REVIEW

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Microglia morphology in the physiological and diseased brain – from fixed tissue to *in vivo* conditions

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Abstract

First mentioned almost 100 years ago, neuroresearch has linked microglia to the initiation and/or maintenance of most central nervous system pathologies. Since their discovery, we learned that the microglia immune status is often correlated directly to their morphology. However, only recently, have we realized that, *in vivo* microglia are extremely dynamic cells capable to respond within minutes. Therefore, the name "resting" microglia is replaced slowly with surveilling microglia. Even more recent, it has been shown that microglia change their morphology and/or immune status depending on the way the tissue is obtained, processed and imaged, making labeling microglia, based on their morphology alone, as active or surveying even more difficult. All these observations correlated with a better understanding of cellular and subcellular process that microglia undergo in pathological conditions, could have profound implications on the time window and/or targets used to ensure the best outcome, when treating brain disease.

Keywords: microglia, morphology, dynamics.

The introduction of microglia in neuroscience

The first mention of microglia, as a distinct cellular element of the central nervous system, was done by Pío del Río-Hortega in 1919, when he used a modified silver carbonate impregnation to label small ramified cells in the brain [1, 2]. However, until the introduction of facial nerve lesion preparation, little progress was done in the microglia field [3]. The facial nerve lesion preparation managed to revolutionize the study of microglial responses to injury in an intact blood–brain barrier (BBB) model, thus opening the modern microglia research. Due to this new method, resident microglia and invading monocytes responses could be distinguished and the cellular bases of the important and irreplaceable role of microglia in de- and regeneration of the brain were established [4].

From their discovery on, microglia were thought to be a special type of brain cell, as they enter the brain, early during development and are not generated there. They are of mesodermal origin and use blood vessels or/and white matter tracts to populate all central nervous system (CNS) regions. They proliferate in the CNS and transform from an amoeboid into a ramified morphological phenotype, each occupying a special domain without interfering with the other microglial cells [4–6]. Before *in vivo* two-photon laser scanning microscopy (2P-LSM), they were described as resting, and microglia in this state were referred to as "resting microglia". But, with the introducing of the cranial window surgery [7–9] and their subsequent direct visualization in a living brain of transgenic animal models [10, 11], using *in vivo* 2P-LSM [12–14], the term should now be changed to "surveilling state microglia", as this cells survey constantly their microenvironment up to four times in one day and are nowhere resting cells [12]. Microglia are linked to most brain pathologies [15–17] and have been described to play important roles in the development and maturation of the brain [18, 19]. They have an intrinsic role in the brain as resident immune cells of the CNS, with key roles in neuroinflammation by producing proinflammatory cytokines and free radicals [20]. They are used as a cellular marker of inflammation, with most papers citing microglia activation as a direct consequence of neuroinflammatory processes [15, 21–23]. The fact that morphological activated microglia have been found in the physiological aging brain might change the view about microglia again [24–26]. Furthermore, recent work has showed that microglia morphology can be affected by the way the tissue is treated, processed and/or analyzed and thereby the state of microglia is influenced [27], making the need for a microglia activation marker more pressing than ever.

Microglia morphology across life span

Microglia populate all regions of the CNS (Figure 1A), however their density varies across different species with 5–12% of all rodent CNS cells estimated to be microglia [28], while this interval is between 0.5–16.6% of all cells

in the human brain [29, 30]. Close quantitative assessment of microglia population has revealed that there is a remarkable consistency in their distribution within a specific brain region [31] but a quick scan of different regions reveals visible differences in microglia densities (Figure 1, B–E).

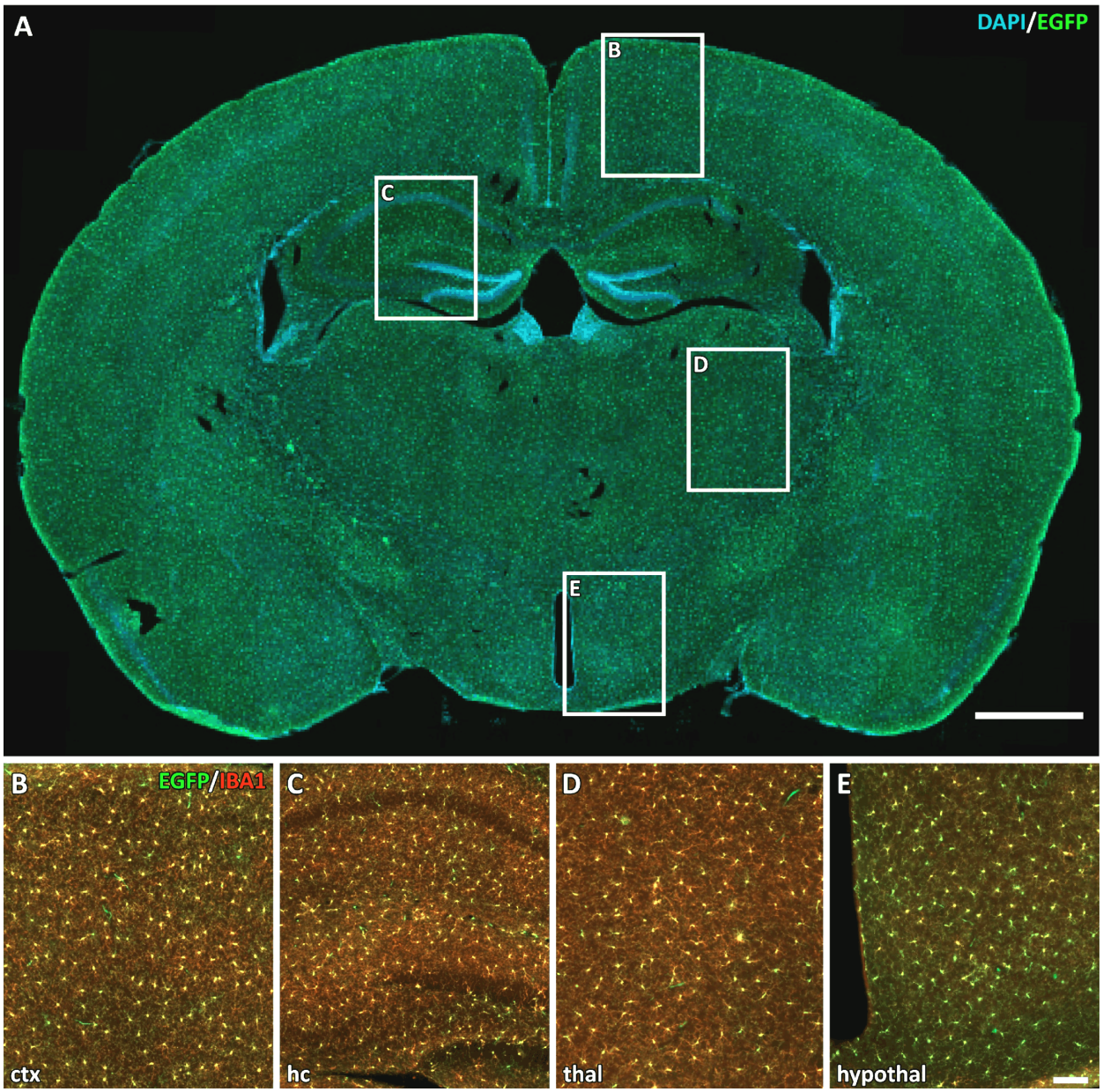

Figure 1 – ***Consistent microglia distribution in an adult mouse brain: (A) Iba1 protein expression colocalized with EGFP expression in microglia (TgH(CX3CR1-EGFP) [10]) in all brain regions with slightly different densities depending on the investigated region, varying from a high density in cortex (B) and hypothalamus (E), to very high like hippocampus (C) or lower in thalamus (D). Iba1: Ionized calcium binding adaptor molecule 1; EGFP: Enhanced green fluorescent protein; CX3CR1: CX3C chemokine receptor 1 (fractalkine receptor); DAPI: 4',6-Diamidino-2-phenylindole; ctx: Cortex; hc: Hippocampus; thal: Thalamus; hypothal: Hypothalamus. Scale bars: 1 mm (A); 100 µm (B–E).***

Although, microglia perform similar functions to macrophages, dendritic cells, and monocytes, they are not considered their CNS equivalent, as microglia are directly involved in physiological brain function [4]. Embryological, CNS-specific macrophages and microglia have different origins. While CNS macrophages (including perivascular macrophages, meningeal macrophages and some choroid plexus macrophages) derive from hematopoietic precursors during embryonic development [32], microglia derive from infiltrating primitive precursors produced by the yolk sac. This macrophage population starts colonizing the brain at approximately E8.5 (embryonic day) in mice in dependence to neuronal expression and secretion of interleukin (IL)-34 [33] and to the brain vasculature [34]. Once the CNS becomes fully isolated by the BBB, no additional recruitment, under physiological conditions, will take place. The microglia population remains relatively stable through a constant process of self-renewal [35]. Because microglia rely on the cerebrovascular network to infiltrate the CNS, reports have linked microglia to angiogenesis [34, 36]. These suggestions came from the observation that microglial depletion results in an altered vascular network within the CNS, with sparser blood vessels compared to physiological

conditions [36, 37]. In addition, microglia and microglial cell-conditioned medium have a positive effect on vessel sprouting [38] but with no direct molecular link yet to be identified.

The fact that microglia enter the brain in an early stage of the development indicates that there is an interlink between CNS development and microglia [25]. This is most obvious if we consider that one of the best-known functions of microglia phagocytoses of inappropriate synaptic connections, a process known as pruning [19, 39]. The exact mechanisms of this process are not completely understood. In general, two main ways are linked to it: First, the classical complement pathway. This serum protein system is an amplification element of the innate immune mechanism, leading to exponential generation of final effectors [40]. The development and study of mice unable to produce the subunit C1q of the C1 complex, allowed researchers to establish the impact of microglia on CNS development. Animals lacking C1q exhibited both an enhanced synaptic connectivity and signs of epilepsy due to insufficient pruning by microglia [41]. The second way microglia could affect CNS development is through the release of plasminogen activator that in turn decreases the levels of synaptic adhesion molecules essential for synaptic activity and stability [42].

Across life span, the microglia population is kept relatively constant, with processes as proliferation and apoptosis being reported as necessary elements of microglia turnover in adult CNS [43]. Microglia develop a heterogeneous morphological phenotype, that seems to be dependent on their location within the CNS [44].

There is an ongoing discussion regarding the impact of aging on microglia population. Some groups have reported no change in the number of ionized calcium binding adaptor molecule 1 (Iba1)-positive cells in the aged rat hippocampus [45], while others describe a decreased number of microglia in the cerebral cortex of analyzed animals [30]. In primates and humans, it seems to be more consistent that microglia change with aging. In old rhesus monkeys, the microglia population is increasing. However, these cells seem to have an altered functionality, with increased phagocytic properties and low degradation capacities [46]. Age-dependent microglia degeneration and their loss of neuroprotective functions play an important role in aging in humans [47]. Microglia dystrophic changes have been linked to the age-dependent onset of sporadic Alzheimer's disease [48] but the underlining cellular and molecular triggers are still unknown. Altered microglia morphology and reduced number of branches have been reported in the human brain during physiological and pathological aging [49]. Because of the tight microglial relationship between morphology and function, a close analysis of morphology might reveal functional activation but it is still not clear if the reported morphological changes are a result of microglia degeneration of they reflect an age-related cytoskeleton with no functional implication [50].

Analyzing microglia morphology

Microglia are an integral part of the nervous system, contributing to its defensive forces and their involvement in different pathologies has been extensively investigated [4, 51]. Although not all research is based solely on microglia morphology to establish inflammation in the brain, the link between the two is so strong that only observing specific microglia morphology can evoke the consideration of pathology, which is probably true in most instances. As such, changes of microglia morphology can be seen as an effective way to assess their immune state, due to the specific morphological changes these cells go through when activated [12, 21]. However, one should consider that microglial cells are highly dynamic both in the physiological and diseased brain, continually changing their morphology [12–14] and a clear-cut point in the immune activation of microglia is difficult to establish.

Several attempts to map a morphological characterization of microglia have been made [52–54]. Although not all are conform with one another, the purpose of this work is not to establish the scientific facts about them but to acknowledge the techniques. Some groups have used classic wide field [55, 56] and fluorescence-based microscopy [57], as a method to look at microglia and their morphology. The analyzed data came from fixed [55, 56], *ex vivo* [57] or *in vivo* tissue [12, 13, 53]. Other laser-based imaging methods have been applied to study cellular dynamics in the brain, such as optical coherence tomography [58, 59], but have so far been ineffective on a cellular/molecular level.

However, most morphological studies have been focused on changes in microglia morphology within neuroinflammation diseases [24, 60, 61] whilst very few have looked at microglia dynamics under physiological conditions [62–64]. Recently, several reports have described microglial active branch remodeling as a response to environmental changes and stress [63, 65], thus demonstrating that the relationship between microglia morphology and function is more complex than initially thought. This lack of underestimation could be due to the static way in which the dynamic microglia cell population was studied [55–57].

Laser scanning microscope technologies, like confocal (cLSM) or 2P-LSM, are highly effective tools to investigate morphological changes in the brain. As these technologies advance, more biological applications are found, with neuroscience as one of the main research branches profiting from these technical developments. Their superior spatial resolution compared with other optical imaging acquisition systems like epifluorescence microscopy makes them ideal for ultrastructural detailing of cellular morphologies [66].

It should be noted that a clear difference in image quality exists between cLSM and 2P-LSM (Figure 2), due to a slight spatial resolution loss in 2P-LSM [67]. However, the huge advantage of 2P-LSM is the possibility of chronic *in vivo* imaging, which allows the observation of morphology, dynamics and proliferation of distinct cells over a long-time span. There are many papers focusing on the technical differences between confocal microscopy and 2P-LSM [68, 69], however the data generated in structural studies reveals different aspects of morphological changes of microglia in the injured tissue [52]. This can be a direct consequence on the method used to generate the structural overview of microglia.

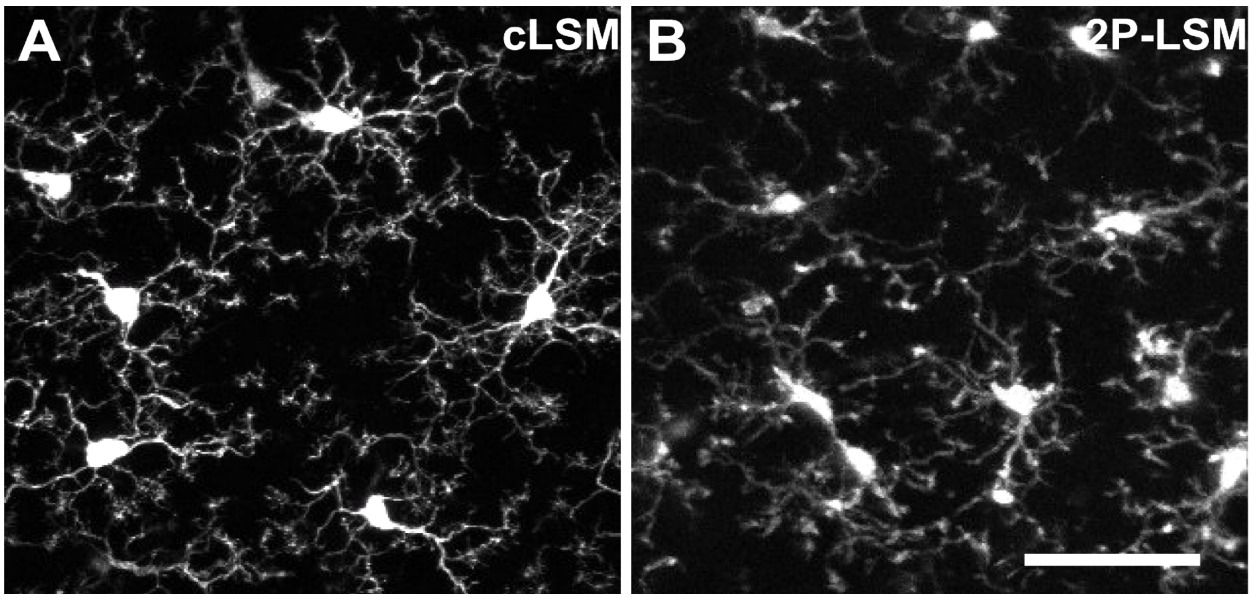

Figure 2 – ***Sample images of microglia acquired with different laser scanning techniques. While the use of a pinhole, in confocal laser scanning microscopy (cLSM) (A), has the advantage of a good signal to noise ratio, the use of two-photon laser scanning microscopy (2P-LSM) (B) permits in vivo data acquisition over a longer time period highly suitable for cells with a high motility like microglia. Scale bar: 50 µm.***

Microglia morphology – new perspectives and milestones

Laser scanning microscopy is one of the most used technologies in neurosciences, generating huge volumes of data. Although *in vitro* applications and analysis of fixed tissue tend to be more in common, data generated by confocal microscopy are often confirmed by *in vivo* 2P-LSM. The better signal to noise ratio obtained with cLSM can be explained in two ways: first, cLSM only uses tissue that has been previously cut in thin slices, whilst *in vivo* experiments use intact brain volume where light is more scattered by continuous blood flow, tissue differences (myelin) and respiration. Second, and probably more important, is that cLSM can use an averaging process and a pinhole to reduce noise, whereas *in vivo* 2P-LSM, due to time limitations, in fast response studies, normally do not use averaging. Due to the nature of the excitation level in 2P-LSM, no pinhole is needed. Although the pixel time for both methods is comparable, the total scanning time per layer is higher in cLSM than 2P-LSM, due to the process of averaging used to obtain a single cLSM image, without problems in fixed tissue. Although it is technically possible to increase the averaging in 2P-LSM, due to the high dynamics of microglia, such an approach is not desirable for *in vivo* applications.

With increasing knowledge about brain function generated by evolving technologies, a tendency of amalgamating information obtained by different acquisition methods is frequent within the scientific community. Often this information can be used to establish validity and helps to confirm new hypotheses. However, even closely related technologies like cLSM and 2P-LSM can generate slightly different data sets. Although overlapping results from the two technologies exist, slight differences can be enough too partially influence conclusions. This is especially applicable in the case of microglia studies, where microglia morphology is linked to microglia immune status. Due to the highly dynamic nature of microglia cells, one should be careful when labeling them as resting or active from an immune point of view solely based on their morphology.

Conflict of interests

The authors declare that they have no conflict of interests.

Author contribution

Laura Stopper and Tudor-Adrian Bălşeanu have equally contributed to this paper.

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