

Feature Review

Cien Años de Microglía: Milestones in a Century of Microglial Research

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The year 2019 marks the 100-year anniversary of the discovery of microglia by Pío del Río-Hortega. We will recount the state of neuroscience research at the beginning of the 20th century and the heated scientific dispute regarding microglial identity. We will then walk through some of the milestones of microglial research in the decades since then. In the last 20 years, the field has grown exponentially. Researchers have shown that microglia are unlike any other resident macrophages: they have a unique origin and distinguishing features. Microglia are extraordinarily motile cells and constantly survey their environment, interacting with neurons, astrocytes, oligodendrocytes, neural stem cells, and infiltrating immune cells. We finally highlight some open questions for future research regarding microglia's identity, population dynamics, and dual (beneficial and detrimental) role in pathology.

In memoriam, Georg Kreutzberg (1932–2019), father of modern microglial research.

Pío del Río-Hortega Discovered and Defined Microglia 100 Years Ago

'As a result of our first observation, we are convinced that this new cell type is a glial cell and we call it microglia due to the tiny size of their soma, which is considerably smaller than that of fibrous and protoplasmic astrocytes. Nonetheless, the subsequent assumption that microglia have a mesodermal origin inspired us also to name them mesoglia' (Río-Hortega, *El "Tercer Elemento" de los Centros Nerviosos. I. La Microglía en Estado Normal*, 1919).

With these words, the scientific field of microglia research was born 100 years ago, amidst a scientific and personal dispute between its scientific father, Pío del Río-Hortega, and the Spanish Nobel awardee Santiago Ramón y Cajal. In the next sections we will describe the origin of the clash between Cajal and Río-Hortega, the state-of-the-art of neuroscience during the early years of the 20th century, and the main findings of Río-Hortega, many of which have only been recently further advanced. We will then recount some of the major findings in the last 100 years of microglial research and we will finish by discussing key topics to be addressed in the future.

Neuroscience at the Beginning of the 20th Century

The early years of the 20th century were a thriving cultural and scientific period in Spain, when Ramón y Cajal created a school of neuroscience research in Madrid, to which many international researchers contributed (Figure 1). In the late decades of the 19th century, neurons and their connections had been established by Cajal as the basis of brain function, in what was called the 'neuron doctrine', defining neurons as the building blocks of the brain. In contrast, Camillo Golgi promoted the 'reticular theory', that considered the brain as a continuous network. For those studies, both Cajal and Golgi were awarded the Nobel prize in 1906. In addition to neurons, 'neuroglia' had been identified by the German pathologist Rudolf Virchow in 1856 [1] and the term 'astrocyte' was introduced by the Hungarian researcher Mihaly Lenhossék in 1895 [2]. In the early 1900s, Cajal became interested in astrocytes, inspired by Nicolás Achúcarro, who joined his laboratory after working with Alois Alzheimer in Munich, Germany. Cajal developed a novel method to stain the brain (the gold chloride sublimate method) that allowed him to obtain superb images of protoplasmic and fibrous astrocytes. The rest of the parenchymal cells remained poorly stained and showed no processes and Cajal named them 'apolar cells' or 'third element' [3].

At this time, Río-Hortega, a young researcher from a remote Castilian village in Spain who professed the highest admiration for Cajal, joined Achúcarro's lab. Río-Hortega's systematic research led him to

Highlights

Microglia were first described by the Spanish researcher Pío del Río-Hortega in 1919.

Río-Hortega's discoveries identified and defined the three types of glial cells of the CNS: astrocytes, oligodendrocytes, and microglia.

The field stagnated until the 1960s, when Georg Kreutzberg discovered the role of microglia in synaptic stripping in pathology.

Microglia were subsequently characterized as pathologic sensors in essentially all brain diseases and orchestrators of the neuro-inflammatory response.

In the last two decades, after the discovery of their extraordinary motility and their unique origin in the yolk sac, microglial research has grown exponentially.

Physiological roles of microglia include synapse monitoring and pruning, as well as modulating neurogenesis, myelination, and vasculogenesis.

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develop novel methods to stain the brain (ammoniacal silver carbonate method) and he was struck to find that the apolar cells consisted in fact of two different cell types, which he named microglia and interfascicular glia (he later renamed them as oligodendrocytes). In his 1919 series of four seminal papers *The 'Third Element' of the Neural Centers*, published in Spanish in *Boletín de la Sociedad Española de Biología*, the scientific journal edited by Cajal, Río-Hortega opposed Cajal's view of the 'third element' and stated that microglia are the phagocytic cells of the brain and are of mesodermal origin [4–7] (for an English translation see [8]).

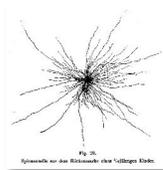
The German School



Rudolf Virchow
(1821–1902)



Mihaly Lenhossék
(1863–1937)



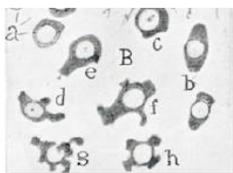
Alois Alzheimer
(1864–1915)



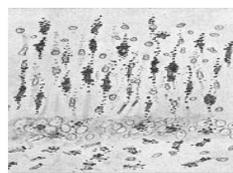
The Spanish School



Santiago Ramón y Cajal
(1852–1934)



Nicolás Achúcarro
(1880–1918)



Pío del Río-Hortega
(1882–1945)



Trends in Neurosciences

Figure 1. Prominent Neuroscientists Who Contributed to the Advancement of Glial Research

In the second half of the 19th century, glial research started to emerge in Europe. In Germany, Rudolph Virchow had coined the term 'glia' back in 1856. Other major players of the German school were Hungarian-born researcher Mihaly Lenhossék, who named astrocytes, and Alois Alzheimer, who in addition to defining the disease that bears his name also studied glial cells in different pathological conditions. In Alzheimer's laboratory, Nicolás Achúcarro studied a type of reactive glia ('rod cells' or *Stäbchenzellen*) in brains of rabid rabbits, and upon his return to Spain in 1910, inspired Santiago Ramón y Cajal to work on glia. It was a mentee of Achúcarro, Pío del Río-Hortega, who finally distinguished the three classes of glial cells, astrocytes, oligodendrocytes, and microglia. Portraits are reprinted from Wikipedia (public domain) and the drawings below them are reprinted with permission from the National Institutes of Health (Virchow [126]), Wikipedia (Lenhossék [2]), the National Library of Medicine (Alzheimer [58]), Juan Río-Hortega (Río-Hortega [4]), and references [127,128] (Achúcarro and Ramón y Cajal).

To fully appreciate the relevance of these statements, it is necessary to explain the complexity of neuroscience research during that period. In those days, each laboratory had its own staining method and animal/disease model and, as a result, researchers faced a confounding nomenclature of brain cells that, in addition to neurons and astrocytes, included 'foam cells', 'rod cells', 'scavenger cells', and 'granuloadipose cells', among others [9]. Adding to the confusion in the nomenclature was the mix of languages used in the scientific communication at the time (primarily German, French, or Spanish). Sharing reagents, recipes, and microscopy preparations was not easy and, in many cases, researchers had to rely on published drawings of the cells rather than photography to compare each others' findings. Río-Hortega's staining, beautiful drawings, and systematic description of the cells under different experimental conditions unified the literature and provided a clear and systematic identification of the three major glial cell types in the brain: astrocytes, oligodendrocytes, and microglia.

Río-Hortega encountered, however, a strong opposition by Cajal. The personal origin of the feud is somewhat obscure but could be related, at least to some extent, to the fact that Río-Hortega's growing laboratory was occupying Cajal's own laboratory, leading to a clash between their members. At the scientific level, the problem was related to the superior staining of Río-Hortega's method, which allowed one to see profusely ramified microglia where Cajal had only seen 'apolar cells' [3]. Further complication arose from Río-Hortega's choice to use the term 'mesoglia' to refer to microglia, which stemmed from his (correct) conjuncture that they are of mesodermal origin, unlike the rest of the brain cells. It is somewhat unfortunate that the term 'mesoglia' had been used a few years earlier by the Scottish researcher William Ford Robertson to refer to small phagocytic cells of mesodermal origin [10]. Cajal, having read a recount of Robertson's paper by the Italian researcher Ugo Cerletti, assigned Robertson the priority, and named the cells 'Robertson-del Río' cells [11]. The dispute between Río-Hortega and Cajal evolved over a few years and it was only in 1924 that the American neurosurgeon Wilder Penfield had the opportunity to directly inspect Robertson's preparations and assess that in fact Robertson had not labeled microglia, but rather oligodendrocytes, which are neither phagocytic nor mesodermal [12]. Since then, Río-Hortega's nomenclature and major findings have been widely recognized to hold true.

Río-Hortega's 1919 Microglial Findings

In the following, we list Río-Hortega's key findings, each followed by related quotes from his 1919 papers [4–8]:

The 'apolar' cell defined by Cajal consists of two cell types, microglia and interfascicular cells (later termed oligodendrocytes), both of which are different from astrocytes:

'(our staining method) convinced us about two things, both equally important: that the "third element" is not, as it is presumed, without processes; and that the "third element" of the white and grey matter are different.

Our research revealed the existence of two different cell types. Many perineuronal satellite cells, which are not astrocytes, are scattered in the grey matter and some of those which are in the white matter are identical in nature and have branched appendages, constituting, for us, the microglia. The «apolar» cells of the white matter, grouped into packages or columns between the bundles of axons, also possess more or less developed processes, but dramatically differ from those of the microglia and in fact are a different cell type distinct from astrocytes. We shall call them interfascicular glia.'

Microglia are of mesodermal origin and related to leukocytes; oligodendrocytes are more related to astrocytes:

'While microglia derive from the mesoderm and are able to phagocytose debris, interfascicular glia (which also have processes) are more alike to astrocytes in their origin and function.'

Microglia phagocytose dendritic spines and cells during development:

'It is very likely that during the development of the complex neuronal network, before young neurons and embryonic neuroglia acquire their final location and shape, some delicate neuronal processes become fragmented and some cells disintegrate or die. These phenomena would require the intervention of microglial macrophages to gather and degrade the cellular debris.'

Microglia are plastic, interact with all other cells of the brain parenchyma, and undergo a morphological activation after damage:

'The nomadic nature of microglia is best observed in neurodegenerative processes, during which the apparent rest they enjoyed in the normal state turns into migratory and phagocytic activity.'

Microglia proliferate in response to damage:

'Microglial proliferation can be easily observed in pathological conditions, during which mitotic and amitotic forms of division coincide with microglial hypertrophy.'

Microglia phagocytose neuronal debris during pathological conditions:

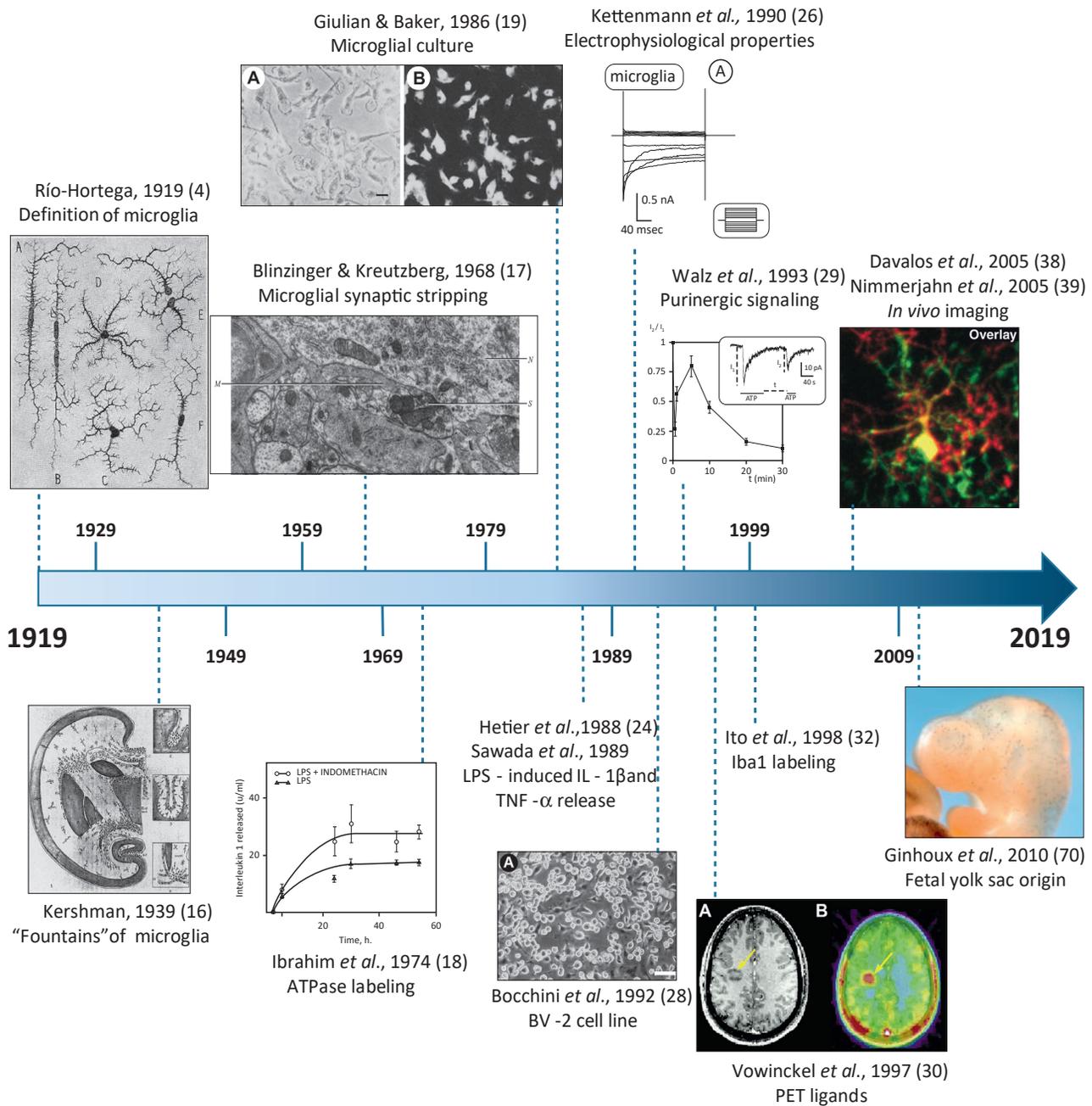
'Microglia acts as a voracious macrophage. A few hours after the injury is produced, they already enclose granulations and thick corpuscles in their cytoplasm (...). The gathering and degradations of cell debris occur, then, in a short period of time.'

Microglial Research was a Tiny Niche within Neuroscience for the First Half Century

After Río-Hortega's definition of microglia, their presence was verified in different brain regions, animal species, and in a variety of pathologies. Wilder Penfield promoted Río-Hortega's findings and analyzed glioma tissue, describing the presence of microglia [13]. Some authors named microglia 'Hortega cells' [14]. In the first review on microglia, Río-Hortega summarized the field in the book series *Cytology and Cellular Pathology of the Nervous System*, issued by Penfield, and this review article in English remained the state-of-the-art for many decades to come [15]. The origin of microglia in the human brain was first analyzed by John Kershman from the Montreal Neurological Institute, who observed the infiltration of microglia during embryonic human development and described hot spots of migrating cells, such as at the choroid plexus, coining the term 'microglia fountains' for these sites [16]. Thereafter, the field of microglial research stagnated for many decades (Figure 2). Over the 23-year span between 1945 and 1968, only 38 publications with the word 'microglia' in their title or abstract were published, based on a PubMed search. In sharp contrast, in 2018, 38 such papers were published, on average every 4 days. The field was slowly revived, starting with the studies performed by Georg Kreutzberg's group in Munich in 1968. Kreutzberg passed away earlier this year, in this centennial anniversary of the discovery of microglia, and we dedicate our publication to him. In the 1960s and 1970s, Kreutzberg's group developed a novel model to study microglial activation in the absence of peripheral monocyte invasion. By lesioning the facial nerve in the periphery, they observed an activation of microglia in the facial nucleus. These cells were involved in the reorganization of the circuitry of the nucleus, which was associated with removal of synaptic terminals on the motoneurons or 'synaptic stripping' by microglia [17]. Another key historical finding was the establishment of novel methods to identify microglia beyond Río-Hortega's silver staining, which works well in rabbit but is not as efficient in mouse or rat, animal models that by the 1960s and 1970s were more widely used. A histochemical identification by ATPase activity was developed, based on the enzymatic activity of ATP degrading enzymes, which are highly expressed by microglia [18].

The Development of a Microglial Culture System Launched a Series of Functional Studies

While the morphologic inspection of microglia in the different healthy or pathologic tissues showed some of their functional features such as migration or phagocytosis, further studies were difficult to perform *in situ*. The establishment of the first cell culture system by Dana Giulian and Timothy Baker



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Figure 2. Microglia: Some of the Key Developments, 1919–2019

After Río-Hortega's discovery, the microglial research field took off very slowly, with a remarkable paucity of published papers until the end of the 1960s, when Georg Kreutzberg discovered that microglia participate in synaptic stripping of the facial nucleus after axiotomy. Since then, microglial research was shaped by development in three major areas: culturing primary microglia and microglial cells, imaging methods for fixed and live cells, and physiological characterization. Since 1999, the field has evolved at an exponential rate [8]. Images are reprinted with permission from Juan Río-Hortega [4], *Archives in Neurology and Psychiatry* [16], *Springer Nature* [17], *Journal of Neuroscience* [19] (Copyright 1986 Society for Neuroscience), and [29] *Journal of Neuroscience Research* [24,26,28,30] and *Science AAAS* [39,70]. Abbreviations: IL-1 β , interleukin 1 β ; LPS, lipopolysaccharide; PET, positron emission tomography, TNF- α , tumor necrosis factor alpha. See [4,16–19,24,26,28–30,32,38,39,70]).

in 1986 to grow isolated and purified microglia was an important step to manipulate and study functional parameters, such as cytokine release. In these studies, microglia were typically isolated from the early postnatal rat brain and showed some of the properties as those of microglia found *in situ* [19]. This culture method rapidly spread among researchers world-wide and resulted in a first wave of functional studies on microglia. To provide some context for the more junior scientists among our readers, at that time, only a few molecules had been cloned, monoclonal antibodies had just started to spread, and modern techniques of molecular biology were not yet available. Using this culture system, it became evident that microglia produce superoxide after stimulation with the yeast-derived glucan zymosan [20]. Cell culture studies also revealed that microglia are capable of expressing molecules for immune interaction, such as MHC-I, which participates in antigen presentation to T lymphocytes [21]. MHC-I was also found in parenchymal cells in postmortem tissue of multiple sclerosis patients using a pan macrophage marker that did not distinguish between intrinsic microglia and monocytes invading from the periphery, but nonetheless suggested that microglia could be the partners of invading T cells in this disease [22].

At that time, in 1988, the Kreutzberg group published the first review (to our knowledge) on microglia since the one by Río-Hortega in 1932, and organized the first conference devoted to these cells (Methods in Microglial Research, 1992) summarizing their known functions and providing a vision of where the field should be going [23]. Microglia emerged as central elements in the pathologic production and release of chemokines and cytokines in the brain. Using bacterial lipopolysaccharide (LPS) as a stimulus, which had been adopted from the macrophage field as an efficient activator, it became evident that microglia are the predominant producer of the cytokine interleukin-1 beta (IL-1 β) in neuroinflammation [24]. Cultured microglia were soon also identified as the major source of another major cytotoxic cytokine, tumor necrosis factor alpha (TNF- α), after stimulation with LPS [25]. At that time, a recently developed electrophysiology technique to study ionic currents in isolated cells, patch-clamp, was applied to study cultured microglia. It was found that microglia express a unique membrane current pattern that distinguishes them from macrophages [26] and it has become evident now that K⁺ channel activity regulates microglial functions [27]. Another major development was the introduction of BV-2 cells, a stable microglial cell line generated by infecting cultured murine microglia with a v-raf/v-myc recombinant retrovirus [28]. BV-2 cells share many properties with cultured microglia, but over the years it became evident that *in vitro* and *ex vivo* microglia differ in many functional aspects. Despite these limitations, this cell line is still in use today (over 400 publications in PubMed using BV-2 cells in 2018). Physiological studies *in vitro* were also extended to demonstrate that microglia respond to neuroactive substances. Among them stand out purinergic receptors, which bind to extracellular purines such as ATP and were identified using the patch-clamp technique [29].

Microglial Research Moves Away from Culture

In vivo studies of microglia were pushed forward by the development of microglial-preferring ligands, such as PK11195, which binds microglial peripheral benzodiazepine receptors (PBR, also known as TSPO, or 18 kDa translocator protein). Using PK11195, the first positron emission tomography (PET) studies in which microglia were labeled in humans came to light, showing that 'activated' microglia were present in areas of demyelination of two multiple sclerosis patients [30]. Nonetheless, TSPO also labels astrocytes and, thus, novel and more specific markers needed to be developed [31]. In 1998, a novel calcium-binding protein, the ionized calcium-binding adaptor molecule 1 (Iba1), was cloned. When antibodies were generated against this protein, a novel and important tool had appeared to reliably identify microglia in tissue, and the Iba1-label has become one of the gold standards to identify microglia [32], although it also labels perivascular, meningeal, and choroid plexus macrophages [33].

Until 2000, the field was dominated by studies of microglia in cell culture and this period was essential to establish many microglial functional parameters (Figure 2). It was evident, however, that cultured cells would never reflect the complexity of signals faced by microglia in the brain environment. A novel labeling method, based on tomato-lectin staining of living microglia in acute brain slices,

eventually allowed recapitulation of the physiological properties of microglia in the tissue [34]. This further confirmed that microglia in a normal tissue environment (as normal as a brain slice can be) express a membrane current pattern that is distinct from the cultured microglia. Under pathological conditions, the cells in the tissue soon acquire the membrane current pattern typical of cultured cells, indicating that culture conditions represent a pathologic environment [35]. In the same year, a knockout mouse line for the chemokine fractalkine receptor (also known as CX3CR1), which in the brain is selectively expressed in microglia, was established [36]. The deletion construct also had a GFP reporter, and the scientific community soon recognized the potential use of heterozygous mice as a microglial-specific reporter line, although some effects due to the lack of one copy of the endogenous locus have been reported [37]. Using this mouse line, two independent studies combined it with *in vivo* imaging techniques and found that microglia rapidly scan the environment with their processes. In response to a laser injury, these processes engulf the damaged tissue within tens of minutes [38,39]. With these imaging techniques, the field took off, and in the past 20 years has evolved rapidly.

Microglia Play Key Roles in Brain Physiology and Pathology

The last decades of microglial research have been focused on figuring out the contributions of microglia to brain physiology and pathology. It is beyond the scope of this article to review thoroughly all functions of microglia in health and disease and we apologize to all our colleagues whose work could not be cited due to space constraints. We will now summarize three major areas of research in the past 20 years: brain physiology, brain pathologies, and microglial identity (Figure 3).

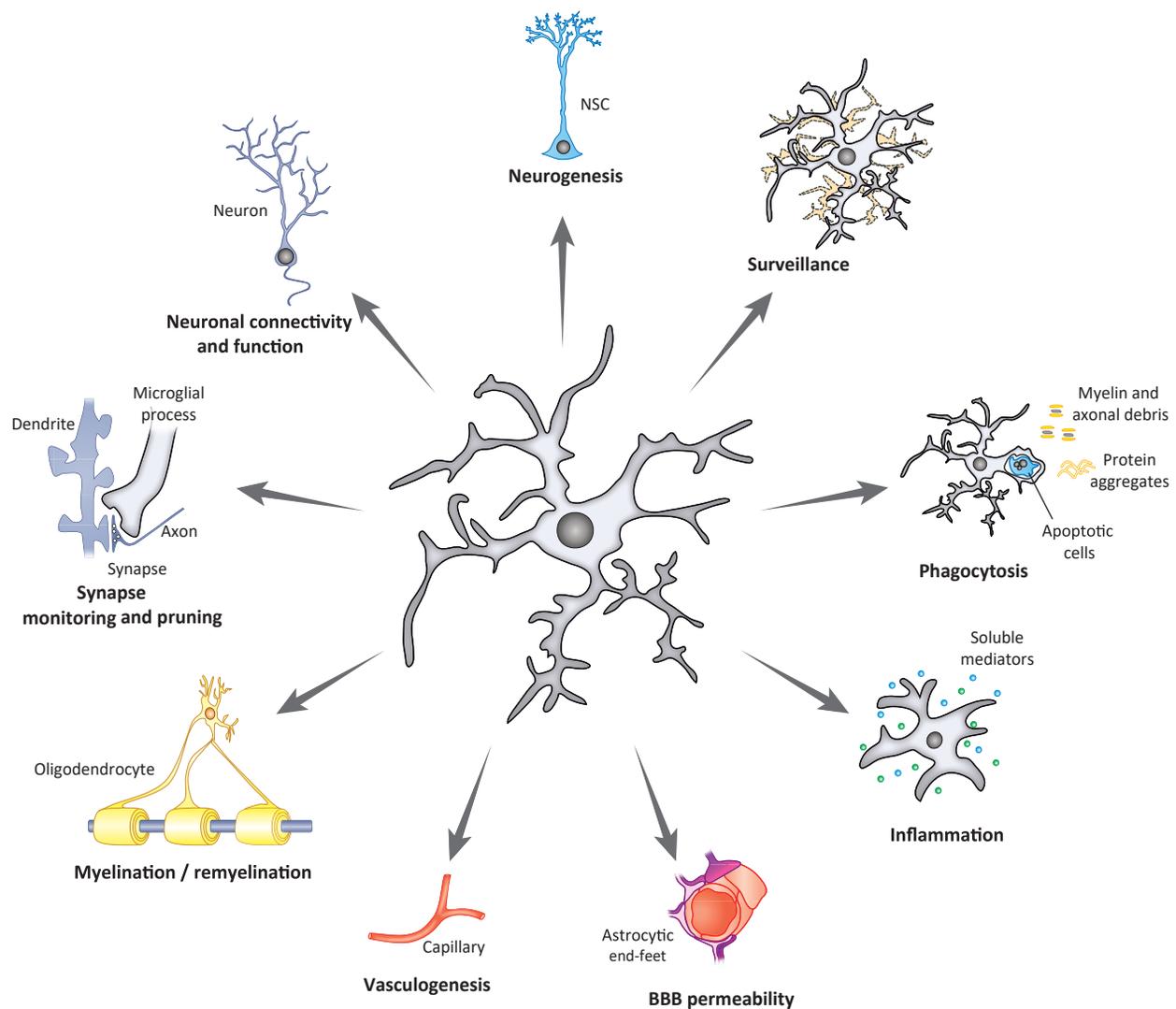
Microglia Have an Active Role in Brain Development and Physiology

The first evidence that microglia actively participate in brain development came from *in vitro* studies in the cerebellum, where it was found that microglia promoted the elimination of Purkinje neurons via superoxide ions produced by microglial respiratory bursts, thereby actively shaping the neuronal circuitry [40]. Since then, microglia have also been shown to limit the production of cortical neurons in rodents as well as in primates [41] and to support survival of layer V neurons in postnatal development through the release of insulin growth factor 1 (IGF1) [42]. In the adult hippocampal neurogenic cascade, microglia remove the excess of newborn cells [43]. However, microglia become detrimental for neurogenesis under inflammatory conditions [44,45].

Microglia not only remove entire apoptotic neurons by phagocytosis, but also selectively remodel synapses, thus participating in brain wiring. Using *in vivo* two-photon imaging of fluorescent-labeled neurons and microglia, it became evident that the movement of the microglial processes is not random but depends on neuronal activity [46] and experience-dependent neuronal activity leads to increased remodeling of synapses [47]. Several pathways participate in microglial-mediated synapse remodeling. Inappropriate synaptic connections are tagged by the complement receptor C1q and C3 and removed by microglial cells during development, as shown in the postnatal retinogeniculate system [48,49] and in dopaminergic forebrain circuits [50]. Synapse remodeling by microglia in the hippocampus is mediated by the fractalkine signaling pathway, with defective microglia-mediated synapse elimination resulting in long-lasting neuronal defects [51]. Microglial brain-derived neurotrophic factor (BDNF) mediates, in part, the crosstalk between microglia and neurons and is essential for motor learning-dependent synapse formation in the motor cortex [52]. In addition to synapse elimination, microglia can contribute to synapse remodeling by promoting filopodia formation, in particular during a critical period at postnatal day 8–10 [53] or by partial nibbling of presynaptic components [54]. An intriguing idea has recently emerged showing that microglia–synapse interaction is coordinated by neighboring astrocytes via release of soluble molecules, such as IL-33 [55].

Microglial Cells Influence the Pathologic Course of Many, if not All Brain Diseases

In the early years of microglial research, microglial activation was often viewed as an all-or-nothing process. This idea stemmed from the early observations by neuropathologists that activated microglia cells are characterized by an amoeboid morphological appearance, as they transform from their ramified phenotype in the normal brain [5]. This morphological transition was observed in diverse



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Figure 3. Microglia: Key Functions

The last two decades of research have focused on defining the roles of microglia in brain physiology and pathology. One salient feature of microglia is their processes' motility, which serves parenchymal surveillance tasks. In pathological conditions, microglia perform classical immune functions, such as release of inflammatory mediators and phagocytosis of cellular debris (apoptotic cells, axonal, and myelin waste). In addition, microglia perform several physiological chores that are to some extent altered in pathological conditions, such as synapse monitoring and pruning. Microglia are capable of interacting with other brain cells, impacting on their function: neurons and their connectivity; neural stem cells (NSCs) and neurogenesis; oligodendrocytes and myelination/remyelination; endothelial cells and vasculogenesis/revascularization; and astrocytes and blood–brain barrier (BBB) permeability.

brain diseases, from inflammatory to neurodegenerative disorders. In a review published in 2007, this view was challenged and it was acknowledged that microglia activation is more diverse and dynamic than ever anticipated, both in transcriptional and nontranscriptional features, as well as in functional consequences, implying that microglia respond differently in different pathologies [56]. Recent studies combining deep single-cell transcriptome analysis, fate mapping, *in vivo* imaging, clonal analysis, and transgenic mouse lines identified microglial subsets in several CNS compartments during neuroinflammation [57–59].

Box 1. Microglial Identity**Are Microglial Cells a Heterogeneous Population?**

Different subpopulations of microglia have been identified within different brain regions, based not only on morphological heterogeneity, but more recently also on their transcriptional profile [58,75,77,78]. However, some key questions remain. First, how dynamic are these populations? Recent ablation-repopulation experiments showed that the microglial identity depends on local cues [83], but it remains unclear, for instance, how much microglia depend on intrinsic versus extrinsic determinants, such as epigenetic instructions or signals coming from the surrounding niche. A somewhat related question is: how does the transcriptional profile of microglia translate into functional heterogeneity?

Are Human Microglia Similar to Microglia from Animal Models?

Most of what we know about microglial function comes from studies based on rodents or from postmortem human samples. Yet, how do murine microglia compare with those in humans? Important differences between murine and human microglia have been revealed using comparative transcriptomics [75,84,85]. Even more importantly, functional analyses of human microglia are still lacking and are urgently required. Since the first protocols for generating microglia from iPSCs were reported just 3 years ago [86–88], human iPSC-derived microglia have become a gold standard for *in vitro* studies of microglia-mediated mechanisms of diseases [89,90]. Importantly, microglial-like cells are present in organoids, opening the door to study human microglia in a complex environment that recapitulates the living brain [91]. However, epigenetic reprogramming due to the culture conditions leads to loss of aging hallmarks, which might significantly contribute to the etiology of the aging-related diseases. Thus, other methods, such as direct conversion, urgently need to be developed to preserve aging-associated features.

How Do Microglia Communicate with One Another and with Other Cell Types?

We still know very little about how microglia regulate their dynamics during adulthood. Perhaps even more importantly, the question of how microglia orchestrate their global behavior has received relatively little attention. Can we take inspiration from insect biology, or other models of complex population behavior, to understand collective behaviors of microglia? Reciprocal interactions between microglia and neurons have just begun to be elucidated and constitute a topic of major interest. By contrast, fairly little is known about microglial interactions with other cell types in the brain, although seminal discoveries in this area are emerging. Future studies are warranted to explore microglial crosstalk with neighboring cells, including oligodendrocytes, neural stem cells, and endothelial cells.

We next discuss briefly some examples of brain diseases that are influenced by microglia, specifically. It was recognized more than 20 years ago that microglia are essential elements for prion disease and dementia associated with HIV infection. In prion diseases, such as bovine spongiform encephalopathy, scrapie, and Creutzfeldt-Jakob disease, the loss of neurons requires the presence of microglia, which respond to a prion fragment by increasing their oxygen radical production [60]. In HIV-related dementia, microglia are the major target cells for HIV-1 infection within the brain parenchyma and they mediate the neuronal damage through chemokine receptors CCR3 and CCR5 [61]. In Nasu-Hakola disease, the microglial-specific transmembrane polypeptide KARAP/DAP12 is mutated, resulting in presenile dementia and demyelination. Mice deficient for KARAP/DAP12 show an altered microglial physiology that results in impaired synaptic function and neuronal plasticity [62]. Amyotrophic lateral sclerosis is a progressive neurodegenerative disease that affects motor neurons. The familial form of the disease is characterized by mutations in a few genes, among those the superoxide dismutase SOD1. By the use of mice carrying a deletable SOD1 mutant gene, it became evident that motor neurons are the primary determinant of disease onset, but that microglia strongly influenced disease progression at later stages [63]. Alois Alzheimer recognized the involvement of glia in the disease that was later named after him as early as in his first histopathological characterization in 1910 [64]. In recent years, it has been widely accepted that microglia accumulate at amyloid plaques and that they are not only bystanders, but have an influence on the progression of the disease [65]. Whereas initial research focused on the capacity of microglia to phagocytose amyloid [66], more recent evidences suggest that microglia are inappropriately activated in Alzheimer's disease (AD)

Box 2. Novel Microglial Functions in Physiology**How Different Are Microglia in the Male and Female Brain?**

Novel evidence indicates clear differences in transcription profiles and functions. Recent work has shown that microglia contribute to sex differences in social behavior [92] and further research will determine to what extent microglia partake in the brain sexual dimorphism. How such intrinsic differences contribute to disease susceptibility also remains to be elucidated [74,93,94].

Microglia as Sensor Unit

Synaptic activity has been described as a major driver for microglial behavior. How does neural activity translate into intelligible signals that microglia can interpret and react to? Neurotransmitter receptors on microglia are likely to play an important role [95,96], but the exact mechanisms remain to be elucidated. In this context, the concept of 'quad-partite' synapse, to which microglia contribute together with astrocytes to the communication between pre- and postsynaptic sites, needs to be extensively substantiated [97].

Reprogramming Microglia: Metabolic and Epigenetic Regulation

Does systemic and brain metabolic activity have an impact on microglia? The role of metabolism in regulating microglial function, so far poorly investigated, is a promising area of research that will shed light on how different metabolic pathways can modulate microglia behavior and *vice versa*, thus affecting basic microglial properties and conferring vulnerability to diseases [98–100]. In macrophages, cellular metabolism plays a central role in epigenetic reprogramming of the cell function [101]. Similarly, epigenetic regulation of microglia is likely to play an important role in the modulation of microglial behavior and may explain the molecular basis of innate immune memory [102–104]. One could hope that in the next few years, reprogramming microglia towards beneficial phenotypes will become feasible.

and mediate synapse loss [67]. Furthermore, single-cell sequencing identified a unique AD-related microglial phenotype, generated by a two-step process involving triggering receptor expressed on myeloid cells 2 (Trem2)-dependent and -independent pathways [59], but it still remains open whether microglial cells are initiators or followers in AD [68].

These are just a few examples of diseases in which microglia play a critical role. Detailed discussion of the implication of microglia in distinct pathological contexts, including chronic pain after nerve damage, psychiatric diseases, and glioma, is out of the scope of this review and is summarized elsewhere [69]. Nevertheless, it is important to highlight that not a single brain disease can be fully understood, diagnosed, or treated, without deciphering the role played by microglia in it.

Microglia Are a Unique Cell Population

Río-Hortega had rightly speculated that microglial cells are of mesodermal origin [6], a highly debated topic at the time [8]. This hypothesis was only recently substantiated by *in vivo* lineage tracing studies showing that microglia derive from primitive myeloid progenitors that arise before embryonic day 8 from the yolk sac [70]. The microglial lineage is similar but not identical to that of macrophages and is driven by the cytokine macrophage colony stimulating factor (M-CSF) [70], as well as the transcription factors Pu.1 (also known as Sfp1), Irf8 [71], and Sall1 [72]. Using single-cell transcriptomic analysis, a recent study indicates that microglia undergo three temporal stages of development that progress in synchrony with brain development and which are each under distinct regulatory circuits [73,74]. Also employing single-cell sequencing and single-cell mass cytometry, other studies have identified subclasses of microglial cells when examining differences in microglia: (i) across regions of the CNS; (ii) at different stages in life, including aging; and (iii) in response to different diseases [58,75–78].

In addition to defining the identity of individual microglial cells, a strong focus lately has been on defining the regulation of microglial population dynamics. Unlike other macrophages, the adult population of microglia does not renew from bone-marrow circulating progenitors, as shown by

Box 3. Microglial Duality in Pathology**Integration of Peripheral and Brain Immunity**

The motility of microglial processes is one of the key features of these cells and a constant source of awe for many microglial researchers. Their fast scanning of the brain parenchyma [38,39], together with their complex 'senses' [105], allows them to quickly recognize disruptions in the homeostasis and has given them the nickname 'brain patrols'. However, not all resident macrophages are similarly motile and it is speculated that the extraordinary motility of microglia may have evolved as an adaptation to neuronal activity. Another point of future research is related to how resident macrophages in the perivascular space, the meninges and choroid plexus, as well as the circulating immune system (monocytes and leukocytes), which infiltrate the brain in most neurodegenerative disease, interact with microglia. Do microglia tolerate invading immune cells or kill them? Do both participate in an evolutionarily integrated immune response? In addition, future research should consider another likely key player in the brain-systemic immune response: the intestinal microbiota [74,106].

Do Microglia Age?

The morphological, transcriptional, and functional changes undergone by microglia during normal aging are now well established [107,108]. What is less clear is how much of these changes are related to cell-autonomous aging versus signals from the aging brain parenchyma. It was recently shown that transplantation of young microglia is sufficient to revert age-related synaptic defects in mice [109] and restoration of phagocytosis efficiency improves cognitive function [110]. The question then is: are microglia a driver for aging phenotypes in the brain? As such, could they be the target for antiaging approaches?

What Role Do Microglia Play in Neurodegeneration?

Large population studies in humans have recently underscored a central role for microglia in AD and multiple sclerosis, indicating that many genetic risk factors associated with neurodegeneration are highly enriched in microglia. Yet, the mechanisms by which dysfunctional microglia may cause neurodegeneration are unclear and need to be disentangled. In fact, microglia are enormously powerful and capable of both beneficial, homeostatic roles in physiology and detrimental roles in disease. This duality can be exemplified in another key role of microglia, the engulfment of cells and cellular debris, including protein aggregates. Whereas homeostatic phagocytosis in physiological conditions is very efficient, it becomes impaired in some pathological conditions and aging [110,111]. In a twisted turn of events, microglia are also capable of engulfing and killing viable cells, a process named phagoptosis [112]. Thus, future research should reveal the mechanisms that turn microglia from a 'friendly neighbor' into a hostile one.

parabiosis experiments [79]. In contrast, microglia are renewed from local proliferation of resident cells, coupling apoptosis and mitosis [80]. Using a novel reporter mouse line, the Microfetti mouse, the turnover rate of the microglial population could be precisely determined, establishing that microglia are long-lived and renew their population three times during the 2-year lifespan of a mouse. New microglia are recruited from the internal pool and there is no apparent contribution from peripheral monocytes [81]. In humans, the turnover is similarly low, with cells living several decades [82], suggesting that that microglia may have a memory of past events imprinted on their epigenome [80].

Concluding Remarks

Since their initial discovery, major advances have been made in understanding the identity and function of microglia. A century of research, while placing microglia at a central stage in neurobiology, has probably generated more questions than answers. Despite the enormous efforts made in the last decades to characterize this population of brain cells, much is still unknown with respect to their function. Hopefully, the fast development of techniques and tools in the coming years will place the new generation of scientists in a privileged position to explore areas we still know little about. Progress in live imaging, in single-cell transcriptomics and proteomics, and in tools aimed at functionally manipulating microglia *ex vivo* and *in vivo* will facilitate rapid advancement of the field. We highlight some of the many open questions that remain (see Outstanding Questions) and also briefly discuss areas of microglial research that are currently particular foci of debate and controversy, thus urgently awaiting to be further elucidated (Boxes 1–4).

Box 4. Novel Tools and Approaches**Can Tools to Manipulate Microglia, Including Viral, Genetic, and Pharmacological Ones, Be Improved?**

Viral tools have proved to be critical for *in vivo* gene manipulation in several models. However, microglial-specific viral infection still faces several difficulties and has so far generally led to unsatisfactory results. Future improvement in viral transduction of microglia *in vivo* is desirable and eagerly awaited [113]. Another area that awaits further development is the generation of additional microglial-specific inducible genetic models, as the only currently available ones are two versions of the CX3CR1-CreER mice (based on a knock-in system that removes one copy of the endogenous locus) [52,114]. However, their absolute specificity for microglia has been recently questioned [115]. On the pharmacological front, drugs targeting M-CSF receptor have been recently developed by the company Plexikon to eliminate and repopulate microglia, with conflicting results about the beneficial or detrimental effects of ablating them [80,109,116–122]. However, dead microglia are unlikely to vanish from the parenchyma without collateral effects and the field will benefit from the development of novel drugs and nanomaterials that allow targeting specific microglial populations or specific microglial functions. Similarly, the unspecificity and low signal-to-noise ratio of TSPO for PET imaging [31] underscores the need for developing imaging tools for *in vivo* identification of microglia and neuroinflammation, particularly in the human brain.

What Can One Learn from Model Organisms?

Neuroscience (similarly to other fields like cancer research) has enormously benefited from research in a variety of model organisms, but microglial research has been largely restricted to rodents (and to some extent, humans). Pioneering work by Francesca Peri and others in zebrafish helped illuminate molecular mechanisms of phagocytosis of apoptotic cells [123,124] and the imaging capabilities in this model could be used to assess many other open questions. Invertebrate models, such as *Drosophila melanogaster*, do not have proper microglia but a sort of mixed-function glial cell population, which nonetheless could be very useful to perform genetic screenings [125]. In addition to mammals, studying microglial in amphibians, reptiles, and birds will provide a much-needed evolutionary perspective of microglial function.

Towards an Integrative View of Microglia through System Biology and Computational Modeling Approaches

Lastly, we would like to draw a vision for a fast-approaching era of integrative knowledge and understanding of microglia using system biology approaches. Integrating microglial epigenetic, transcriptomic, proteomic, and metabolomic data collected under different conditions will be a daunting bioinformatic task, but one that will nonetheless serve to provide a wider and deeper perspective of microglia. Similarly, the development of computational models of microglial behavior, such as process motility, population dynamics (mitosis vs apoptosis), or interaction with other elements of the brain parenchyma, will inform us of how these processes are regulated and dysregulated in different conditions and will shed light on their ultimate impact in brain physiology.

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Outstanding Questions

Microglia are present throughout vertebrates and related cell types are present in invertebrates. How did the relevance of microglia for brain function change during evolution?

Genome-wide association studies suggest the participation of microglia in most brain diseases. Are microglia responders or initiators of brain pathologies?

Are there yet-to-be-discovered functions of microglia in the healthy and diseased brain? What would novel genetic, viral, pharmacological, and nanomaterial-based approaches reveal about microglia?

What can be learnt about human microglia using induced pluripotent stem cells (iPSCs) and organoids? How can culture conditions be optimized to mimic the brain environment? How different are microglia between men and women? This knowledge will be used to steer treatments aimed at microglia, for which it is essential to develop biomarkers that faithfully represent microglial activity in the living human brain.

How is microglial identity and function regulated at the single cell and population level? Microglia seem to integrate signals coming from their surrounding niche, the peripheral immune system, and the microbiota, but how is this integration accomplished at the epigenetic, transcriptional, and metabolic levels?

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