen and Denk 2005; Svoboda and Yasuda 2006).

4.2.1 Two-Photon Laser Scanning Microscopy: Introduction to the Technique

Fluorescence microscopy is largely based upon the ability of specific molecules called fluorophores to absorb and then emit light. In single-photon fluorescence microscopy, one photon delivers enough energy to excite an electron of a fluorophore to a higher energy state (Fig. 4.1a). Part of this absorbed energy is released by light emission that occurs when the excited electron returns to its basal state as the fluorophore relaxes. In 1931, Maria Göppert-Mayer first described the principle of multi-photon excitation (Göppert-Mayer 1931), such that if two or more photons arrive at a fluorophore simultaneously, their energies could be combined in a sum that, if sufficient, would excite an electron to its higher energy state, after which it can relax to its ground state and emit light, namely produce fluorescence (Fig. 4.1a). Sixty years later, Winfried Denk invented the first microscope equipped with a Titan-Sapphire laser, which experimentally accomplished two-photon excitation (Denk et al. 1990). The space and time requirements for two-photon excitation are very strict, as two photons must arrive at the same molecule within 10^{-18} s. To meet this time requirement, Titan-Sapphire lasers have undergone extensive

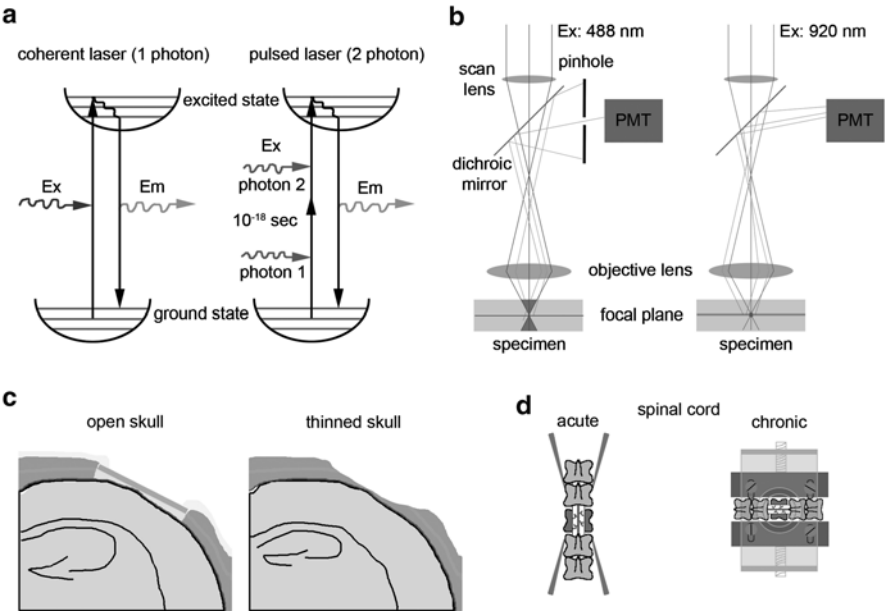

Fig. 4.1 Two photon in vivo imaging of the central nervous system. (**a**) Jablonski diagrams illustrating single versus two-photon excitation of a green fluorophore. *Left panel*: For single photon excitation, coherent laser light of higher energy and shorter wavelength (e.g., *blue* 488 nm) than the emission wavelength (*green* 530 nm) is used. *Right panel*: For two-photon excitation pulsed infrared laser light of lower energy and longer wavelength (e.g., *red* 920 nm) than the emission wavelength of the fluorophore (*green* 530 nm) is used. Two photons have to spatio-temporally coincide to combine their energy and elevate the electron to a higher energy state within 10^{-18} s. (**b**) Exemplary light paths of a confocal and a two-photon microscope. All fluorophores of the specimen within the light-cone of the objective are excited with coherent laser light in a confocal microscope. The pinhole in front of the photomultiplier detector (PMT) prevents out of focus light from reaching the PMT. Two-photon excitation occurs only at the focal point, preventing out of focus excitation of the fluorophore. Hence, no pinhole is necessary and all emitted photons reach the PMT. (**c**) The two widely used surgery techniques chronic window or "open skull" and "thinned skull". A circular coverslip is glued with dental acrylic as skull replacement (*left panel*). For thinned skull the bone stays intact and is thinned to about 50 μm (*right panel*). (**d**) Two options for two-photon in vivo imaging of the spinal cord. After performing a laminectomy on a single vertebra the vertebral column is stabilized by two external holders (*left panel*). For a chronic window, two metal bars laterally stabilize the vertebral column after the laminectomy. A glass coverslip is held in position by a placeholder (*right panel*)

development over the past two decades to pulse light with a repetition rate of 70–100 MHz and a pulse width of 70–100 fs (10^{-15} s). Additionally, to accomplish a high enough abundance of photons for two-photon excitation it is necessary to concentrate the laser light in space, namely increase the density of photons available to excite a fluorophore molecule. High numerical aperture (NA) objective lenses contribute to this end. In recent years, most major microscope manufacturers have constructed high NA (>0.95) objective lenses, with magnifications ranging

from ×16 to ×60 and working distances of up to 8 mm. These are typically water-immersion lenses that have been optimized for infrared light and manufactured specifically for use with two-photon microscopy of living tissues or organs immersed in physiological solutions.

In single photon excitation, like that used in conventional confocal microscopy, specimens are illuminated with coherent laser light, leading to fluorescence generation throughout the depth of the specimen (Fig. 4.1b). To accomplish sufficient spatial resolution, the abundance of fluorescence generated from the tissue above and below the focal plane is prevented from reaching the light detector by the use of mechanical aperture regulators called pinholes. In contrast, the strict time and space requirements for successful two-photon excitation result in fluorescence generation only at the focal plane of the lens that is used to focus the excitation beam within the specimen (Fig. 4.1b). As a result, two-photon excitation only happens at the focal point, making pinholes redundant. This is also important, because less tissue is exposed to high amounts of energy, thereby reducing the amount of photobleaching and photodamage of the imaged tissue. Conversely, the use of pinholes in confocal microscopy results in slightly better resolution along the z-axis, which is a slight disadvantage of two-photon microscopes. Nonetheless, two-photon microscopes have numerous advantages over confocal microscopy. For example, since there is no out-of-focus light generated, two-photon microscopes are designed to collect all fluorescence-generated photons, which translates into enhanced detection sensitivity (Fig. 4.1b). Furthermore, the long-wavelength infrared light generated by two-photon lasers is scattered less by biological tissue, resulting in better depth of penetration than any other light microscopy approach. Collectively, these features suggest that two-photon microscopy is the most appropriate and advantageous technique for imaging microglia in animal models in vivo, especially at the single cell level, in both physiology and disease.

Even as such, since it still requires light penetration for both fluorescence generation and detection, light scattering within living tissues is the main limiting factor for two-photon microscopy, with a penetration limit of ~1 mm below an organ's surface. One way to overcome this limitation and image deeper into living tissues is to use fiber optics. Fiberscopes with small diameters of 1–2 mm may be inserted in living organs at depths of more than a millimeter, as needed (Flusberg et al. 2005). The technique of microendoscopy for in vivo imaging of deep brain structures such as the hippocampus was greatly advanced by Mark Schnitzer's laboratory that fabricated grin-lenses, suitable for two-photon microscopy (Barretto et al. 2011). The same group also built a miniaturized fluorescence microendoscope that can be implanted in the brain and thus allow in vivo imaging in freely moving animals, with single cell resolution (Flusberg et al. 2008; Ziv et al. 2013). Imaging in head-fixed mice has also been exploited as an approach to study cellular responses in awake animals, using two-photon microscopy (Dombeck et al. 2007; Lovett-Barron et al. 2014). Though most of these approaches have been used to study neuronal activity in vivo, expanding them to studies of microglia in deeper brain structures, and in animals free from the possible effects of anesthesia, remain interesting directions for the future.

4.2.2 *In Vivo Imaging Methods for the Mouse Brain and Spinal Cord*

The breakthrough of two-photon microscopy generated a need for developing new methods to apply this major technological advance to imaging cells in living organisms. In the past two decades, many laboratory animal species have been used for in vivo imaging, including *Drosophila* flies, zebrafish, *Caenorhabditis elegans* (*C. elegans*) worms, mice, rats, cats, and primates. Microglia, for example, have been imaged in vivo mostly in mice, both in the brain and, more recently, in the spinal cord. Proper exposure and immobilization of the imaged tissues are essential for acquiring motion-artifact free, high-resolution images. As a result, several approaches have been exploited for stabilizing the skull and the vertebral column while having optical access to image the cerebral cortex and the spinal cord, respectively (Fig. 4.1c, d; Holtmaat et al. 2005; Davalos et al. 2008).

Two main approaches have been used for in vivo imaging of the mouse cortex: the thinned skull and the chronic cranial window (Fig. 4.1c; Xu et al. 2007; Holtmaat et al. 2009). After skin removal and exposure of the skull, a high-speed dental drill is typically used to remove the upper layers of bone. Subsequently, the skull is very carefully thinned with a surgical blade to a thickness of 20–50 μm (Grutzendler et al. 2002), allowing sufficient light to get through for imaging up to a depth of ~300 μm. For the chronic cranial window, a circular part of the skull is removed using a dental drill. A glass coverslip with optimal optical characteristics replaces the removed skull, allowing imaging up to a depth of 600–700 μm in the cerebral cortex. Each approach has advantages and disadvantages that should be carefully considered depending on the experimental aims. The thinned skull procedure has the advantage of keeping the skull intact and the pial surface injury and inflammation to a minimum (Xu et al. 2007). This may be particularly important for studying acute microglial behavior in the brain as microglia quickly respond to tissue damage as will be discussed later in this chapter. On the other hand, depth penetration and the ability to repeatedly reimage through the same thinned window are limited. Since the thinned part of the bone regrows, the skull-thinning procedure must be repeated every time the same cortical area or individual cells need to be reimaged, over time-periods ranging from days to weeks or months. With every attempt to re-thin the bone, the possibility of causing pial damage and inflammation becomes greater, making it difficult to image through the same thinned window more than three to four times in total. Alternatively, the complete removal of a bone flap to generate a chronic cranial window enables unrestricted repetitive and deep imaging up to a period of a year or even longer, after an initial healing period that limits the applicability of this method for acute studies. This is usually a 2- to 3-week period, which is required for the increased amount of inflammation that this technique introduces to subside and allows for useful imaging data collection thereafter. To overcome these limitations, a combination of both techniques was recently described using a polished and reinforced thinned skull (PoRTS) or thinned-skull cortical window (TSCW) (Drew et al. 2010; Marker et al. 2010). This approach requires placing

a coverslip on top of a thinned and polished area of the skull and, though more technically demanding, can significantly improve the accessibility and durability of thinned skull preparations for long-term imaging of the intact mouse brain.

In contrast to the skull, the spinal cord lies within the flexible structure of the vertebral column, close to the lungs and the heart. This location makes stabilization a major challenge for in vivo imaging of the spinal cord. The first attempt to image the spinal cord in vivo on a regular microscope stage required an elaborate experimental setup with several serial laminectomies, a perfusion system, and animal intubation that allowed breathing to be stopped for each image acquisition (Kerschensteiner et al. 2005; Misgeld et al. 2007). This approach also required extensive image post-processing to align frames that had shifted position during animal breathing intervals. This method was appropriate for imaging and realigning single axons in the spinal cord, but was limiting for use with more dense cellular populations, such as microglia. These limitations were overcome in a more recent method that accomplished stable in vivo imaging of a spinal cord segment using a spinal stabilization device to immobilize the vertebral column (Davalos et al. 2008; Davalos and Akassoglou 2012). In this method, after the paravertebral muscles are separated from the vertebral column, a laminectomy is carried out on a single vertebra. The spinal column, head, and tail base of the animal are then clamped in a way that provides space underneath the body for breathing movements (Fig. 4.1d; Davalos et al. 2008; Davalos and Akassoglou 2012). Since the exposed spinal cord is not sealed with a coverslip during this procedure, repetitive imaging in the spinal cord requires suturing and reopening the musculature over the exposed spinal cord window. This allows for only a limited number of reimaging attempts, as scar tissue can form over time. Recently, however, an implantable window was developed, which is placed over the exposed spinal cord segment with a coverslip that—at least to some degree—limits tissue regrowth (Fig. 4.1d; Farrar et al. 2012). This approach allows for repetitive and unrestricted access to the spinal cord over extended periods of time.

4.2.3 Mouse Models to Study Microglia by In Vivo Imaging

In vivo imaging has been facilitated tremendously by the availability of transgenic animals that express fluorescent proteins in specific cell types (Tsien 1998; Feng et al. 2000). This technology made possible the direct observation and in vivo tracking of many cell types—including microglia—under physiological and pathological conditions (Helmchen and Denk 2005; Germain et al. 2006; Misgeld and Kerschensteiner 2006; Svoboda and Yasuda 2006; Ishii and Ishii 2011; Kawakami et al. 2012). Using fluorescent proteins to image microglia has proven difficult, as microglia are derived from the monocytic lineage and, therefore, share a large number of markers with peripheral cells of the same origin. Additionally, a unique microglial promoter has yet to be identified, so there currently is not a transgenic or knockout mouse line available that can accomplish specific and exclusive genetic

manipulation, including fluorescent protein expression, only in microglia. Nevertheless, since microglia are the only monocytes normally residing in the brain and spinal cord, there are a few mouse lines that have been used as fluorescent reporters of microglia.

The first and most widely used mouse line for in vivo imaging of microglia is the CX3CR1-GFP line that was generated by Steffen Jung in Dan Littman's laboratory (Jung et al. 2000). In this line, the coding sequence for the enhanced GFP is knocked into the *Cx3cr1* locus, partially replacing the *Cx3cr1* exon. Therefore, in homozygosity, this results in GFP expression and a loss of function of the *CX3CR1* protein. The *Cx3cr1* gene is expressed by myeloid precursors in the bone-marrow, circulating monocytes in the blood and dendritic cells throughout the body, but only by microglia in the physiological CNS. While homozygote mice are viable and have bright expression of GFP, they are typically used for studying the effects of *Cx3cr1* deletion. Heterozygote mice that bear one copy of both the *Cx3cr1* and the GFP gene were recently shown to have altered synaptic transmission, learning, and memory (Maggi et al. 2011; Rogers et al. 2011). Nevertheless, as a reporter line, the heterozygous CX3CR1-GFP mice have proven an invaluable tool for in vivo imaging studies of microglia in the CNS.

Two additional mouse lines that further exploit the *Cx3cr1* locus and may also be used as reporters for microglia were recently generated by two different groups. They are the CX3CR1-Cre and CX3CR1-CreER mouse lines (Parkhurst et al. 2013; Yona et al. 2013), in which the coding sequence for the Cre-recombinase is introduced into the *Cx3cr1* locus. The tamoxifen-inducible CX3CR1-CreER line offers the advantage of driving the Cre-recombinase expression in mice through the administration of tamoxifen. When these mice are bred to a reporter mouse line expressing floxed forms of a fluorescent protein, such as the Rosa26-YFP or others, the expression of that fluorescent protein is turned on specifically in CX3CR1-expressing cells. Additionally, using these mouse lines conditional targeted deletion of floxed genes in microglia can be carried out.

An additional microglial reporter line is the one expressing GFP under the *Iba1* promoter (Iba1-GFP) (Hirasawa et al. 2005). These mice were generated via pronucleus injection and carry a transgene that consists of the 5′ non-coding region upstream of the promoter of the ionized calcium-binding adapter molecule 1 (Iba1), exon 1 and the beginning of exon 2. The coding sequence of *Gfp* replaces most of the exon 2 sequence, but all interferon-γ response elements (IRE) are preserved and the genomic *Iba-1* locus also remains functional in this transgenic mouse line. Iba1-GFP mice exhibit GFP expression also outside the brain (e.g., macrophages, liver, and foreleg) and are not used as commonly as the CX3CR1-GFP mice.

Another transgenic mouse line uses the c-fms promoter, also named macrophage colony-stimulating factor receptor (M-CSFR or CSF-1R) or CD115, for GFP expression in monocytes and macrophages (Sasmono et al. 2003; Geissmann et al. 2010). M-CSFR-GFP mice express GFP in microglia and other mononuclear cells. Similar to Iba-1-GFP mice the transgene is randomly introduced into the genome leaving the *M-csfr* gene intact. They have been used to study microglia in the aging and prion-infected brain, as well as to study the role of microglia for neurogenesis

(Sierra et al. 2007, 2010; Ebert et al. 2009; Erblich et al. 2011; Gomez-Nicola et al. 2013). However, whether the expression of GFP is sufficient for two-photon in vivo imaging remains to be determined.

CD11b-DTR transgenic mice may also be used as a microglial reporter. In these mice the diphteria toxin receptor is expressed under the CD11b promoter together with GFP (Duffield et al. 2005). This line has GFP expression in all CD11b-expressing leukocytes, which include monocytes, neutrophils, natural killer cells, granulocytes, microglia and macrophages, a significant percentage of bone marrow cells, and a small percentage of spleen cells. This mouse was recently used to study neurotoxicity of microglia during development (Ueno et al. 2013). Interestingly, the depletion of all CD11b-positive cells, which is accomplished by administration of diphteria toxin, was found to be transient in this mouse model, implicating that the expression of GFP may have a similar time-sensitive expression pattern. Whether the amount of GFP expression is sufficient for two-photon in vivo imaging also remains an open question.

In summary, there are several reporter mouse lines that can be used to study different aspects of microglial function using in vivo two-photon microscopy. Heterozygous CX3CR1-GFP mice provide the best model to visualize microglial morphology, to date. In the future, the generation of a mouse reporter line that specifically and exclusively labels only microglia and not peripheral cells of the monocytic lineage will be instrumental for deciphering the specific roles of CNS-resident microglia in physiology, as well as in neurological disease.

4.3 Histological, In Vitro, and Ex Vivo Studies of Microglia

4.3.1 Histological Studies of Microglia

Prior to the development of the technological and genetic tools that made in vivo imaging possible, studies were performed either in fixed tissue using classic histological staining or immunohistochemical techniques, or by in vitro imaging of cultured cells or ex vivo preparations of CNS tissues. Since the invention of the light microscope, scientists have been compelled to describe the morphological and structural characteristics of cells and tissues and use this information to propose potential functions and cell–cell relationships in different organs. Using a modified silver carbonate impregnation technique to label cells in situ allowed Pio del Rio Hortega to draw microglial cells in their highly ramified morphology as commonly seen in the physiological brain. These drawings were impressively accurate representations of the cellular morphology of microglia in an unchallenged setting. Using his advanced staining technique, Pio del Rio Hortega managed to conduct and publish a number of studies between 1919 and 1927 which put him in a position to describe and propose some of the fundamental principles of microglial biology that remain unchallenged to date (Kettenmann et al. 2011). Interestingly, several studies of the same era also proposed that some brain glial cells were of mesodermal origin

and infiltrated the brain at an early developmental stage (Kettenmann et al. 2011). This concept was only recently confirmed for microglia using elegant genetic fate mapping approaches (Chan et al. 2007; Ginhoux et al. 2010; Kierdorf et al. 2013) and for reviews see (Prinz et al. 2011; Saijo and Glass 2011).

In more recent decades, immunohistochemical studies of healthy and diseased tissue have identified several morphological phenotypes of microglia, which were further characterized as distinct functional states of the cells. The amoeboid microglia which are first seen upon infiltration and differentiation of pial macrophages and peripheral monocyte progenitors during the early developmental stages were shown to be the precursor form of adult microglia (Kaur et al. 2001; Zusso et al. 2012). In the adult CNS, under physiological conditions, microglia bear long processes with many finer branches that have given them the name "ramified" microglia (Fig. 4.2a, c; Kettenmann et al. 2011). Ramified microglia are ubiquitously deployed throughout the CNS, in a mostly regular manner, where they each cover their own territory (Lawson et al. 1990). Interestingly, their distribution varies considerably among different brain regions. For example, they are denser in the hippocampus, basal ganglia, and the substantia nigra than in fiber tracts, the cerebellum, and much of the brainstem. Additionally, they appear to be denser in gray rather than white matter (Lawson et al. 1990). Unlike ramified microglia, the reactive or activated microglia have enlarged, mostly rounded cell bodies and a few short or no processes (Fig. 4.2b, d; Kreutzberg 1996). Activated microglia are typically found around sites of traumatic injury and in most pathological CNS conditions. Some of these distinct microglial phenotypes have been shown to interconvert in vitro, in response to stimulation with cytokines, growth factors, astrocyte condition media, or other stimulating agents like lipopolysaccharide (LPS) and adenosine triphosphate (ATP) (Suzumura et al. 1991; Stence et al. 2001), implying the dynamic capacity of these cells to adapt to changes in their environment.

4.3.2 Studies of Microglia in Cell Culture Conditions In Vitro

Beyond the morphological characterization of cells in situ, however, histological analyses can only allow speculative assessments of cellular functions and cell–cell relationships. In order to confirm or analyze the specific function of a particular cell type, experimental observation and manipulation of living cells is required. The development and continuous improvement of cell culture conditions over the past few decades has allowed numerous studies of living cells in vitro, both in immortalized cell lines and in primary cultures. Although the isolation of primary microglia was already described in 1930, microglia-derived immortalized cell lines have only been established in the last three decades (Rodhe 2013).

Microglial cells in culture express several of the typical markers of the monocytic lineage like CD11b, CD68, FcR (Fragment crystallizable region Receptor), major histocompatibility complex (MHC) class I and II, and many others (Duke et al. 2004; Rodhe 2013). Studying microglial cells in culture has demonstrated some of

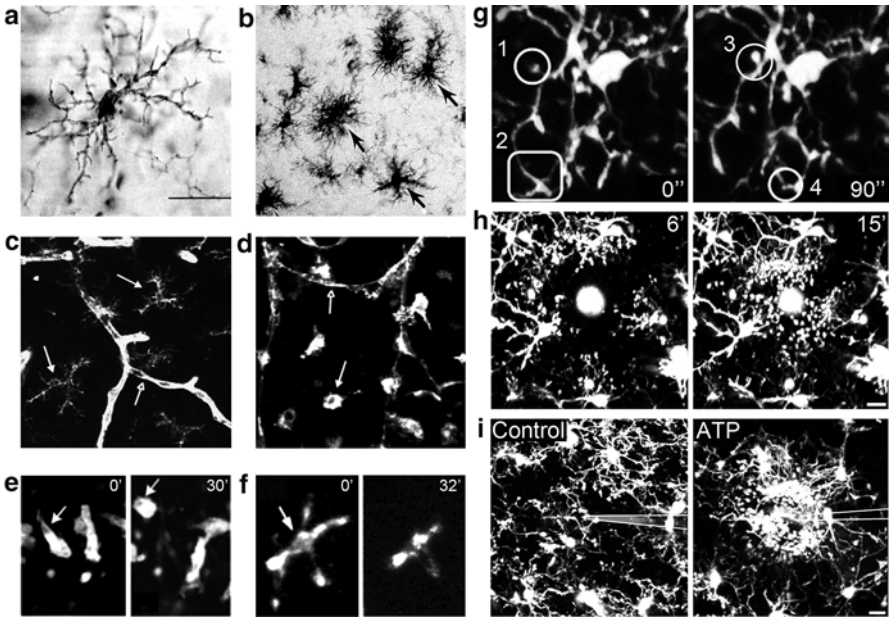

Fig. 4.2 Imaging of microglia in fixed tissue, in vitro, in brain slice cultures, and in the intact brain in vivo revealed different aspects of microglial biology. (**a**) Ramified microglial cell from a 64-year-old human brain and (**b**) activated microglia with a "bushy appearance" from a 41-year-old human brain, stained with the monoclonal antibody LN-3. Adapted from (Streit 2013) and reprinted with permission from Oxford University Press (**c**, **d**) Confocal microscopy images of hippocampal brain slices from a postnatal day 7 rat, stained with FITC-isolectin B4 (IB4), which labels both microglia and blood vessels. Highly ramified microglial cells (*solid arrows*), as well as blood vessels (*open arrows*), are seen when the slice was fixed, stained, and imaged immediately after preparation (**c**), whereas microglia appear rounded or amoeboid when the slice was first left in culture for 1 day (**d**); notice that the blood vessels have also begun to collapse at this point in culture. Adapted from (Dailey and Waite 1999) and reprinted with permission from Elsevier. (**e**) Live-cell imaging of IB4-labeled microglia in culture shows robust changes in shape and movement among different amoeboid cells, over a 30 min time-lapse. Adapted from (Dailey et al. 2006) and reprinted with permission from Springer. (**f**) Ex vivo confocal imaging of microglia in a postnatal day 5 mouse brain slice demonstrates the process of process retraction as cells in the slice get activated. The first time-point in the time-lapse (0 min) was collected within 45 min of sacrificing the animal; while half of the cell's processes are already retracted another 32 min later, all processes are absorbed in the cell body (*arrow*) within 1–2 h from the time of sacrifice (not shown). Adapted from (Stence et al. 2001) and reprinted with permission from Wiley and Sons. (**g–i**) In vivo imaging of GFP-expressing microglia in the cortex of an adult *CX3CR1*$^{GFP/+}$ mouse. (**g**) Time-lapse imaging of the same microglial branches demonstrated rapid extension and retraction of fine microglial processes over seconds. *Circles* and *rounded box* indicate four representative processes that change in length and shape over time. (**h**) Time-lapse in vivo imaging of microglial responses to a localized ablation (bright sphere, ~15 μm in diameter, in the *center*) delivered using the two-photon laser inside the mouse cortex. Nearby microglial processes responded immediately with bulbous termini and started extending within just a few minutes toward the ablation until they formed a spherical containment around it (not shown); at the same time, the same cells retracted those processes that lay in directions opposite to the site of injury. (**i**) Microglial response to local injection of adenosine triphosphate (ATP) at the 30 min time point after insertion of the injecting glass electrode (*white lines*) through a craniotomy. Whereas a control microelectrode containing artificial cerebrospinal fluid (ACSF) and rhodamine-dextran caused little or no microglial response (*left*), similar electrodes containing 10 mM ATP induced rapid extension of microglial processes towards the tip of the electrode (*right*). Panels g-i adapted from (Davalos et al. 2005)

their classic known abilities, such as their membrane ruffling upon stimulation, chemotactic migration towards a concentration gradient, activation and secretion of cytokines in the culture medium following stimulation, and phagocytic behaviors (Smith et al. 1998; Hide et al. 2000; Honda et al. 2001; Shigemoto-Mogami et al. 2001; Wollmer et al. 2001; Xiang et al. 2006; Ohsawa et al. 2007; Jeon et al. 2012). In vitro studies have the advantage that they can be performed under well-defined conditions, allowing the experimental manipulation of cells by transfection of exogenous genes and addition of stimulatory or inhibitory agents. As a result, in vitro cell culture studies have elucidated both cellular and molecular properties and capabilities of microglia under specific experimental conditions. However, as is the case with all cell types, both immortalized and primary microglial cultures have significant limitations with respect to the artificial environment that cells grow in, their viability, and their relevance to cells in their physiological environment.

4.3.3 Ex Vivo Studies of Microglia in Acute or Organotypic Slice Cultures

To overcome some of the in vitro limitations, microglia have been studied in their endogenous microenvironment, mostly within acutely extracted tissue from brain slices. In the field of neuroscience, brain slice preparations have been used in electrophysiological, morphological, biochemical, and pharmacological studies of brain structures. For example, imaging studies performed in brain slices have expanded our understanding of dendritic calcium dynamics and the biochemical signals regulated by neuronal activity (Svoboda et al. 1997; Nagerl et al. 2004; Lohmann et al. 2005; Yasuda et al. 2006; Muller et al. 2012). Brain slice preparations provide precise control of experimental conditions, such as temperature, pH, oxygen, carbon dioxide (CO_2), and ionic and drug concentrations, while maintaining cells in the context of the tissue that surrounds them. As such, these preparations have facilitated the examination of electrophysiological properties and metabolic parameters of intact neuronal and glial cells, which can easily be located, identified, and accessed within a brain slice. A technical advantage of brain slices over in vivo studies is that they can be performed without contamination from anesthetics, muscle relaxants, or intrinsic regulatory substances (Wang and Kass 1997). They also offer great stability for imaging purposes and electrophysiological recordings due to the lack of movement artifacts that are generated from the heartbeat and respiration pulsations.

On the other hand, brain slices also have inherent limitations. Although the architecture and connectivity of the spared tissue can be maintained and kept functional within a distance from the edges of a slice and for a certain period of time, brain slices lack inputs and outputs from distant connections that have been severed. In addition, both acute and organotypic brain slices have a limited lifespan, with the first being viable for a few hours and the latter for a few days to weeks, depending on the preparation and the goal of the study (Lossi et al. 2009). Moreover, since

blood circulation through the vascular system cannot be maintained, even evolved artificial bathing media that have improved the viability of slices lack blood-derived factors and the physiological signaling that occurs between vascular, neuronal, and glial cells. The effects of decapitation ischemia produced by the loss of blood supply and the stress that the tissue undergoes until bathed in media are not well understood, and probably significantly underestimated (Mayevsky 1978). Most importantly for microglial studies, slicing an acutely extracted brain delivers extensive tissue injury, especially at the top and bottom surfaces of the slice. Although this may not appear to affect neuronal and other glial cell populations that may continue to function "physiologically" as long as they are located further away from the slice surfaces, the same is not true for microglia. Microglia have myeloid origins and are, thus, immune cells programmed to respond to tissue damage in the brain or the spinal cord (Thomas 1992; Kreutzberg 1996; Gonzalez-Scarano and Baltuch 1999; Raivich et al. 1999). As tissue injury is an unavoidable effect of the preparation procedure, brain slicing causes microglia to become activated and undergo a series of changes in their cellular morphology, behavior, and gene expression profile, the degree of which depends on the severity of the incurred injury (Fig. 4.2c, d; Davis et al. 1994; Stence et al. 2001; Petersen and Dailey 2004). Some of the most commonly observed functions of activated microglia include cell proliferation, migration, phagocytosis, and up-regulation of typical immunological markers and antigen-presenting cell capabilities (Kreutzberg 1996; Aloisi 2001; Carson 2002). Several of these functions were first observed and described before the advent of two-photon microscopy, by ex vivo imaging studies performed in brain slices using white light or fluorescence microscopy (see below).

4.4 Imaging Studies of Microglial Morphology and Activation In Vitro or Ex Vivo

The ability of microglia to perform highly active movements has been evident since the earliest imaging studies were performed in dissociated cell cultures (Fig. 4.2e; Booth and Thomas 1991; Ward et al. 1991; Haapaniemi et al. 1995; Tomita et al. 1996; Takeda et al. 1998). Imaging microglia by time-lapse confocal microscopy in acutely extracted mouse brain slices led to a detailed description of the morphological transformation that they undergo from their presumed sessile ramified phenotype in the unperturbed brain (Stoll and Jander 1999) to that of fully activated cells that migrate to sites of injury, typically towards the surfaces of the slices (Czapiga and Colton 1999; Stence et al. 2001). This transformation included almost complete withdrawal of microglial "resting" processes before activated microglia were able to migrate, proliferate, and phagocytose dead or injured cells and cellular debris (Fig. 4.2f; Stence et al. 2001). Interestingly, under acute slicing conditions, preexisting ramified branches were found to be incapable of extension and only underwent a slow retraction, sometimes until complete withdrawal, while new "reactive" processes had to form for activated microglia to phagocytose injured cells or debris

(Stence et al. 2001). Similarly, different microglial motility behaviors were described in mouse brain slices towards recently deceased versus preexisting dead cells (Petersen and Dailey 2004).

In another confocal time-lapse video microscopy study, microglial migratory patterns were studied in acute mouse brain slices in which a stab wound injury was delivered 1–7 days prior to slice preparation. As a result, microglia in the injured hemisphere had sufficient time to become activated by the injury in vivo, and their motility patterns were compared to those of cells in the contralateral, uninjured hemisphere (Carbonell et al. 2005). Microglia activated by the stab wound injury exhibited increased migratory activity for 1–4 h after acute brain slice preparation from mice sacrificed 1 and 3 days after injury. These motility patterns were not directed but instead appeared autologous and non-biased and were hence characterized as a "random walk" (Carbonell et al. 2005). The behavior of microglia in organotypic slice cultures was shown to vary significantly over time and with the preparation methods, as discussed above, but also between animal species and medium contents (Czapiga and Colton 1999).

The analysis of "snapshots" of biological events by classic histological approaches could hinder the full appreciation of the sequence or causality between ongoing cellular processes, responses to stimuli, and transient cell–cell interactions (Davalos and Akassoglou 2008). Although some of these phenomena can be studied ex vivo, the preparation and conditioning of slice cultures inherently activates microglia, the first responders to brain injury (Dailey and Waite 1999). Therefore, careful consideration of the physiological relevance of observed microglial responses in slice studies is required, as many of the cells are exposed and hence react to the massive tissue injury in their vicinity. In contrast, imaging microglia in their intact environment represents a reliable approach to investigate their interactions with the surrounding tissue, in the absence of underlying injury signals.

4.5 Redefining the Role of Microglia by Imaging Them In Vivo for the First Time

4.5.1 Baseline Process Dynamics

For many decades, the highly branched microglia in the unperturbed adult brain were considered to be largely inactive. As a result, ramified microglia were characterized as "resting", underscoring the limited functional role that was attributed to the cells under normal conditions compared to their activated state (Streit et al. 1988; Davis et al. 1994; Stoll and Jander 1999). The concept of "resting" microglia in the physiological brain was challenged by the first in vivo imaging studies of microglia in the mouse cortex, published in 2005. By performing transcranial two-photon imaging in *CX3CR1*$^{GFP/+}$ mice, Davalos et al. and Nimmerjahn et al. showed that the higher order processes of ramified microglia demonstrate a highly motile

behavior, while their cell bodies and main processes remain relatively stationary (Fig. 4.2g; Davalos et al. 2005; Nimmerjahn et al. 2005). These real-time recordings of the continuous cycles of small extensions and retractions of the finer microglial processes were the first in vivo demonstration of the tissue surveillance function of microglia in the unperturbed mammalian brain, in real time. Moreover, this was a completely unexpected result that generated extensive interest in the physiological significance of microglia and is currently being investigated by many groups globally.

4.5.2 Rapid Response to Focal Brain Injury

The first imaging study of microglial behavior in a living organism was performed in the leech, examining their responses to massive nerve injury. By labeling only cell nuclei and using ultraviolet illumination for fluorescence video microscopy, invertebrate microglia were found capable of rapidly migrating along axonal tracks towards nerve lesions from distances often further than 200 μm away (McGlade-McCulloh et al. 1989). These migratory responses were similar to those described in mouse brain slices that were discussed above and seem to be typical for microglia challenged with massive neuronal injury. The ability of microglia to rapidly contain localized damage in their vicinity was also first described by in vivo two-photon microscopy in *CX3CR1*$^{GFP/+}$ mice.

By taking advantage of the focal properties of the two-photon laser, Davalos et al. and Nimmerjahn et al. introduced small focal injuries in the mouse cortex, delivered either to the brain parenchyma or on cortical blood vessels. In both cases, neighboring microglia exhibited very rapid responses by extending their processes directly towards the sites of injury and physically containing them (Davalos et al. 2005; Nimmerjahn et al. 2005). Microglial processes showed enlarged bulbous termini within seconds and began extending within only a few minutes (Fig. 4.2h). Interestingly, when challenged with two injuries very close to each other, individual microglial processes of even the same cell can differentially respond by extending towards the nearest injury site (Davalos et al. 2005). These studies showed that acute microglial responses to localized injury delivered in the living brain were significantly different from those previously described in ex vivo setups, thereby challenging the concept that a "reactive transformation" was required for microglia to respond to traumatic injuries. Injuries that can be contained locally do not necessarily require process retraction, migration, and proliferation of microglia, as described in brain slice experiments. Also, preexisting processes of the ramified microglia are capable of morphological activation within seconds after they are challenged and can individually respond to nearby damage without delay (Davalos and Akassoglou 2008). In line with these observations, it was recently shown in situ that microglial phagocytosis of apoptotic neurons in the physiological brain is fast and efficient, can be performed by ramified cell processes, and does not require

pre-activation of the cells by an inflammatory or a traumatic challenge (Sierra et al. 2010). Finally, microglial process extension was always directed towards the sites of injury (Davalos et al. 2005; Nimmerjahn et al. 2005). Cell body migration was not observed over the first 10 h after laser-induced injury (Davalos et al. 2005), but likely occurs over longer periods, since an increase in microglial numbers was reported after reimaging in vivo the same sites 1–3 days after injury (Kim and Dustin 2006), unlike what was previously shown ex vivo following stab wound injury in mice (Carbonell et al. 2005).

4.5.3 Discrepancies Between In Vitro and In Vivo Imaging Studies of Microglial Behavior

Though similar approaches might have proven more reliable for studying other cell types, attempting to predict microglial behavior in the intact CNS based on what had previously been described either in culture or in brain slices proved largely unsuccessful. The conflicting findings likely represent the different cellular and molecular properties that microglial cells have to acquire under artificial conditions in vitro, compared to those in the intact CNS. Interestingly, dissimilarities in the findings from in vitro and in vivo studies of microglial biology are not only limited to imaging studies of cell morphology and behavior. Gene expression profiles in cultured microglia differ significantly among cells that were extracted using different isolation methods, and often more so if compared to microglia in vivo. A comprehensive and comparative transcriptional profiling following LPS stimulation of microglia in vitro and in vivo was done for 480 inflammation-related genes and analyzed by custom-spotted microarray analysis (Lund et al. 2006). The outcome of these studies was correlated with data from a proteomics analysis and genome spanning Affymetrix chips to evaluate differences between methodologies and to obtain additional information on the regulation of non-inflammatory genes and proteins (Lund et al. 2006). This thorough comparison of the effects of the most commonly used activating molecule for microglia showed that although most inflammatory genes induced in vitro were also upregulated in vivo, several discrepancies were identified between the two experimental settings. These differences may offer the molecular basis of the observed functional and behavioral differences for microglia in vivo and are likely due to regulatory factors and compensatory mechanisms present in the CNS but not in a dish. Nevertheless, the identification of such discrepancies and limitations does not discredit the observations made in vitro or ex vivo, but highlights the importance of fully acknowledging the applicability and limitations of each experimental design. Accurate interpretation of the results acquired under specific experimental conditions is imperative for placing study findings in the appropriate context, especially for cells as responsive to changes in their microenvironment as microglia.

4.6 Regulation of Microglial Process Dynamics In Vivo

The surprising features of the observed microglial behaviors in vivo created the need to study their molecular properties and signaling mechanisms in their natural habitat inside the living brain (Davalos and Akassoglou 2008). Among the first questions asked were about the mechanisms that regulate microglial tissue surveillance, and about the nature of the molecules involved in attracting microglial processes towards sites of damage. Several molecules have been shown to activate microglia, induce morphological changes, and attract them in a concentration gradient-dependent manner in vitro. However, most studies have addressed these issues on a time scale of several hours to days, in accordance with typical immunological response paradigms. The constant motility of microglial processes in unperturbed conditions implies a mechanism that is acutely regulated within the physiological brain. Moreover, the rapid directional convergence of microglial processes towards a localized injury implies the presence of a gradient of one or more highly diffusible and abundant molecules that can mediate this phenomenon.

4.6.1 Regulation of Rapid Microglial Responses to Localized Injury

Davalos et al. demonstrated that extracellular ATP is sufficient for mediating rapid microglial responses by directly injecting ATP in the living cortex and imaging microglial responses in vivo (Fig. 4.2i). Using several pharmacological inhibitors applied directly on the exposed cortex, they showed that ATP signaling through P2Y receptors is essential for mediating microglial responses to local ATP application, as well as to focal brain injury (Davalos et al. 2005). This result was genetically confirmed by another in vivo imaging study that used microglia lacking the P2Y12 receptor and showed that they are incapable of responding to laser-induced injury in vivo (Haynes et al. 2006). Indeed, ATP is a highly diffusible small molecule that is also highly concentrated intracellularly, but not present in the cerebrospinal fluid, making ATP a good candidate for explaining the rapid microglial process responses as they were imaged in real time in vivo, following tissue damage.

Since these responses required process extension lasting for 30–60 min until the injury was contained, how could a single blast of ATP from the injured cells sustain microglial responses to the site of injury? One possibility is that the surrounding tissue plays a role in preserving and regenerating the signals that fuel the observed microglial response to damage. Indeed, pharmacologically blocking ATP release from astrocytes in vivo was sufficient to arrest microglial process extension to laser injury, implying a role for astrocytes in mediating the rapid and widespread microglial response to focal brain injury (Davalos et al. 2005). The ability of ATP to act as a chemoattractant of microglia in vitro had previously been recognized (Honda et al. 2001), and there is extensive literature describing the activating effects of

purinergic signaling on microglial cell lines, primary cells, and microglia in brain slices (Hide et al. 2000; Honda et al. 2001; Shigemoto-Mogami et al. 2001; Xiang et al. 2006; Ohsawa et al. 2007; Inoue 2008; Koizumi et al. 2013). However, the study of this phenomenon inside the intact brain, where all cellular constituents are present, allowed the identification of this intriguing cell–cell interaction between astrocytes and microglia and revealed their orchestrated responses to brain injury (Davalos et al. 2005). Follow-up studies done in acute brain slices were able to recapitulate aspects of the rapid microglial responses to ATP and characterize some of the molecular downstream players involved in them. For example, the generation of outward potassium currents, the phosphoinositide 3-kinase (PI3K) pathway (Wu et al. 2007), and the activation of integrin-β1 (Ohsawa et al. 2010) were shown to be involved in the directional process extension by microglia in brain tissue. The role of ATP signaling in microglia is discussed in further detail in Chap. 3.

A similar mechanism of ATP-dependent rapid microglial responses to focal laser injury was also described in white matter by in vivo imaging in the dorsal mouse spinal cord (Lee et al. 2008). This study revealed that the nitric oxide (NO) pathway is also involved in regulating rapid microglial responses to injury. Using a combination of local inhibition or activation experiments for both the NO and ATP pathways, Dibaj et al. demonstrated that blocking the NO pathway arrests microglial responses, which can be triggered by donors and downstream compounds of the NO pathway. Interestingly, though the ATP effect on microglia was found to be independent of the NO pathway, a substantial purinergic influence on the NO-mediated microglial attraction was evident (Lee et al. 2008). The involvement of the NO pathway in mediating microglial migration to neuronal injury had previously been demonstrated in the leech nerve cord, in an elegant study combining a similar array of pharmacological treatments and low-light fluorescence video microscopy to track individual Hoechst-stained microglia as they migrated to a crush site (Chen et al. 2000). Finally, volume-sensitive chloride channels were shown to be required for the rapid process outgrowth of microglia in response to laser-induced damage in brain slices (Hines et al. 2009). The same study showed that the microglial containment of injury sites prevented lesion expansion at least in the acute brain slice setting, suggesting a beneficial role for this rapid and localized microglial response following brain injury (Hines et al. 2009).

4.6.2 Regulation of Microglial Baseline Process Motility

The striking observation that microglial processes are so dynamic, even under physiological conditions, has attracted a lot of attention since it was first described by performing in vivo imaging in the mouse cortex. Similar baseline motility observations were made in vivo in the developing zebrafish embryo (Peri and Nusslein-Volhard 2008), as well as in mouse spinal cord and retinal explants under physiological conditions (Davalos et al. 2008; Lee et al. 2008). This implies a function of fundamental significance for all anatomical sites of the CNS. What is the

biological significance of the "baseline motility" pattern of microglial processes? Are they simply performing surveillance of the extracellular space to ensure structural integrity of their immediate neighbors, or are they participating in physiological brain functions? What are the molecular signals and downstream mechanisms that mediate these small extension and retraction events? Do these events play different roles in the developing, adult, and diseased CNS? Such questions became the subject of several studies in postnatal development, adult neuronal plasticity, and neuronal circuit function and dysfunction. While several of these matters are discussed in depth in separate chapters of this book, we will highlight some of the findings that emerged from in vivo imaging studies.

One hypothesis is that microglial processes extend rapidly in response to neuronal synaptic activity in their vicinity. This is certainly a reasonable assumption, since microglial baseline process dynamics are very transient, similar to events occurring in nearby synaptic clefts. However, reducing neuronal activity with tetrodotoxin (TTX, a sodium channel blocker) had no significant effect on baseline microglial dynamics in vivo, while enhancing synaptic activity with a γ-aminobutyric acid (GABA)-receptor inhibitor had a slightly stimulating effect at increasing their sampled area (Nimmerjahn et al. 2005). In both cases, the velocity of microglial process changes was also unaffected, suggesting that microglial tissue surveillance may not be directly modulated by neuronal activity in their vicinity. This finding was in agreement with a study in acute hippocampal slices where simultaneous electrophysiological recordings and time-lapse confocal microscopy showed that glutamate, GABA, or long-term potentiation did not induce chemotaxis in microglia (Wu and Zhuo 2008).

Interestingly, sensory deprivation by binocular enucleation in mice induced a reduction of neuronal activity, which was followed by a prominent retraction of microglial processes in the visual cortex over a few hours (Wake et al. 2009). This study performed the first in vivo imaging analysis of the interaction between microglia and axonal boutons or dendritic spines, the sites of synaptic connections between neuronal cells. By crossing the Iba1–GFP mice with a Thy1-driven neuronal reporter line (the GFP-expressing M-line (Feng et al. 2000)), Wake et al. detailed the frequency and duration of direct contacts between microglial processes and pre- and post-synaptic neuronal structures. They found a clear reduction in the number of contacts with pre-synaptic axon terminals following a reduction of neuronal activity, implying that at least part of the microglial baseline surveillance activity is directed toward synapses, and may depend on their functional status (Wake et al. 2009). Interestingly, following an ischemic challenge, microglial processes made prolonged contacts with axon terminals that lasted for >60 min (versus ~5 min in normal conditions), and sometimes resulted in the disappearance of the neuronal structures (Wake et al. 2009).

The possibility that microglia may be involved with the removal of neuronal processes was explored in another elegant study that used in vivo two-photon microscopy in combination with immunocytochemical electron microscopy (EM) and serial section EM followed by tedious three-dimensional (3D) reconstruction analyses. Tremblay et al. crossed CX3CR1-GFP with the Thy1-YFP H-line

(Feng et al. 2000) to label microglial and neuronal processes with different fluorescent proteins and record their interactions over time, in the presence or absence of sensory experience in the visual cortex of juvenile mice (Tremblay et al. 2010). In normal conditions, microglial processes interacted mostly with small and transient dendritic spines, which were typically lost over 2 days. Following light deprivation, however, microglial processes became less motile and shifted their preference towards larger dendritic spines that persistently shrank (Tremblay et al. 2010). The EM analysis confirmed the extensive envelopment of synaptic elements and the presence of phagocytic structures sometimes with ultrastructural features of axon terminals and dendritic spines inside of microglial processes, particularly under conditions of sensory deprivation, in the juvenile mouse cortex (Tremblay et al. 2010). Interestingly, similar inclusions were also observed in the aged brain (Tremblay et al. 2012). Overall, these findings imply a role for microglia in synaptic stripping throughout life.

Microglia are indeed essential for sculpting the developing mammalian brain, by contributing to the elimination of newborn cells, neurons, and synapses, both in the early postnatal days and in adulthood (Marin-Teva et al. 2004; Sierra et al. 2010; Paolicelli et al. 2011). In accordance with the in vivo observations, another elegant set of studies in the developing retino-geniculate system has demonstrated in situ that sculpting of this network requires microglial engulfment and remodeling of synaptic connections in a complement C1q and C3/C3R-dependent manner (Stevens et al. 2007; Schafer et al. 2012). Interestingly, the initiation of the complement protein expression seems to be regulated by transforming growth factor β (TGFβ) (Bialas and Stevens 2013), and there is a clear link between the complement-mediated synaptic pruning by microglia and neuronal activity (Schafer et al. 2012). For further information about the role of microglia in development, please refer to Chaps. 7, 8, and 9. Overall, there are several indications linking the microglial baseline process motility with synaptic activity and neuronal network modulation, but we are only beginning to uncover the cellular and molecular mechanisms involved in these processes and the potential impact they may have for neuronal function.

4.7 Imaging Studies of Microglia in the Retina

The retina is considered a part of the CNS and it is often preferred by investigators as an experimental model due to its simple cellular architecture and accessibility, compared to the brain and spinal cord. Proper preparation of whole retinal explants seems to introduce less tissue damage to the retina itself than brain slicing, an attribute that is likely responsible for the recapitulation in these preparations of several microglial behaviors previously described in the cortex in vivo. For example, ex vivo imaging of microglia in retinal explants using time-lapse confocal imaging showed similar baseline extension and retraction process dynamics to the adult unperturbed cortex (Lee et al. 2008). In response to laser injury, ramified microglia responded by rapid processes extension, as well as cell migration (Lee et al. 2008).

Abolishing the fractalkine receptor (CX3CR1) in microglia diminished both the responses to injury and the baseline dynamics of microglial processes in retinal explants (Liang et al. 2009). This finding implies that these microglial responses may be regulated through the fractalkine ligand–receptor interaction expressed in neurons and microglia, respectively. Moreover, both the morphology of microglia and their process dynamics were differentially regulated by different modes of neurotransmission in retinal explants. Specifically, there was an increase in baseline motility by glutamatergic and a decrease by GABAergic neurotransmission (Fontainhas et al. 2011). However, similar to experiments in the mouse cortex, these neurotransmitters could not induce microglial responses directly; instead, they appeared to be mediated indirectly via extracellular ATP released in response to glutamatergic neurotransmission through probenecid-sensitive pannexin hemichannels (Fontainhas et al. 2011). Interestingly, the baseline dynamics of microglial processes were also reduced in the mouse cortex, in the presence of pharmacological inhibitors of purinergic receptors (Davalos et al. 2005). Overall, these observations imply that retinal explants might offer a good ex vivo alternative for studying microglia in a controlled setting that may still preserve some fundamental aspects of the in vivo condition. Nevertheless, since retinal explants are still ex vivo preparations devoid of blood circulation, they also only remain stable for limited periods of time.

The retina has also been imaged in vivo in CX3CR1-GFP mice using confocal scanning laser ophthalmoscopes (cSLO). Highly motile microglia were shown to migrate from distances up to 400 μm towards laser-induced injuries, starting from a few minutes to hours, and persisting at the injury sites for at least a few weeks (Eter et al. 2008; Paques et al. 2010). Similarly, longitudinal cSLO imaging was used to track microglial responses in models of retinal ganglion cell damage. Following optic nerve crush injury and acute elevation of intraocular pressure, microglia transformed from a ramified to an amoeboid morphology and significantly increased in numbers over the first week post-injury. These microglia gradually returned to the ramified morphology and near-baseline cell densities over 3 additional weeks (Liu et al. 2012). These results show that the massive microglial responses that were previously described in brain slice experiments also occur in vivo, but only under conditions of extensive tissue injury that require recruitment of distant cells to contain such widespread trauma. They also underscore the adaptability of the brain's resident immune cells to different types of damage as well as their ability to return to a nonactivated state once a particular insult has been addressed.

On the other hand, even microglia are limited in their abilities to address some challenges, especially those that are of a chronic nature, such as in the context of neurological disease or normal aging. For example, ex vivo imaging in a retinal explant identified a number of morphological and functional impairments in aged microglia that were also recently confirmed in vivo, in the mouse cortex (Hefendehl et al. 2014). Unchallenged cells presented with significantly smaller and less branched dendritic arbors and exhibited slower process motilities (Damani et al. 2011). In addition, while young microglia extended processes toward extracellularly applied ATP, aged microglia became less dynamic and ramified. In response to laser-induced injury, aged microglia demonstrated slower responses acutely, which

were sustained for longer than those of microglia in younger retinas (Damani et al. 2011). The compromised responses of senescent microglia both in terms of tissue surveillance and in response to injury reveal potential roles for microglia in age-dependent neuroinflammatory degeneration of the CNS.

4.8 In Vivo Imaging Studies of Microglia in CNS Disease

Microglia have been implicated in the pathogenesis of neurodegenerative and neuroinflammatory diseases, such as Alzheimer's disease (AD), Huntington's disease (HD), age-related macular degeneration, multiple sclerosis (MS), amyotrophic lateral sclerosis (ALS), and several others (Perry et al. 2010). The role of microglia in MS and AD will be discussed in detail in Chaps. 16 and 18, respectively. In the following paragraph, we will primarily focus on imaging studies of microglia related to these diseases. The involvement of microglia in pathologies of both the brain and spinal cord can be acute or chronic, and depending on the disease or tissue context, microglia may exert harmful or beneficial effects. As discussed above, microglia can contain injuries and limit the damage to surrounding healthy tissue, by clearing cellular debris in the eye, the spinal cord, or the cortex. Their accumulation around lesions can also be found in most neurological diseases. For example, microglia reside around amyloid beta (Aβ) plaques in AD, and they are found in increased numbers in prion disease, in the substantia nigra in Parkinson's disease, in MS lesions, etc. However, whether their chronic activation in many of these pathologies is protective or damaging remains largely unknown. In recent years, animal models for most neurological diseases have been developed and tremendously improved, and although they still present limitations, some can recapitulate several aspects of the human conditions to a satisfactory degree. Given the very dynamic nature of microglial responses to their microenvironment, using two-photon microscopy to follow microglial responses over the course of disease in available animal models represents a powerful approach to further elucidate their role and understand how to treat them in the context of CNS disease.

4.8.1 In Vivo Imaging of Microglia in Neurodegeneration: Alzheimer's Disease

AD is characterized by the accumulation of Aβ in plaques and by the intracellular aggregation of hyper-phosphorylated protein tau (Haass and Selkoe 2007; Palop and Mucke 2010). In AD, increased numbers of activated microglia have been identified around Aβ plaques (Fig. 4.3a) and in proximity of hyper-phosphorylated tau-bearing neurons (Hanisch and Kettenmann 2007; Kettenmann et al. 2011), suggesting that AD exhibits a prominent neuroinflammatory component comprising the innate immune system. Two-photon in vivo imaging has had a significant impact on AD

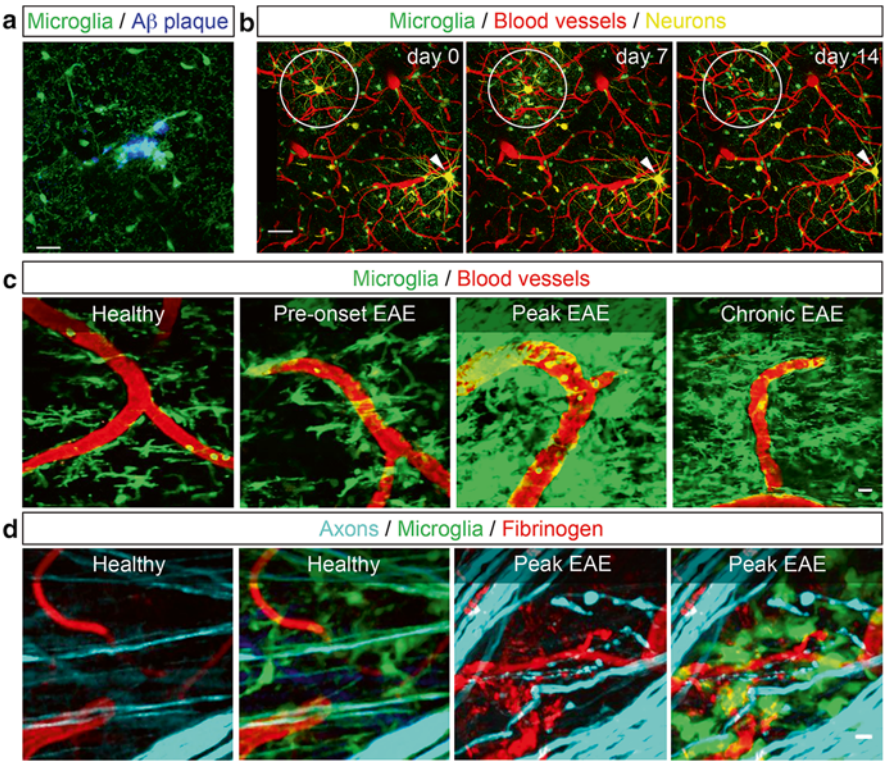

Fig. 4.3 In vivo imaging of microglia in mouse models of neurological disease. (**a**) Microglia (*green*) accumulate around amyloid beta plaques that are stained with MethoxyX04 (*blue*). (**b**) Microglia (*green*) increase in density around neurons that subsequently disappear (*yellow*) suggesting that they are involved in neuronal loss in a mouse model of Alzheimer's disease. Blood vessels (*red*) are labeled with an intravenous injection of Texas-red dextran. Adapted from (Fuhrmann et al. 2010). (**c**) Microglia (*green*) cluster around blood vessels (*red*, labeled as in **b**) at different stages of neuroinflammatory disease. Microglial perivascular clusters start forming before the onset of clinical signs, become very pronounced at the peak of experimental autoimmune encephalomyelitis (EAE), and continue forming and resolving even through the chronic phase of the disease. (**d**) In the spinal cord of healthy *CX3CR1*$^{GFP/+}$*Thy1-CFP*$^{+}$ mice ramified microglia (*green*) appear evenly distributed among healthy intact axons (*cyan*) and blood vessels (*red*, labeled by intravenous administration of fibrinogen solution). In contrast, at the peak of disease, blood-brain barrier disruption results in perivascular fibrin deposition in the perivascular space of leaky vessels; these areas of fibrin deposition define the sites of perivascular microglial clusters and the areas where neuronal damage (the main culprit of multiple sclerosis) is evident, in the form of swelling, bending, and fragmentation of axons. Adapted from (Davalos et al. 2012). Scale bars: 20 μm (**a**, **c**), 10 μm (**b**), 5 μm (**d**)

research and our understanding of the role of microglia in this disease. Meyer-Luehmann et al. investigated the formation of new plaques and showed in a semi-quantitative way that microglia were rapidly recruited to newly formed Aβ plaques, using in vivo labeling of Aβ with the blood-brain barrier (BBB)-permeant dye MethoxyX04 (Meyer-Luehmann et al. 2008). In another study,

Bolmont et al. (2008) observed plaques that increased or decreased in size over a 4-week period. In this study, the authors performed an in-depth in vivo imaging analysis of the relationship between microglia and plaques in an APP/PS1 transgenic mouse model of Aβ deposition crossed with the IBA1-GFP microglial reporter (Bolmont et al. 2008). They found a positive correlation between the size of amyloid plaques and the number of microglia associated with them. Moreover, they analyzed the migration of microglia in the proximity of Aβ plaques and found that while the majority of microglial cell bodies were sessile, a small proportion of cells were moving. Specifically, during a 24-h period, they observed microglia migrating towards Aβ plaques by first extending a main process and then subsequently pulling the soma along the axis of this process towards the plaque. In the proximity of Aβ plaques, Bolmont et al. found an increase in total microglial process length, which was significantly reduced for cells directly contacting the plaque. The authors found no difference with respect to the baseline motility of microglial processes in AD mice (Bolmont et al. 2008).

Interestingly, Bolmont et al. also examined whether microglia are involved with the phagocytosis of Aβ. They showed that microglial lysosomal-associated membrane protein 1 (LAMP-1)-positive lysosomes contained some MethoxyX04-labeled Aβ, suggesting that microglia can uptake Aβ, and thereby providing an in vivo assay to measure Aβ-phagocytosis (Bolmont et al. 2008). Liu et al. (2010) later demonstrated that microglia are unable to phagocytose fibrillar Aβ, since MethoxyX04-labeled plaques did not diminish in size over time, but instead found newly formed protofibrillar—presumably oligomeric—Aβ within microglial lysosomes. Moreover, they concluded that the microglial fractalkine receptor CX3CR1 is involved in regulating Aβ-uptake, since CX3CR1-deficient mice exhibit an increased amount of Aβ inside microglia and a diminished number of amyloid plaques. However, decreased plaque size (Aβ clearance) after microglial recruitment was not observed by Meyer-Luehmann et al., suggesting that microglial phagocytosis is not effective, or at least cannot compensate for continuous deposition. Overall, though the process of engulfment of Aβ has not been recorded in real time in the living brain to date, increasing the phagocytic capacity of microglia may represent a credible target for the treatment of AD. Indeed, microglia were involved in antibody-mediated clearing of Aβ in mouse models of AD, but their contribution was found to be limited (Garcia-Alloza et al. 2007; Koenigsknecht-Talboo et al. 2008). However, the treatment periods in these studies were limited to several days, and the time point of treatment may also be critical. Thus, whether boosting the phagocytic activity of microglia, without changing the inflammatory profile, represents a valuable strategy to treat AD remains unresolved.

Another method in which to target microglia for the treatment of AD may be to improve the fine-process motility of microglia in response to tissue damage, as this process was shown to be impaired in the proximity of Aβ plaques (Krabbe et al. 2013). While many studies have investigated the relationship of microglia and Aβ, there is only one study that has analyzed the relationship of microglia and neurons in a mouse model of AD, particularly the 3xTg-AD which display both Aβ accumulation and tau hyperphosphorylation. Herein, Fuhrmann et al. used two-photon in vivo imaging to show that microglia migrated towards neurons in advance of their

elimination, in the somatosensory cortex still devoid of Aβ plaques at 4–6 months of age (as confirmed in situ using correlative histological analyses of the same neurons imaged in vivo), and subsequently displayed a reduction in fine process motility once the neurons were lost (Fig. 4.3b; Fuhrmann et al. 2010). Additionally, they found that the CX3CR1 was involved in this process, since CX3CR1-deficient mice were rescued from microglia-mediated neuronal loss (Fuhrmann et al. 2010). The mechanisms underlying this loss of neurons are still unclear and could involve microglial release of cytokines, chemokines, and neurotoxins.

Several in vivo imaging studies have now been performed using mouse models of AD to investigate Aβ plaque formation, stability and growth, the kinetics of dendritic spine loss in relation to the proximity of Aβ plaques, etc. However, the potential contribution of microglia to amyloid deposition, plaque formation and/or clearance, and the potential role of microglia in shaping neuronal networks in AD are still very poorly understood. Besides the classical aspects of AD like synapse-, neuron-loss, Aβ-deposition, tau-hyperphosphorylation and gliosis, future studies using in vivo imaging approaches may focus also on new risk factors identified for AD (Seshadri et al. 2010) and help design new strategies for patient treatments.

4.8.2 In Vivo Imaging of Microglia in Neuroinflammation

The involvement of microglia with neuroinflammatory diseases such as MS and ALS has been known for decades. However, in vivo imaging has only recently been employed to study the role of microglia in neuroinflammation at different stages of disease in available mouse models. In the experimental autoimmune encephalomyelitis (EAE) animal model of MS, mice are immunized with a myelin antigen, resulting in the development of autoimmune disease that simulates several of the pathological characteristics of human MS, such as demyelination and axonal damage in the CNS. Davalos et al. evaluated the progression of microglial responses at different stages of EAE by performing longitudinal in vivo imaging in the spinal cord of CX3CR1-GFP mice, using a previously developed imaging method (Davalos et al. 2008; Davalos and Akassoglou 2012). They found that microglia are the first cellular responders in EAE, as they begin accumulating around blood vessels several days before the onset of clinical signs of disease (Davalos et al. 2012). The number of microglial clusters was also shown to increase at the peak of disease and was correlated with disease severity (Fig. 4.3c). Longitudinal in vivo imaging of the same spinal cord areas in the same mice at different stages of EAE revealed that microglial cluster formation and resolution is an ongoing process; some microglial clusters grow in size and some eventually get resolved, while new ones appear around different vessels during various disease stages (Davalos et al. 2012). Herein, time-lapse imaging of forming clusters showed the directed microglial cell body migration and process extension towards blood vessels for cells that were previously further away, while those that were already close to a given vessel maintained

their vascular association. Moreover, in vivo imaging of dye leakage demonstrated that microglial clusters specifically formed around vessels with a disrupted BBB and, as a consequence of BBB disruption, perivascular fibrin deposition was shown to be essential for microglial clustering in the perivascular space (Davalos et al. 2012). Finally, by crossing the CX3CR1-GFP reporter with a Thy1-CFP mouse line, they were able to record the interactions of GFP-labeled microglia with CFP-labeled axons in vivo, at the peak of EAE (Fig. 4.3d). These experiments showed abrupt physical manipulations of axons passing through perivascular microglial clusters, and showed for the first time axonal fragments still expressing CFP being detached and taken up by microglial cells (Davalos et al. 2012). By performing in vivo imaging and correlated histology in the same spinal cord areas, combined with genetic or pharmacological inhibition approaches, this study provided a mechanistic link between the leakage of the blood protein fibrinogen at sites of BBB disruption and the development of axonal damage through the perivascular activation of microglia at these sites, in a mouse model of MS.

A mutation in the superoxide dismutase-1 enzyme (SOD1-G93A) has been associated with the sporadic form of ALS in humans (Synofzik et al. 2010). A similar phenotype of progressive degeneration of motor neurons and pyramidal tracts was also seen in mice bearing the mutation ($SOD1^{G93A}$ mice). In this mouse model of ALS, Dibaj et al. examined the interactions between GFP-expressing microglia and YFP-expressing cortico-spinal tract axons in the lateral column of the spinal cord also by using in vivo two-photon microscopy. They found that microglia progressively reduced their ramification and baseline process motility velocity as a function of disease progression (Dibaj et al. 2011). The number of amoeboid microglia also increased in areas of axonal degeneration as the disease progressed to a more advanced stage with more prominent and widespread axonal pathology. In addition, when challenged with a focal axonal injury in their vicinity, microglia demonstrated a diverse profile of acute responses at different disease stages. At disease onset, they appeared hyper-activated and capable of significantly more robust responses towards the injury sites than healthy controls, while their responses were significantly reduced in advanced disease stages (Dibaj et al. 2011). Interestingly, in clinically unaffected areas of the spinal cord, microglial responses were comparable to control, irrespective of disease stage. This in vivo imaging study in a mouse model of ALS revealed that microglia undergo a transition to a progressively more activated phenotype with disease progression that not only influences their morphological appearance, but also determines their ability to perform tissue surveillance and contain injuries in the CNS. Overall, the application of in vivo imaging to studying microglial responses in animal models of neuroinflammatory disease has helped elucidate anatomical and structural characteristics of their activation and identify key pathological culprits and consequences of their sustained activation at different stages of CNS disease.

4.9 Conclusion

In the past decade, microglial biology has essentially been redefined, as a large number of unexpected physiological and pathological functions for these cells have emerged. In vivo imaging studies have been at the forefront of this dramatic expansion of our understanding of microglia. The constant development of new microscopy technologies, mouse models and imaging methodologies promises to further expand our access to uncharted territories of the CNS, such as image deeper brain structures or in awake animals, and shed more light on the extensive functional repertoire of microglia. Our ability to follow the natural defenders of the brain over the course of a disease is an important step towards characterizing and, eventually, modifying their behavior in therapeutic ways. In vivo imaging is essential for capturing the true potential of microglia, which is only beginning to be discovered.

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