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Microglia states and nomenclature: A field at its crossroads

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## Microglia states and nomenclature: A field at its crossroads

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### SUMMARY

Microglial research has advanced considerably in recent decades yet has been constrained by a rolling series of dichotomies such as “resting versus activated” and “M1 versus M2.” This dualistic classification of good or bad microglia is inconsistent with the wide repertoire of microglial states and functions in development, plasticity, aging, and diseases that were elucidated in recent years. New designations continuously arising in an attempt to describe the different microglial states, notably defined using transcriptomics and proteomics, may easily lead to a misleading, although unintentional, coupling of categories and functions. To address these issues, we assembled a group of multidisciplinary experts to discuss our current understanding of microglial states as a dynamic concept and the importance of addressing microglial function. Here, we provide a conceptual framework and recommendations on the use of microglial nomenclature for researchers, reviewers, and editors, which will serve as the foundations for a future white paper.

### NAMES, NAMES, NAMES

If the names are unknown, knowledge of the things also perishes.<sup>1</sup>

—Carolus Linnaeus

And yet, we humans instinctively tend to name things and use that name to define their properties. Biologists are no exception: from the time of 18<sup>th</sup> century father of taxonomy Carolus Linnaeus, the main purpose of biology has been categorizing the natural world as a way of understanding it. Naming species and grouping them together into taxa served to define evolutionary relationships; even today taxonomy and phylogeny are closely interrelated. But we must never forget that nomenclatures and categories are artificial constructs and that biology is seldom black and white but rather an extended continuum of greys. While giving names is natural and useful, we need to be aware that categorization constrains our thinking by forcing us to fit our observations into established classes. As sociologists say, “categorization spawns expectations.”<sup>2</sup> This semantic issue has already been acknowledged by immunologists because, in fact, the given names have connotations that often imply a specific function.<sup>3</sup> In this paper, we extend similar initiatives on macrophages,<sup>4</sup> dendritic cells,<sup>3</sup> interneurons,<sup>5</sup> and astrocytes<sup>6</sup> to discuss the widespread

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#### SUPPLEMENTAL INFORMATION

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problems associated with categorization of microglia using outdated terms such as “resting versus activated” (Box 1) or “M1 versus M2” (Box 2).

Dichotomic, rigid categories convey a dualistic idea of good versus bad microglia and may actually impede scientific advancement. Widely used terms, such as “neuroinflammation” as a synonym of microglial reactivity (Box 3) and naming a panoply of presumed microglial populations and assumed functions arising from single-cell transcriptomics, are misleading and increasingly problematic, especially to those entering the field of glial biology and neuroimmunology. This nomenclature does not address the important question: what are the specific functions of microglia in the contexts of development, health, aging, and disease? It is now clear that microglia exist in diverse, dynamic, and multidimensional states depending on the context, including local environment (Figure 1). We define dimensions as the key variables driving the phenotypic transformations of microglia. These variables are molecularly distinct signaling pathways regulated at multiple levels (e.g., transcriptional, epigenetic, translational, metabolic) that each give rise to distinct microglial functions or properties. In this manner, categorizing microglia based on a historical, one-dimensional nomenclature in the absence of functional data will constrain and stifle future progress and innovation.

To examine and address these issues, we assembled a team of international experts who have made major contributions to microglia research, inclusive of various groups, and balancing gender, geographical distribution, and seniority. Authors from the fields of neuroscience, neurobiology, immunology, neuroimmunology, oncology, and neuropathology, from both academia and industry, discussed their perspectives on the current and future challenges in defining microglial states and nomenclature. A questionnaire (Data S1) was created to collect all the authors’ opinions on several nomenclature issues and the importance of directly addressing microglial function. The responses to the questionnaire, an online meeting held in June 2021, and an open session held at the EMBO meeting Microglia 2021 were used as a backbone to develop this paper.

Herein, we summarize our current knowledge about the identity of microglia and discuss best practices for how to define and study microglial state dynamics. We then outline “classical” microglial nomenclatures, highlighting some of the key discoveries that led to the above classifications and their limitations. We intentionally focus on citing studies related to the nomenclature rather than providing a comprehensive review of the history of microglial research, as it has been done elsewhere.<sup>7,8</sup> We discuss the overall limitations and conclude with recommendations for the proper usage of microglial nomenclature as research evolves, provide a conceptual framework for discussing microglia, and offer perspectives on the future questions, gaps in knowledge, and challenges to tackle as a field.

## **MICROGLIAL IDENTITY: WHAT WE MEAN ABOUT WHEN WE TALK ABOUT MICROGLIA**

The origin and identity of microglia was, for many years, a matter of debate. In the dim and distant past, Ramón y Cajal’s disciple, Pío del Río-Hortega, suggested that these cells were of mesodermal origin.<sup>9</sup> However, over time, an ectodermal origin was also proposed,<sup>10</sup>

sparkling controversy until the 1980s. The mesodermal origin took solid hold later with the advance of technical approaches revealing more similarities than differences with the functions and features of macrophages. In 1999, microglia were reported to appear in the brain rudiment as early as embryonic day 8 (E8) in mice and proposed to originate from yolk sac progenitors.<sup>11</sup> The recent combination of fate mapping studies and transplantation approaches this debate, revealing key aspects of microglial identity and plasticity. In mice, unlike other model organisms such as zebrafish,<sup>12,13</sup> microglia are now considered to originate from a pool of macrophages produced during primitive hematopoiesis in the yolk sac, which start invading the neuroepithelium at E8.5.<sup>14–17</sup> In humans, microglial precursors invade the brain primordium around 4.5 to 5.5 gestational weeks.<sup>18</sup>

One key signaling pathway critical for microglial development and maintenance is the colony stimulating factor receptor (CSF1R). Ligands of CSF1R that sustain this pathway include two cytokines with different origins and primary sequences but similar tridimensional structures and binding to CSF1R: IL-34 and CSF1.<sup>19</sup> IL-34 is produced by neurons, while CSF1 is secreted primarily by oligodendrocytes and astrocytes. Accordingly, the two ligands have distinct and non-overlapping functions in the establishment and maintenance of microglia within the gray and white matter.<sup>20</sup> Microglia have the capacity for self-renewal in certain contexts, allowing them to repopulate the CNS within 1 week of depletion, even when more than 99% of microglia are ablated with CSF1R antagonists<sup>21,22</sup> or diphtheria toxin.<sup>22</sup> This process, termed “microglial repopulation” or “microglial self-renewal,”<sup>23–25</sup> is different from “microglia replacement,” which, in contrast, occurs when endogenous microglia are replaced by exogenous cells that can include bone-marrow-derived myeloid cells,<sup>26–29</sup> peripheral blood cells,<sup>28,30</sup> stem-cell- or induced-pluripotent-stem-cell (iPSC)-derived peripheral blood cells,<sup>31</sup> across various experimental or pathological conditions.<sup>31–33</sup> Our current definition is that mammalian microglia are yolk-sac-derived, long-lived cells within the CNS parenchyma that persist into adulthood and self-renew without any contribution from bone-marrow-derived cells at a steady state.

The identification of microglia is currently based on the expression of specific genes highly enriched in microglia, which represent their transcriptional identity and are commonly employed as “microglial markers” (Table 1). However, the expression of each marker alone is not sufficient to define microglial identity, as levels of expression may change depending on microglial adaptation to local signals. The present consensus is that mammalian microglia can be identified by the expression of transcription factors like Pu.1<sup>16</sup>, cytoplasmic markers such as ionized calcium-binding adapter molecule 1 (IBA1), and surface markers including the purinergic receptor P2YR12, trans-membrane protein 119 (TMEM119), and CSF1R.<sup>34</sup> Based on these markers, genetic tools (such as Cx3cr1<sup>CreERT2</sup>, P2ry12<sup>CreERT2</sup>, Tmem119<sup>CreERT2</sup>, and Hexb<sup>CreERT2</sup> mouse lines) (Table 2) are available that allow for more specific manipulation or visualization of microglia, although they could also target other populations, including border-associated macrophages (BAMs), also named CNS-associated macrophages (CAMs), and other glial cells.<sup>35–40</sup> Most recently, a new binary transgenic model relying on co-expression of Sall1 and Cx3cr1 has been introduced that specifically targets microglia in a non-inducible way.<sup>41</sup>

Nonetheless, many of these markers are downregulated in pathological states and can be expressed by other brain macrophage populations such as BAMs residing in the perivascular space and leptomeninges,<sup>42,43</sup> which also derive from the yolk sac.<sup>44</sup> In addition, caution must be exercised, because many classical microglial markers can also be expressed by cells originating from monocytes or iPSCs, and therefore their presence does not imply *bona fide* microglia. These cells should be more accurately described as monocyte-derived microglia-like or iPSC-derived microglia-like cells (iMGL cells).

As resident macrophages of the brain parenchyma, microglia participate in many critical CNS functions ranging from glio-, vasculo-, and neurogenesis to synaptic and myelination through their process motility, release of soluble factors, and capacity for phagocytosis (Figure 2). These functions have been revealed using several constitutive and inducible knockout models for microglial-specific genes<sup>45</sup> and by microglial-depletion paradigms in animal models,<sup>46</sup> particularly rodents and zebrafish.

The key role of microglia in maintaining CNS health is also supported by the severe phenotype displayed by patients lacking microglia due to loss-of-function *CSF1R* mutations. Heterozygous mutations, particularly in the kinase domain of *CSF1R*, are associated with adult-onset leukoencephalopathy with axonal spheroids and pigmented glia (ALSP; OMIM: 221820) characterized by reduced microglial numbers and white matter atrophy that result in progressive cognitive and motor impairment, dementia, and early death.<sup>47</sup> Additionally, biallelic mutations are reported to cause complete absence of microglia with developmental brain malformation, hydrocephalus, bony lesions, and early death.<sup>48,49</sup> This phenotype, however, seems in apparent contradiction with the reported absence of gross neurological abnormalities at birth observed in mice with genomic deletion of FIRE, an intra-intronic super enhancer in the *Csf1r* gene enhancer region, whose brains lack microglia,<sup>50</sup> though more nuanced analyses are needed. Nonetheless, FIRE mice have premature lethality and increased amyloid pathology as early as 5 months of age.<sup>51</sup> The source of discrepancy between the developmental impact of *CSF1R* mutations in humans and mice is not yet fully understood. One possibility is that microglial developmental functions are partly redundant, modified by other environmental factors, or compensated in their absence by other cell types, such as astrocytes.<sup>52</sup> It will be important to determine how microglia communicate with other glial cells and immune cell populations to support CNS maturation and function in the future.

## **(RE)DEFINING MICROGLIAL STATES: DAMs, HAMs, WAMs, AND MORE**

Core markers of cellular identity are useful to identify microglia but are not necessarily informative about the functional “state” of microglia, which depends on the context (i.e., the physiological conditions in which microglia are found at any given CNS region and time). Microglia have a complex “sensome,”<sup>53</sup> a series of surface receptors that allow them to detect changes in their environment. Microglial states are thus dynamic, and the outcome of the cell’s epigenome, transcriptome, proteome, and metabolome yields discrete morphological, ultrastructural, and/or functional outputs (Figure 3). Microglia are anything but static, as they are exceptionally responsive to alterations in their local environment. In the mature healthy CNS, the distribution of microglia is largely uniform and generally

regular with little overlap between adjacent territories.<sup>54</sup> The cell bodies are largely sessile, but their processes are constantly moving and scanning the brain parenchyma.<sup>55,56</sup> Microglial functions adapt to their location and reciprocal interactions with nearby cells and structures. Their morphology, ultrastructure, and molecular profile are similarly dynamic and plastic, resulting in many different cell states. As Conrad H. Waddington, founding father of systems biology, eloquently described: “Cells are residents of a vast ‘landscape’ of possible states, over which they travel during development and in disease”.<sup>57</sup>

Single-cell technologies, multi-omics, and integrative analyses of gene and protein expression have helped to not only locate cells on this landscape but also provide new insight into the molecular mechanisms that shape the landscape and regulate specific cell states in a given context (e.g., development, adult, disease, or injury model, etc.). Many diverse and context-dependent microglial states have been observed across species and models. Some examples of these states are the disease-associated microglia (DAMs), originally associated with Alzheimer’s disease (AD) pathology models;<sup>58</sup> microglial neurodegenerative phenotype (MGnD) documented across several disease models;<sup>59</sup> activated response microglia (ARMs) and interferon-responsive microglia (IRMs) in an AD pathology mouse model;<sup>60</sup> human AD microglia (HAMs);<sup>61</sup> microglia inflamed in multiple sclerosis (MS) (MIMS);<sup>62</sup> and lipid-droplet-accumulating microglia (LDAMs) in aging mice and humans,<sup>63</sup> brain tumors (glioma-associated microglia, GAMs),<sup>64</sup> amyotrophic lateral sclerosis (ALS)-associated signature,<sup>65</sup> and Parkinson disease (PD) microglial signature.<sup>66</sup> In the developing and aging brain, the white matter-associated microglia (WAMs),<sup>67</sup> axon tract-associated microglia (ATMs),<sup>68</sup> and proliferative-region-associated microglia (PAMs, related to phagocytosis of developing oligodendrocytes)<sup>69</sup> may share some features with the core DAM signature. In the developing human CNS, microglia also express some of the DAM/MGnD/ARM-like profiles.<sup>70</sup>

While gene expression signatures indicate biological pathways, the functional implications of these states and relationship to one another remain unclear. In fact, the ever-growing list of branding clusters in single-cell RNA sequencing (scRNA-seq) experiments and use of acronyms is not consistent across research groups and could hinder future advance of the field without validation and functional experiments to understand their meaning. Moreover, transcriptomic signatures depend on tissue dissection and gating strategies that can lead to isolation artifacts,<sup>71–74</sup> which, when layered with the technical limitations of single-cell sequencing, can make it difficult to assign state identity across different studies. Another source of complexity comes from evident interspecies differences,<sup>75–77</sup> which can further hamper comparisons. Advances in computational tools and approaches, which enable the alignment and integration of single-cell datasets, can help solve some of these issues, providing a powerful way to determine microglial-state similarities across contexts.<sup>78,79</sup>

A practical limitation of solely defining functional states by their transcriptional signature is that mRNA expression may not directly predict protein levels.<sup>80</sup> Protein expression signatures obtained by methods, such as single-cell mass cytometry, have their own technical limitations<sup>81</sup> but may better represent true cell states.<sup>82,83</sup> Importantly, mRNA or protein expression alone does not necessarily predict microglial function, although they can be used to generate functional hypotheses that need to be experimentally tested. There are many

methods that allow for the classification of microglia based on their constituent states, including gene expression, protein expression, post-translational modifications, mRNA profiling, morphology, and ultrastructure. All these approaches can vary in coverage (e.g., expression of a single-cell versus whole-transcriptome profiling), which has created overall confusion and mislabeling in the field. Presumably, each microglial state is associated with unique or specialized functions, although the unique roles of any observed state have so far remained elusive. Thus, it is critical that we begin to define microglial states taking into account their specific context within and between species, across sex, space, and time (e.g., CNS region and biological age) as well as layers of complexity (e.g., epigenetic, transcriptional, translational, and metabolic signatures), which ultimately determine together the cell's phenome (i.e., motility, morphology, and ultrastructure) and function (Figure 5).

One major conceptual limitation of the various “one-off” microglial acronyms (e.g., DAMs, MGnD, etc.) is that they suggest stable states or phenotypes of microglia associated with a disease context, such as neurodegeneration. Intuitively, this classification system is similar to the concept of neuronal cell types, where neurons cluster into distinct subtypes based on their gene expression or neuroanatomy. However, contrary to microglia, neuronal groupings are considered fixed and terminally differentiated.<sup>5</sup> We do not know how temporally or spatially dynamic microglial states may be, as microglia are remarkably heterogeneous and plastic. Therefore, these cells are probably not permanently “locked” into any single functional state. From the evidence available so far, microglial states appear dynamic and plastic, possibly transitory, and strongly dependent on the context.<sup>84</sup> New tools including imaging reporters for microglial states are needed to track transitions within individual cells over time and across the lifespan, following different challenges and perturbations, as well as in response to treatment.

## MICROGLIAL HETEROGENEITY: IT ALL DEPENDS ON THE CONTEXT

The term “homeostatic” is used to refer to microglia in physiological conditions, but there are different interpretations of this nomenclature when describing microglia in health and disease. While homeostatic relates to the “physiological” context assessed in space and time, it does not necessarily correspond to a unique molecular profile because, even without any perturbation, microglia display diverse morphological and functional states depending on the signals from the CNS microenvironment. This continuous microglial sensing results in multiple transcriptional signatures from development to aging depending on the specific local signals or challenges to the brain at each developmental stage.<sup>53</sup> A less responsive microglial state, which in other contexts would be considered more homeostatic, might be less effective at responding to damage or pathological cues in aging and disease contexts. For example, in aging and neurodegenerative disease, microglia may have reduced ability to rapidly respond to brain challenges (i.e., removing toxic amyloid, infected, damaged, or degenerating neurons), leading to CNS dysfunction and disease progression. Microglia from adult TREM2 knockout mice have been described as “locked in a homeostatic state” as they are less responsive to challenges (such as amyloid) and do not adopt a transcriptional DAM signature in disease contexts.<sup>85,86</sup> From this example, the term “homeostatic” is not informative if not well defined and placed in the context of function.



Key modifying factors that lead to microglial heterogeneous states include age, sex, circadian time, local CNS signals, and peripheral cues, such as the changes in the microbiota<sup>87,88</sup> or other systemic diseases (e.g., asthma)<sup>89</sup> in addition to the pathophysiological state of the CNS and overall organism (discussed in more depth in the next section). Age, indeed, has a key influence on the microglial homeostatic state, which goes through several distinct temporal stages (embryonic, perinatal, adult, and aging microglia), each notably characterized by an enrichment of defined regulatory factors and gene expression profiles.<sup>68,90</sup> After the initial establishment of microglial identity by a network of developmentally programmed and environment-dependent transcription factors,<sup>75,90</sup> microglia become extremely heterogeneous in their transcriptome during early postnatal development, as determined by scRNA-seq.<sup>68,69,91</sup> In contrast, microglia display a more limited transcriptomic heterogeneity in the adult CNS, where the different microglial scRNA-seq clusters fall into a transcriptional continuum instead of representing distinct states.<sup>68,69,91</sup> Relatively small transcriptional differences may, however, lead to relevant functional differences, as exemplified by the functional variations between hippocampal and cerebellar microglia.<sup>92,93</sup>

Sex differences due to sex chromosomes and/or gonadal hormones may also impact microglial states in different contexts. A growing body of evidence shows that male and female microglia differ in their transcriptomic, proteomic, and morphological profiles across brain colonization, maturation, and function in health and disease.<sup>88,94–96</sup> Of note, the microglial sex-specific transcriptomic signatures appear to be intrinsically determined, being maintained when microglia are transplanted into the brains of mice from the other sex.<sup>96</sup> Sexually differentiated roles of microglia could critically influence a variety of biological processes, in a time-dependent manner, and, thus, emerge as key disease modifiers across various pathological conditions with sexual dimorphism in prevalence, manifestation, and response to treatment.<sup>97</sup> A well-characterized example for sex-specific divergence is the purinergic receptor P2X4R, identified as the male-biased microglial mediator of chronic pain.<sup>98</sup> Sex differences in sexually dimorphic responses in physiology and pathology likely arise from a combination of Y-chromosome-specific genes, sex hormones, neuronal circuit-related factors, and epigenetic mechanisms.<sup>99</sup>

Regardless of the reduced heterogeneity in the mature adult (compared to embryonic) CNS,<sup>7,68,90</sup> microglia do differ among CNS areas in terms of their morphology and ultrastructure, transcriptional, proteomic, epigenetic profiles, and functional specialization, suggesting that microglial states are modulated by local cues.<sup>83,100,101</sup> However, local CNS signals are not sufficient to determine microglial identity because macrophages engrafted in the brain parenchyma can acquire a microglia-like morphology without reaching a transcriptomic signature identical to host microglia, even after prolonged CNS residence,<sup>26,102,103</sup> supporting the idea that microglia are distinct from peripherally derived macrophages, even when they colonize a similar niche. In addition, these findings suggest that once their identity is established, microglia assume different functional states in response to local CNS signals. Therefore, both the developmental genetic programs and CNS environment (nature and nurture) collaborate to dynamically determine microglial functional states.



Microglia not only respond to local cues within the brain, but they also receive continuous inputs from the periphery, including signals from the gastrointestinal tract.<sup>104</sup> In this context, the role of the host microbiota is gaining momentum in controlling microglial maturation and function in the CNS,<sup>88</sup> with growing evidence that microbiota-derived short-chain fatty acids represent major mediators of the gut-brain axis.<sup>87,105</sup> Another example of crosstalk between microglia and the periphery is the so called “sickness behavior,” as a result of the central response to peripherally released cytokines produced by peripheral immune cells and tissue-resident macrophages detecting specific pathogen-associated molecular patterns (PAMPs).<sup>106</sup> This complex and coordinated response, in which the functional role of microglia remains poorly understood, gives rise to adaptive behavioral strategies, including lethargy. Acute systemic inflammation, nevertheless, was extensively shown to impact on microglia<sup>107,108</sup> and induce a microglial state associated with robust IL-1 $\beta$  production.<sup>109</sup>

The concept of the brain as an immune-privileged organ has been challenged and definitely revisited in recent years. Indeed, peripherally produced cytokines and immune cells access the CNS and patrol the perivascular space in disease but also in health, thus playing important roles in coordinating central and peripheral immune responses.<sup>110</sup> It was also suggested that microglia require resident CD4<sup>+</sup> T cells in the healthy developing brain for proper maturation and complete fetal-to-adult transition.<sup>111</sup> Microglia and T cell crosstalk was shown to help maintain homeostasis in the CNS, with dysfunctional regulation occurring in diseases, such as MS,<sup>112</sup> ALS,<sup>113</sup> AD,<sup>114</sup> and encephalitis.<sup>115</sup> It will be important to continue investigating the influence of the peripheral immune system, including B cells, natural killer cells, and other cells, on microglial states and function in both health and disease.

## MICROGLIAL STATES IN THE DISEASED CNS

Microglia are keen responders and critical players in numerous neurodevelopmental, neurological, and neurodegenerative conditions, as thoroughly reviewed elsewhere. Altered microglial states have been described in the diseased human brain and across various animal models of disease pathology based on morphology and gene expression signature. In addition, these states also differ depending on the timing (i.e., disease stage), genetic background, and local environment. Context-dependent signals vary dramatically during disease progression; they range from apoptotic cells, extracellular debris, toxic proteins (i.e., amyloid,  $\alpha$ -synuclein), and signals resulting from blood-brain barrier disruption and altered function of neurons and other glial cells. Microglia respond to these challenges by changing their molecular profile, morphology, and ultrastructure (Box 3), as well as motility and function.

The expression of core microglial markers is also altered over the course of disease, including downregulation of the homeostatic microglial signature. A prototypical example is P2RY12, one of the most widely used markers to discriminate microglia from other macrophages, with its reduced expression being one of the salient features of the microglial response to AD pathology and other disease conditions,<sup>116</sup> as shown in several mouse models of disease (Figure 4). The apparent contradiction that core markers do not have a steady expression, as could perhaps be expected, is likely reflecting the functions

those proteins have and how they change in the diseased brain. For instance, P2RY12 upregulation in epilepsy may relate to microglial sensing ATP and nucleotides released during seizures.<sup>117</sup> This seeming paradox strengthens the fact that determining microglial expression profile is far from attributing any function to microglia, as it may only be suggestive of a potential functional identity, which, with unanimous consensus from all the authors, requires experimental validation using appropriate animal models and mutagenesis while using analyses that preserve the environmental influences shaping microglial function.

A microglial state that has received particular focus is the one denoted by the DAM signature, initially identified in a mouse model with mutations within five AD genes (5XFAD)<sup>58</sup> and later detected in other AD mouse models and samples from human AD (reviewed in Chen and Colonna<sup>116</sup>) and MS patients.<sup>62,118</sup> Single-cell transcriptomic profiling of human microglial nuclei revealed a tau-associated microglia cluster that had not been identified in mice,<sup>119</sup> reinforcing the idea that more human studies are needed. The shared DAM signature includes downregulation of CX3CR1 and P2RY12 and upregulation of APOE, AXL, SPP1, and TREM2,<sup>116</sup> and it has been recently shown that it comprises two ontogenetically different cell lineages, both expressing TREM2, resident microglia and invading monocyte-derived cells (termed disease inflammatory macrophages, DIMs) that accumulate during aging.<sup>120</sup> Many questions remain open regarding the functional significance of the DAM signature.

Are DAMs beneficial, detrimental, or both? Several studies, in both mouse and human stem-cell-differentiated microglia, demonstrated that the transition to a DAM state is dependent on TREM2.<sup>58,59,85,121</sup> How the TREM2 receptor drives the DAM transcriptional phenotype remains unclear, although the TREM2-ApoE signaling pathway is necessary for the switch from homeostatic to MGnD.<sup>59</sup> Further investigations are required to fully elucidate the role of TREM2. For instance, is TREM2 a key sensor for amyloid- $\beta$  and other AD-related pathology, or does its loss of function cause developmental defects in microglia that render them unable to change state? Is TREM2 controlling the microglial state by regulating their energetic and anabolic metabolism?<sup>122,123</sup>

New bulk and single-cell epigenetic approaches<sup>75,124–129</sup> will help answer these questions and ultimately may provide a means to toggle microglial states at will, enabling the field to finally understand the function of distinct microglial states and their impact in different contexts. Additionally, many genes of the DAM signature were identified across various contexts. For example, a common set of markers including (but not limited to) an upregulation of TREM2, APOE, CD11c, CLEC7A, and LPL and downregulation of TGF $\beta$ , CSF1R, P2RY12, and TMEM119 has been recently used to denote a microglial state that associates with myelinating areas in the developing brain but also with aging and several models of degenerative diseases, such as AD, ALS,<sup>130</sup> and MS.<sup>58,67,131</sup> These observations raise the question as to whether the DAM is a signature strictly associated with certain diseases, as the name implies, or perhaps represents a more universal core signature that appears in response to various challenges and may differ between the young/developing versus aged/diseased CNS and across distinct regions. Most likely, the same states that are beneficial in certain contexts may be detrimental in others, strictly depending on the complex interactions between microglia and their surrounding environment. One of the most

relevant questions to be addressed is to which extent microglial states identified in the mouse brain are conserved and functionally relevant in the human brain.

## NOMENCLATURE TROUBLES

Our current understanding of the plasticity of microglial states is at odds with the simplistic scenario established using outdated microglial nomenclature (resting versus activated and M1 versus M2; Boxes 1 and 2). Thus, a systematic, careful naming approach would greatly benefit microglial biology. As a first step to guide the field regarding the use of nomenclature, we generated a questionnaire (Data S1) and collected the responses from the co-authors.

Surprisingly, there was more consensus than disagreement that the current nomenclature has severe limitations, and a more useful conceptual framework is needed to properly understand microglial states. There is also agreement that this framework is a first important step to guide the field and should be revisited every 5 to 10 years by an international panel of experts as new discoveries are made. There is also a broad agreement that microglial responses should be framed in a multidimensional space and should not be simplified as dichotomic good versus bad (Figure 1). Another point of strong agreement: abandon M1/M2 (and similar) nomenclature once and for all and generally avoid using the vague term “neuroinflammation.” Most agree that inflammation is not always detrimental but, instead, represents an adaptive response to damage that can sometimes get out of control (Box 4). Quite importantly, a vast majority of authors support the use of “markers” (genes or proteins) to identify cell populations, but not as a readout of cell functions, which need to be addressed directly.

Nonetheless, there were a few points that are still under intense debate. The term “resting” microglia is strongly avoided by some authors, whereas others acknowledge that they still use it even with its limitations for lack of a better term. “Homeostatic” has more acceptance, although it is recognized that it is based on a very particular gene signature not shared by microglia across all physiological contexts, such as embryonic and postnatal development, and that several homeostatic states likely exist. Thus, the term “homeostatic” should always be accompanied by an accurate description of the context.

The opinion on use of the term “DAM,” on the other hand, is highly polarized. Many authors consider that a core set of transcripts in this signature is common to several pathological conditions and some physiological processes, including the development of white matter, whereas an equal number of authors state there is not enough evidence for “DAM” to be a universal signature of microglial response to damage. Finally, the extent to which microglia are unique or similar to other brain-associated or tissue macrophages is evolving with new data and profiling methods: most agree that because of their lineage, microglia are, to some extent, similar to other macrophages but have unique functions resulting from their longer residence in the CNS environment.

## RECOMMENDATIONS: DO'S AND DON'TS

Based on the collective opinions from the authors, we provide a series of recommendations for researchers, reviewers, and editors. As the field has not yet reached a consensus on several nomenclature topics, including the appropriate use of descriptors for microglial states, it is premature to provide clearer recommendations. Nevertheless, we aim to raise awareness on these issues and stimulate the launch of further initiatives that will guide the field and allow to develop more specific guidelines.

### Classic nomenclature

- Consider microglia as highly dynamic and plastic cells that display multivariate morphological/ultrastructural, transcriptional, metabolic, and functional states in both the healthy and pathological CNS.
- Describe microglia using as many layers of complexity as possible: ontogeny, morphology/ultrastructure, motility, -omics, and function, always placing them into a species and spatiotemporal context (Figure 5).
- Refer to microglia in basal conditions as “homeostatic” instead of “resting” microglia, considering the limitations discussed above (i.e., that these terms refer to microglia under physiological conditions and not to the function of microglia). Use the term “surveillant/surveilling” to refer to microglia that are engaged in surveillance, but not as a synonym of microglia under normal physiological conditions.
- Refer to microglia in your experimental condition as “reactive to” or “responding to” while describing the particular signals they respond to (i.e., the context) instead of using the widely used broad term “activated,” as microglia are active in both health and disease.
- Disregard simplistic, dichotomic categorizations by providing the observed data and its context.
- Describe profiles of cytokine expression, considering that microglial complexity cannot be reduced to oversimplified and polarized “pro-inflammatory” versus “anti-inflammatory” categories. Similarly, do not use M1 versus M2 classification.
- When using the term “DAM,” do not use it as a universal term applicable to all diseases, models, or challenges. The jury is still out to test whether its full or core signature is common to all or a subset of pathologies, particularly in the human brain.

### Introducing new terminology

- Until a consensus is reached about true subtype(s) of microglia, with defined ontogeny, physical niches, functions, and transcriptional profiles (whether permanent or transient), use the term “state” rather than “subpopulation.”

- Use combinations of gene or protein “markers” to identify putative subpopulations but be aware that their expression is plastic and may change over time and under different experimental conditions. Use fate mapping approaches with lineage tracing to track individual microglial cells and assess possible intrinsic differences as well as changes in their state over time.<sup>84,132</sup>
- In scRNA-seq studies, describe the transcriptional signatures (sets or modules of expressed genes) that can be compared with other studies.<sup>116,133</sup> To describe groups of transcriptionally similar cells in terms of signature, use the term “cluster.”
- Avoid the use of acronyms wherever possible, and only use these once multiple laboratories have defined a stable state with a clearly defined functional role.
- If new terminology needs to be introduced, follow FAIR principles: findable, accessible, interoperable, and reusable (<https://neuronline.sfn.org/professional-development/data-sharing-principles-to-promote-open-science>). An example of naming cell lines following these principles can be found here.<sup>134</sup>

### Microglial markers and function

- Use integrative methodological approaches that allow probing of microglia using different levels of analysis (Figure 5).
- Follow updated consensus guidelines when using methodologies such as scRNA-seq,<sup>135</sup> qRT-PCR,<sup>136</sup> or digital PCR.<sup>137</sup>
- Do not use morphology or gene/protein expression as a substitute for directly assessing cell function. Morphology and expression can be used to generate hypotheses about function that need to be specifically tested.

### Grammar quandary

- “Microglia” as a population is a plural noun in English but a singular noun in Latin-derived languages, which occasionally causes confusion. In English texts, microglial cells should always be referred to in the plural form unless referring to an individual cell. For example, “microglia are brain cells” but “this microglia is adjacent to a neuron”.

## FUTURE QUESTIONS AND CHALLENGES

### From words to action

A key challenge in the field is to match microglial morphological, ultrastructural, transcriptomic, proteomic, metabolomics, and emerging lipidomic changes with functional responses (Figure 3). In the current single-cell era, an overwhelming wealth of data has been generated, profiling the expression of millions of microglia in different organisms, at different ages, across diverse brain regions. Yet, such “omics” identities are not necessarily linked to functional states and often lack spatial resolution. Additionally, many widely used microglial markers are sense genes, whose expression and activity at the microglial

membrane may reflect functional adaptations to a changing environment and are possibly more indicative of the microglial functional state than the transcription profile.

Transcriptional analysis will benefit from ribosome profiling by RiboSeq<sup>138</sup> and from gene-trap insertion profiling by TRAP-Seq.<sup>139</sup> Proteomic approaches combined with *in situ* studies will provide better information in this respect, bridging the gap between expression and function. Further integration of complementary approaches, such as spatial transcriptomics, imaging mass cytometry, and correlative or conjugate electron microscopy in combination with other single-cell approaches, will provide a more comprehensive characterization of microglia. Ultimately, functional studies using specific pharmacological and transgenic approaches in animal models, as well as human-derived cells and organoids, are indispensable to understand the multiple roles of microglia within specific spatiotemporal contexts of health and disease.

### **How are microglial states coordinated?**

Even as we acquire more data about microglial states, there are still key questions remaining unanswered. To which extent are microglial states plastic and reversible? What is the relationship between microglial state and cellular function? These varied single-cell characterizations ultimately need to be linked to particular functions to become relevant to development, health, and diseases. How do these states come about? How do signals from the CNS environment get integrated in microglia to produce specific states? New imaging tools and reporters that enable tracking and manipulation of specific microglial states are needed to address these questions.

### **How similar are peripherally derived macrophages and microglia?**

A burning question that surely requires further investigation is related to the identity and function of microglia versus other brain macrophages. Although recent studies have provided evidence for an intrinsic unique core signature of microglia, their functional resemblances and differences remain undetermined. For instance, could engrafted parenchymal macrophages functionally replace the resident microglia despite having a different molecular identity, and could they serve as therapeutic vectors?

### **The devil is in the details**

Another major caveat is that microglia are incredibly reactive cells, and evidence indicates that artifacts are often introduced during sample processing for a variety of methodologies, such as RNA profiling, immunohistochemistry, fluorescence-activated cell sorting, *in vivo* imaging, and so on. Hence, we may be missing or confounding important pieces of information because we unintentionally introduce changes in the parameters we are trying to measure. In addition, these artifacts are likely to generate variability across laboratories using different protocols. A future challenge is to increase reproducibility of data across laboratories by coordinating a shared database of protocols and analysis pipelines curated using STAR Methods guidelines. In addition, in the current single-cell multi-omics era, the challenges in big data analysis are exponentially growing.<sup>140</sup> Statistical methods (including multivariate statistics)<sup>141</sup> and artificial-intelligence-based data mining approaches (such as

machine learning)<sup>142</sup> will have to be introduced to uniformly process and integrate large datasets, as well as extract the biological relevance of the findings.

### **Diversity as a source of richness**

Many transcriptional states have been reported during embryonic development, aging, and disease. How many different microglial states can be identified? Within the homeostatic microglia, how many states exist? How do microglia navigate among their many states? Are they related through a transcriptional continuum or perhaps as a hub-and-spoke set of states, as has been proposed for macrophages?<sup>4</sup> How dynamic are these states? And how spatially defined are they? Future research will need to address these important questions.

### **Male versus female microglia**

Sex differences have been reported to affect the brain colonization, maturation, structure, transcriptomic, proteomic, and functional profiles of microglia in a time-dependent manner. To what extent these differences may regulate the susceptibility to neurological diseases remains a fascinating question that urgently awaits answers. Investigating the molecular and cellular mechanisms underlying sex-mediated differences in microglial states would advance our understanding of microglial implication in diseases with clear sex-related differences in their prevalence, symptoms, and progression, as well as response to treatments.

### **Relevance to humans**

It will be imperative to study developmental and functional differences between human and animal model microglia. To date, most of the studies on microglia were conducted in mice, and a direct comparison among brain regions is still missing. Whether microglial states identified in mice also exist in humans is still under debate. Translating and validating these findings across species is critical and will help prevent failure of clinical trials that stem from animal model limitations. In addition, most human microglial studies were performed in Caucasians, and only recently data from other groups, such as African American individuals, are becoming available.<sup>143</sup>

### **Toward a unified nomenclature**

The conclusion of this paper is that the community has not yet reached an agreement on what defines microglial identity compared to other cell types, nor consensus on the number, dynamic nature, or definition of microglial states. The community advocates for creating harmonized, curated databases and guidelines for introducing novel terminology; following STAR methods; and sharing data as early as possible. Until such consensus is reached, the community urges all microglial studies to present data with all their layers of complexity and carefully define the context examined to offer clarity instead of confusion, thereby contributing to a more thorough understanding of the many facets of microglial biology. To establish new guidelines for microglial states and nomenclature, we call for a community-based approach, whereby the issues and progress are discussed openly in workshops and meetings, with input from diverse researchers across fields and career stages. A useful model to look after are the 10 Human Leukocyte Differentiation Antigen workshops that have taken place since 1982, in charge of renaming cluster of differentiation (CD) antigens (<https://>



[www.sinobiological.com/research/cd-antigens/hlda1](http://www.sinobiological.com/research/cd-antigens/hlda1)). We lastly advocate for the creation of an international panel/committee of experts in charge of overseeing the guidelines and establishing a specific roadmap to write a white paper in the nearest future.

We would like to conclude with the words of Río-Hortega, who sarcastically identified the problems of microglial nomenclature already 100 years ago: “If we were fond of introducing new nomenclature to describe microglia, as many modern histologists are, who think that enriching nomenclature resolves problems, we would find for microglia names that would indicate their origin, or morphology, or function, in addition to classify all the shapes that acquire when moving and evolving—resulting in the same absurdity that occurs in some branches of Histology and, particularly, Hematology.”<sup>144</sup>

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## DECLARATION OF INTERESTS

B.A. is the shareholder and member of scientific advisory board of Tranquis Therapeutics. K.B. is an employee and shareholder of AbbVie. M.C. receives research support from Vigil, is a member of the scientific advisory board of Vigil, and has a patent on TREM2. S.C. is a recipient of research funding from Eli Lilly and Company. C.C. is a member of the advisory board of Exalys Therapeutics and is the recipient of a research grant from IONIS therapeutics. B.D.S. is occasionally consulting for different companies. He is founding scientist of Augustin TX and of Muna TX. He is also shareholder of Muna TX. C.H. collaborates with Denali Therapeutics. C.H. is chief advisor of ISAR Bioscience and a member of the advisory board of AviadoBio. J.K. is a scientific advisor and collaborator with PureTech. T.M. is a cofounder of REGAIN Therapeutics, owner of a provisional patent on compositions and methods for treatment and/or prophylaxis of proteinopathies, and owner of a provisional patent on preventing or reverting abnormal amyloid deposition. R.M. has scientific collaborations with Alector, Nodthera, and Alchemab and is a consultant for Sanofi. B.M. has received consultancy fees from AstraZeneca. A. Sierra is a recipient of a research grant from Hoffmann La Roche.

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**Box 1.****Resting versus activated microglia**

The development of specific silver staining techniques in 1919 allowed Río-Hortega to clearly identify microglia and study their response to experimental manipulations.<sup>7,145</sup> Early on, Río-Hortega appreciated the striking morphological transformation of microglia following brain damage, but it was in the mid-1970s that the terms “resting” and “activated” microglia first appeared in the literature. These terms were used to morphologically describe cells with an affinity for silver staining that were observed in physiological (“resting”) versus pathological (“activated”) conditions. This nomenclature consolidated in the 1980s and became widely used during the 1990s,<sup>146</sup> in parallel with the development and use of histochemical and immunohistochemical techniques, such as lectin staining,<sup>147</sup> detection of phosphatases and phosphorylases,<sup>148</sup> and antibodies against the complement receptor CR3.<sup>7</sup> These techniques and nomenclature were pivotal in determining that “resting” microglia were unrelated to astrocytes, as some studies had wrongly concluded,<sup>149</sup> and that “reactive” microglia shared many characteristics with the blood-borne monocytes.<sup>10</sup> As shown by a PubMed search with microglia in all fields, there were only few papers published on the topic before the 1990s, and then a steady increase until the beginning of our century, followed by an exponential growth.<sup>150</sup> There is a first inflexion point in 2005, with the seminal discovery using non-invasive two-photon *in vivo* imaging that microglia are extremely dynamic in the absence of pathological challenge, continuously surveying the parenchyma with their highly motile processes.<sup>55,56</sup> The development of non-invasive methods was necessary for our understanding of microglial roles in the healthy brain (reviewed in Tremblay<sup>151</sup>). In 2005, microglial extreme dynamism in the intact brain was examined for the first time, through the skull of CX3CR1-GFP mice in which microglia are fluorescently labeled.<sup>55,56</sup> As a result, microglia are now considered to be the most dynamic cells of the healthy mature brain.<sup>151</sup> This seminal discovery prompted the renaming of quiescent or resting microglia as surveying<sup>56,152</sup> or surveillant (from the verb to survey)<sup>153</sup> microglia and also led to the proposal of the concept that microglia are never resting.<sup>154</sup> Together, these and other *in vivo* two-photon imaging data put into serious doubt the concept of “activated” microglia, which suggests a unique form of response, as in fact microglia are always active, constantly responding (in different ways depending on the context) to the changes in their CNS environment, even under normal physiological conditions. Therefore, microglia do not switch from “resting” to “activated” in response to trauma, injury, infection, disease, and other challenges. Rather, microglia are continuously active and react to the stage of life, CNS region, species, sex, and context of health or disease by adopting different states and performing different functions. Thus, although still widely used, “resting” and “activated” microglia are labels that should be discontinued.

**Box 2.****M1 versus M2 microglia**

Another terminology emerged in the early 2000s from immunologists classifying macrophages based on findings obtained using *in vitro* models: “M1,” the classical activation, considered pro-inflammatory and neurotoxic, as well as closely related to the concept of “activated” microglia, and “M2,” or alternative activation, considered anti-inflammatory and neuroprotective.<sup>155</sup> These responses were related to those of T helper lymphocytes (Th1 and Th2) based on their *in vitro* activation by specific immune stimuli that activated differential metabolic programs and changes in cytokine expression.<sup>156</sup> An associated term is “M0” microglia, which describes their state when cultured in the presence of transforming growth factor  $\beta$  (TGF $\beta$ ) and CSF1 to mimic *in vivo* counterparts.<sup>157</sup> The terms became widely adopted in microglial research, and the 2010s saw a boom of papers phenotyping macrophages and microglia into “M1” and “M2” based on the expression of markers related to these categories, used to indirectly assume a detrimental (“M1”) or beneficial (“M2”) microglial role.<sup>156</sup> In many cases, editors and reviewers have asked authors to comply with this nomenclature. However, it soon became evident that macrophage responses are more complex than simply “M1” and “M2.”<sup>158</sup> In the case of microglia, the advent of single-cell technologies provided clear evidence that microglia in the living brain do not polarize to either of these categories, often co-expressing M1 and M2 markers,<sup>159</sup> despite the continued use of M1 and M2 in the literature. We thus recommend strictly avoiding M1 and M2 labels and using more nuanced tools to investigate microglial function (reviewed in Devanney et al.<sup>160</sup>).

**Box 3.****Microglial morphological responses across species**

Microglial cells display a profusion of morphologies that have fascinated researchers since the early days of Río-Hortega. Many were tempted to equate morphology with function. Ramified microglia were traditionally associated with the “resting” state, although we now know that ramified microglia actively play many functions during normal physiological conditions. In contrast, “reactive” microglia (rounder cell body, generally with fewer and shorter processes) were called “activated” and equated with an inflammatory response. Only recently, however, a mechanistic link between microglial reduced branching and increased release of the inflammatory cytokine interleukin 1 $\beta$  (IL-1 $\beta$ ) was reported.<sup>161</sup> Activation of P2Y<sub>12</sub> by tissue damage signals potentiates the tonically active potassium THIK-1 channel, expressed in microglia, leading to both decreased microglial ramifications and activation of the inflammasome machinery processing IL-1 $\beta$  precursors into their mature form.<sup>161</sup> Another morphology associated with functional changes is “ameboid” microglia, which were thought to be more “phagocytic,” but it is clear now that ramified microglia execute phagocytosis through their terminal or “en passant” branches notably during adult neurogenesis,<sup>162,163</sup> while in disease conditions such as epilepsy, ameboid microglia can display reduced phagocytosis.<sup>164</sup> Therefore, morphological changes should not be interpreted in functional terms but rather taken as a suggestion prompting further investigation of the relationship between microglial structure and function. While the categorization described above is now outdated, the analysis of microglial morphology is considered valuable and still often used across animal model and human postmortem brain studies. Studies in postmortem brain samples have revealed that human and mouse microglia can adopt similar morphologies. Using the now outdated terms “ramified,” “primed” (larger cell body, ramified processes), “reactive” (ameboid, few ramified processes), and “ameboid” (less than two unramified processes), microglia were described in middle-aged individuals.<sup>165</sup> In addition, “rodshaped” microglia (elongated cell body, polarized processes) were found to become more abundant with aging.<sup>166</sup> Similarly, “dystrophic” microglia, presenting apparently fragmented (but still intact at the ultrastructural level) processes were reported in aging.<sup>167,168</sup> These different morphological types observed in humans were previously described in rodent models (reviewed in Savage et al.<sup>169</sup>). Nevertheless, a more sensitive quantitative microglial morphological assessment using a computational pipeline involving cluster analysis revealed differences between mouse and human, with distinct clusters found to be unique to each species.<sup>170</sup> Subsequently, a high-throughput comparative morphology analysis revealed a generally conserved evolutionary pattern, with some intriguing differences observed between the leech, zebrafish, axolotl, turtle, chicken, gecko, snake, bearded dragon, bat, boar, sheep, whale, hamster, rat, mouse, marmoset, macaque, and human and across brain regions between mouse and human.<sup>76</sup> While detailed comparative ultrastructural analyses of microglia between species are currently lacking, the state of “dark microglia” (named based on their increased electron density giving these cells a dark appearance, compared to other microglial states) discovered in 2016, which is defined using electron microscopy by its markers of cellular stress in contexts of aging

and disease, was found to be conserved across mouse, rat, and human.<sup>171,172</sup> New strategies are currently being developed to provide morphological data analyses based on automated pipeline, thus overcoming feature-selection-based biases.<sup>173</sup> Future studies will show how these varied morphologies correlate with transcriptional and proteomic profiles and what they imply for the cell's function. At the molecular level, recent single-cell transcriptome analyses also revealed that human microglia show multiple clusters that indicate a greater heterogeneity than in other mammalian species such as the mouse.<sup>76,91</sup>

**Box 4.****Microglia and the term “neuroinflammation”**

There is a long historical literature stating that inflammation is an important part of recovery from infection, injury, and disease, and it is the lack of resolution of this inflammatory response that is problematic in the context of CNS cell “reactivity.” Therefore, when the term “neuroinflammation” is encountered in the literature, the reader must be aware that it means different things depending on the context.

While the term “neuroinflammation” is widely used in the field as a synonym of microglial “activation,”<sup>174</sup> its definition also varies dramatically among authors, according to our survey. Below are representative definitions which are currently used by the authors:

- a. Neuroinflammation is inflammation of neural tissue particularly mediated by glial cells.
- b. Neuroinflammation is strictly limited to conditions in which leukocytes enter CNS, e.g., in stroke and MS.
- c. Neuroinflammation is a mixed cellular response to brain infection or damage involving innate and adaptive responses of resident brain cells and circulating immune cells.
- d. The term neuroinflammation is too unclear and imprecise and should be avoided.

Considering that different definitions are used across authors, our main recommendation for the field is to liberate neuroinflammation from microglia and microglia from neuroinflammation and to use both terms rigorously. The consensus among authors is 4-fold. First, protection against tissue damage and extreme departures from homeostasis as well as repair (i.e., “inflammation”) encompasses, in the CNS, a highly complex set of local responses and equally complex interactions with circulating immune cells or with immune cells residing in brain-blood and brain-cerebrospinal fluid interphases. In other words, “neuroinflammation” is not a substitute for “microglial reaction.” Second, there are numerous transcriptional states of microglia, astrocytes, and oligodendrocytes. The functional outcomes of cells undergoing these transcriptional states remain incompletely understood. Furthermore, it is uncertain which transcriptional states are transient or represent durable cell-fate choices. It is also unknown whether changes in states during diseases are “inflammatory” or dedicated to maintaining microglial homeostatic functions. Taking these considerations together, one should exercise extreme caution in simplifying these phenomena as “neuroinflammation,” as at least some of these phenomena may represent alternative homeostatic or non-inflammatory reactive states. Third, it is not appropriate to imply that neuroinflammation is invariably deleterious. Rather, it should be recognized that each inflammatory response may exert adaptive or maladaptive effects, contingent on context. To be more specific, research is necessary to explore functions and distinct actions of cytokine-enriched microglia secretomes beyond binary characterizations such as “pro-inflammatory” and “anti-inflammatory.” Fourth,



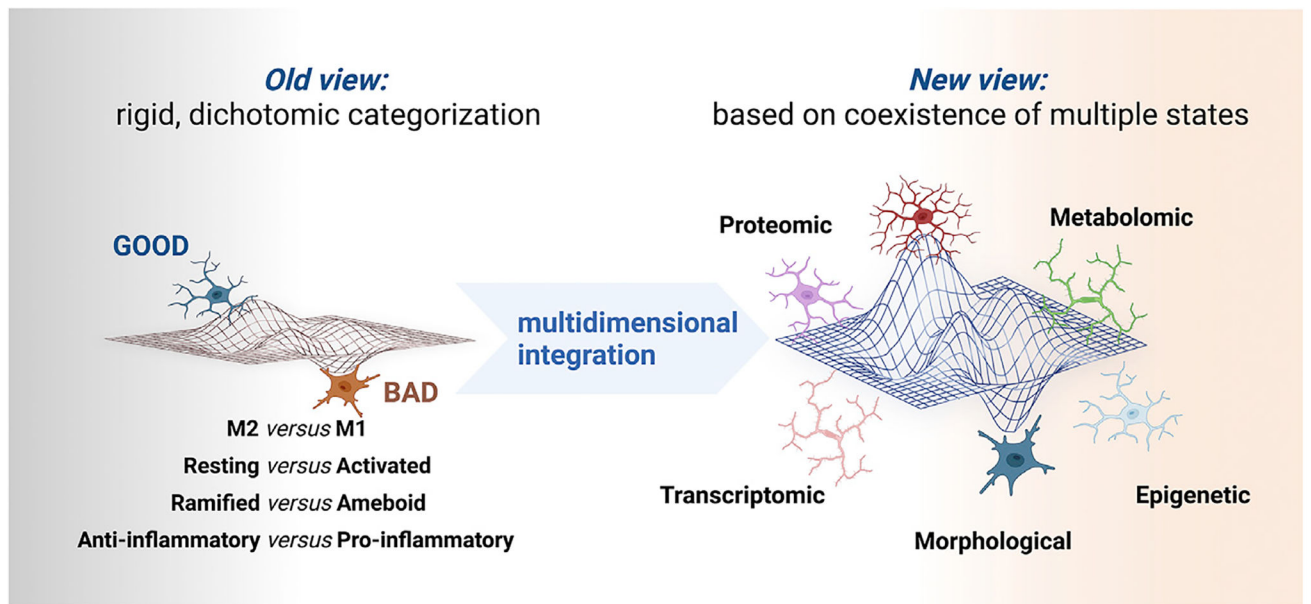
with regards to nomenclature, we recommend the use of modest and precise terms to describe specific phenomena such as: microglial reaction, astrocytic reaction, molecules involved, loss of barrier function at the blood-brain barrier, etc. All in all, the main message we wish to convey is that inflammation associated with the CNS follows unique rules that need to be fully discerned experimentally and not simply extrapolated from observations in non-nervous tissue.

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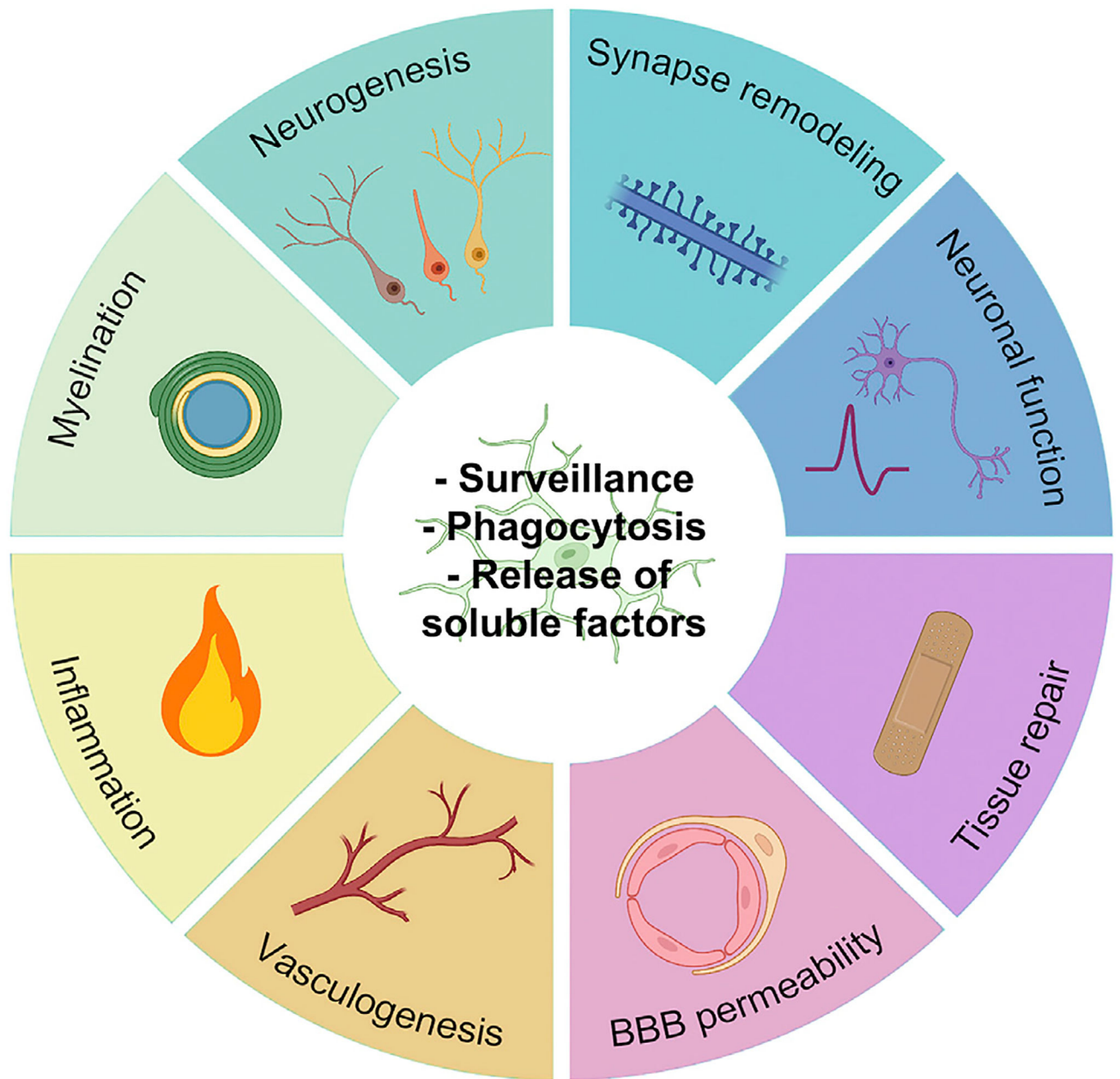
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**Figure 1. Microglial nomenclatures: Past and future**

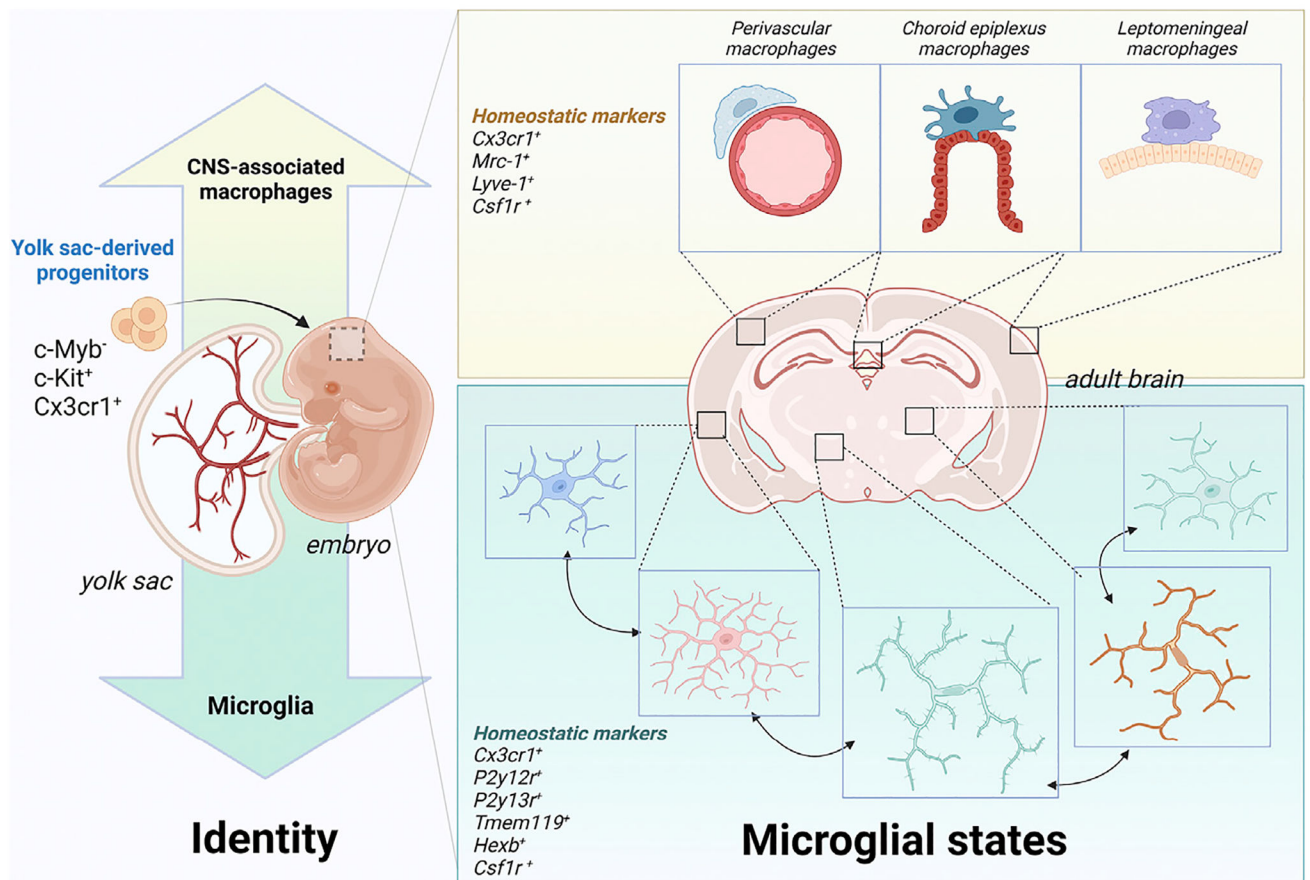
Microglia have been traditionally framed into dichotomic categories, but our current integration of epigenetic, transcriptomic, metabolomic, and proteomic data favors a multidimensional integration of coexisting states.



**Figure 2. Microglial core properties and functions**

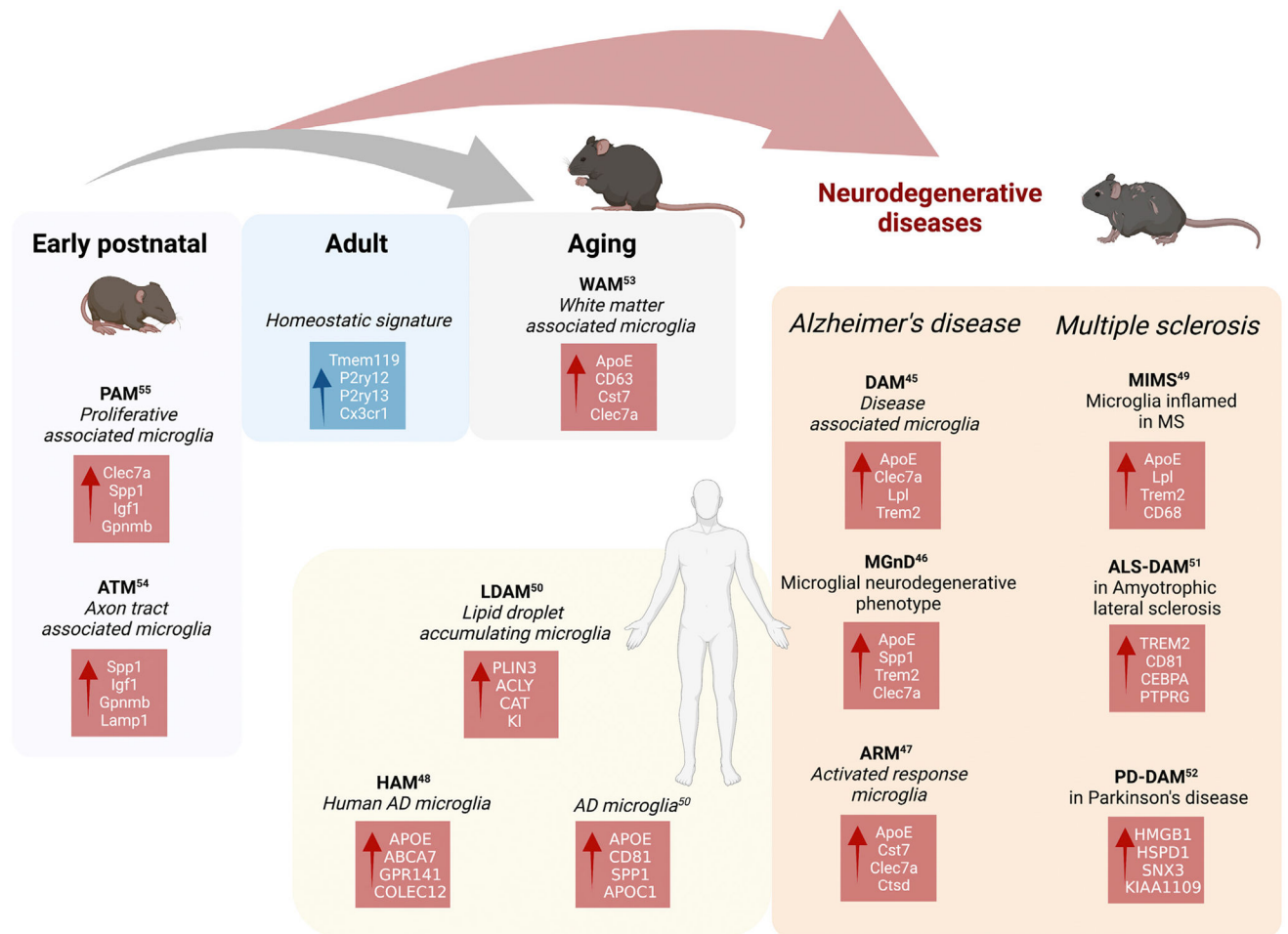
Phagocytosis, surveillance, and capacity for releasing soluble factors (inner circle) are core properties through which microglia contribute to key biological functions (outer circle).

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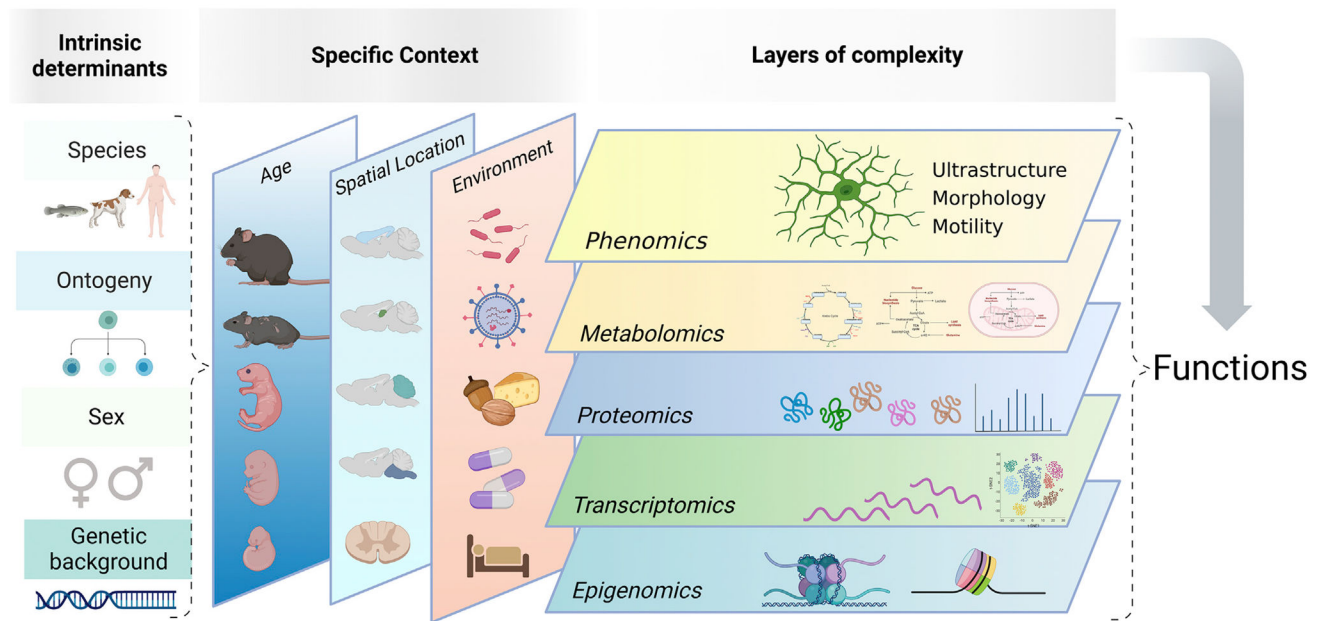
**Figure 3. Microglial identity and states**

The identity of microglia, compared to other CNS-associated macrophages in the perivascular space, choroid plexus, and leptomeninges, is established early on from yolk-sac-derived progenitors. Once they colonize the brain parenchyma and differentiate, they can adopt multiple states depending on the particular spatiotemporal context, as shown in more detail in Figure 5. Created with [BioRender.com](https://www.biorender.com).



#### Figure 4. Microglial transcriptomic signatures

Recent scRNA-seq studies have identified many microglial transcriptional signatures including, but not limited to, PAM and ATM in development; DAM, MgnD, ARM, and MIMS in disease models of AD, MS, ALS, and PD; and WAM, LDAM, and HAM in aging, in both mice and human. The key upregulated (red) and downregulated (blue) genes in each signature are indicated. Created with [BioRender.com](https://www.biorender.com).



**Figure 5. Microglial states defined by their intrinsic and extrinsic determinants, spatiotemporal context, and layers of complexity**

Microglial states depend on intrinsic determinants (such as species, ontogeny, sex, or genetic background) as well as the specific context they inhabit, including age, spatial location, and environmental factors (such as nutrition, microbiota, pathogens, drugs, etc.). All together, these factors impinge on microglia at multiple levels (i.e., epigenomic, transcriptomic, proteomic, metabolomics, ultrastructural, and phenomic), which ultimately determine microglial functions. Created with <https://BioRender.com>



Table 1.

Main antibody markers used to visualize microglia in rodents and humans from early embryonic development to adulthood and aging. Other proteins expressed by microglia but whose specificity is not confirmed include APOE, CLEC7A, ITGAX, and LPL.

Marker	Specificity	Labeled states	Staining patterns	Main applications	Reference
F4/80 (EMR1)	macrophages including microglia	homeostatic conditions and disease associated, expressed in rodents but presence not yet confirmed in human.	does not provide a detailed cellular visualization, especially in homeostatic conditions, because of its low basal expression. its expression varies significantly between species and is low in human macrophages.	brightfield or fluorescence analysis of microglial density, distribution, and categorization into morphological states.	Lawson et al., <sup>175</sup> Gautier et al., <sup>176</sup> Waddell et al. <sup>177</sup>
CX3CR1	macrophages including microglia	homeostatic conditions and disease associated, but downregulated by the DAMs, MGMD, dark microglia, and other pathological states.	CX3CR1-GFP reporter line generally used for visualization, with or without GFP immunostaining.	brightfield or fluorescence analysis of microglial density, distribution, and categorization into morphological states.	Keren-Shaul et al., <sup>58</sup> Krasemann et al., <sup>59</sup> Jung et al., <sup>178</sup> Wolf et al., <sup>179</sup> Bisht et al. <sup>180</sup>
IBA1	macrophages including microglia	homeostatic conditions and disease associated, used to study microglia in early embryonic and postnatal development, conserved across several species including human.	provides exceptional visualization of microglial cell body and processes, including distal extremities, diffuses throughout the cytoplasm. staining can, however, be discontinuous in aging.	brightfield or fluorescence analysis of microglial density, distribution, and morphology. ultrastructural studies.	Keren-Shaul et al., <sup>58</sup> Geirsdottir et al., <sup>76</sup> Tischer et al., <sup>168</sup> Inat et al., <sup>181</sup> Ito et al., <sup>182</sup> Shapiro et al., <sup>183</sup> Wake et al., <sup>184</sup> Tremblay et al., <sup>185</sup> Lier et al. <sup>186</sup>
MerTK	macrophages including microglia	homeostatic conditions and disease associated, expressed in health and across various contexts of disease, notably in association with the phagocytosis of newborn neurons, amyloid, and myelin.	partial visualization of microglial cell bodies and diffuse staining of their processes preventing a complete morphological visualization.	brightfield or fluorescence analysis of microglial density, distribution, and morphology. categorization into morphological states possible in combination with IBA1.	Fourgeaud et al., <sup>187</sup> Savage et al., <sup>188</sup> Healy et al., <sup>189</sup> Huang et al. <sup>190</sup>
CD11b/c	macrophages including microglia	homeostatic conditions and disease associated, used to study microglia in early postnatal development, conserved across species including human.	visualization of microglial cell body and low basal expression in adult microglia. staining is mainly restricted to the plasma membrane.	brightfield or fluorescence analysis of microglial density, distribution, and morphology. ultrastructural studies of subsets downregulating IBA1.	Bisht et al., <sup>180</sup> Robinson et al., <sup>191</sup> Milligan et al., <sup>192</sup> McKay et al., <sup>183</sup> Blackbeard et al., <sup>194</sup> Marshall et al. <sup>195</sup>
P2RY12	largely microglia specific (not expressed by monocytes), but state dependent	homeostatic marker, strongly downregulated in disease-associated and reactive states (but upregulated in <i>status epilepticus</i> ). used to study microglia in early postnatal development, conserved across several species including human.	visualization of microglial cell body and processes. staining can localize to the plasma membrane or diffuse throughout the cytoplasm and can be more profuse than IBA1 depending on staining conditions.	brightfield or fluorescence analysis of microglial density, distribution, and morphology. ultrastructural studies.	Avignone et al., <sup>117</sup> Peng et al., <sup>196</sup> Haynes et al., <sup>197</sup> Sipe et al. <sup>198</sup>
TMEM119	largely microglia specific, but state dependent	homeostatic conditions and disease associated, but downregulated on reactive microglia in some contexts (e.g., traumatic brain injury and ischemia, MS). developmentally regulated, conserved across species including human.	partial visualization of microglial cell bodies and diffuse staining of their processes preventing a complete morphological visualization.	brightfield or fluorescence analysis of microglial density, distribution, and morphology. morphological analysis or categorization into morphological states.	Kanamoto et al., <sup>199</sup> Bennett et al., <sup>200</sup> Satoh et al., <sup>201</sup> van Wageningen et al., <sup>202</sup> Gonzalez Ibanez <sup>203</sup>

Marker	Specificity	Labeled states	Staining patterns	Main applications	Reference
TREM2	macrophages including microglia, state dependent	microglial subsets in early postnatal development, aging, and disease conditions (e.g., microglia involved in synaptic pruning or associated with amyloid plaques in AD pathology), shown to label monocytes or neurons instead of microglia in human.	visualization of microglial cell body and processes, staining diffuses throughout the cytoplasm.	states possible in combination with IBA1. brightfield or fluorescence analysis of microglial density, distribution, and categorization into morphological states, ultrastructural studies of pathological states downregulating IBA1.	Bisht et al., <sup>180</sup> Savage et al., <sup>188</sup> Satoh et al., <sup>201</sup> Chertoff et al., <sup>204</sup> Fahrenhold et al. <sup>205</sup>

Table 2.

Main mouse lines used to visualize microglia from early embryonic development to adulthood and aging. Other proteins expressed by microglia but whose specificity is not confirmed include APOE, CLEC7A, ITGAX, and LPL.

Mouse line	Specificity	Labeled states	Expression patterns	Main applications	Reference
CX3CR1-GFP	macrophages including microglia	homeostatic conditions and disease associated, but downregulated in DAM, MGNd, dark microglia, and other pathological states.	visualization of microglial cell body and processes. Fluorescence diffuses throughout the cytoplasm. bright enough for two-photon <i>in vivo</i> imaging. a limitation is that the heterozygous mice used for <i>in vivo</i> imaging are partially deficient in fractalkine signaling, with possible outcomes on the brain and behavior. <sup>206</sup> The homozygous mice are knockout for CX3CR1 and used to study the outcomes of fractalkine receptor deficiency.	two-photon <i>in vivo</i> imaging or fluorescence analysis of microglial density, distribution, dynamics, interactions with other parenchymal elements, and categorization into morphological states. ultrastructural studies using staining against GFP.	Davalos et al., <sup>55</sup> Nimmerjahn et al., <sup>56</sup> Jung et al., <sup>178</sup> Bisht et al., <sup>180</sup> Tremblay et al., <sup>185</sup> Paolicelli et al. <sup>207</sup>
Iba1-EGFP	macrophages including microglia	homeostatic conditions and disease associated. downregulated in some contexts (e.g., obesity and aging) and in some pathological states (e.g., DAM, dark microglia). used to study microglia in early embryonic and postnatal development. conserved across several species including human.	visualization of microglial cell body and processes. Fluorescence diffuses throughout the cytoplasm. less bright than fluorescence in CX3CR1-GFP mice but generally sufficient for two-photon <i>in vivo</i> imaging of cell body and proximal processes. these mice are not partially deficient in IBA1 in their heterozygous state, which is a main advantage.	two-photon <i>in vivo</i> imaging or fluorescence analysis of microglial density, distribution, dynamics, interactions with other parenchymal elements, and categorization into morphological states. ultrastructural studies using staining against GFP.	Bisht et al., <sup>180</sup> Wake et al., <sup>184</sup> Hirasawa et al. <sup>208</sup>
Fms-EGFP or CSF1R-EGFP; CSF1R-FusionRed	Macrophages including microglia. CSF1R is expressed by most microglia.	Homeostatic conditions and disease-associated, but considered to be downregulated in DAM and other pathological states.	Fluorescence is less bright than in CX3CR1-GFP mice, and generally sufficient for two-photon <i>in vivo</i> imaging. It also allows for fluorescence-activated cell sorting and fluorescence imaging when combined with immunostaining. These mice are not partially deficient in CSF1R in their heterozygous state, which is a main advantage.	Fluorescence-activated cell sorting and fluorescence analysis of microglial density, distribution, dynamics, interactions with other parenchymal elements, and categorization into morphological states when combined with immunostaining.	Grabert et al., <sup>34</sup> Sierra et al., <sup>162</sup> Sasmono et al. <sup>209</sup>
HEXB-TdTomato	largely overlaps with IBA1 staining but restricted to microglia. Does not label CAMs and other border-associated macrophage populations.	expression appears stable in homeostatic conditions and disease-associated states. The labeled microglia are also depleted by CSF1R inhibition.	visualization of microglial cell body and processes. Fluorescence diffuses throughout the cytoplasm. bright enough for two-photon <i>in vivo</i> imaging. a limitation is that the heterozygous mice used for <i>in vivo</i> imaging are partially deficient in HEXB. However, their microglial gene expression patterns do not appear affected.	two-photon <i>in vivo</i> imaging or fluorescence analysis of microglial density, distribution, dynamics, interactions with other parenchymal elements, and categorization into morphological states.	Masuda et al. <sup>38</sup>