

Chapter 2

Historical Context

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Abstract The term ‘microglia’ was first introduced into the scientific literature almost a century ago. The various eras of microglia research have not only been defined by the number of reports subsequently generated but, more critically, by the concepts that have shaped our present-day views and understanding of microglia. Key methods, technologies, and models as well as seminal discoveries made possible through their deployment have enabled breakthroughs, and now pave the way for lines of investigation that could not have been anticipated even a decade ago. Advances in our understanding of microglial origin, forms, and functions have relied fundamentally on parallel developments in immunology. As *the* ‘neuro-immune’ cells of the brain, microglia are now under the spotlight in various disciplines. This chapter surveys the gradual processes and precipitous events that helped form ideas concerning the developmental origin of microglia and their roles in health and disease. It covers first the dawning phase during which the early pioneers of microglia research discovered cellular entities and already assigned functions to them. Following a recess period, the 1960s brought a renaissance of active interest, with a development of tools and models—and fundamental notions on microglial contributions to central nervous system pathologies. These seminal efforts laid the fundament for the awakening of a sweeping research era beginning with 1980s and being spurred on by a blast of immunological discoveries. Finally, the chapter stresses the advancement of molecular, genetic as well as imaging approaches to the study of microglia with the turn of the millennium, enabling insights into virtually all facets of microglial physiology. Moving forward it is clear that the future holds substantial promise for further discoveries. The next epoch in the history of microglia research has just begun.

Keywords Microglia • Developmental origin • Roles • Physiology • Pathology • Methods • Technologies • Models

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Bullet Points

- This chapter surveys the historical context that surrounds microglial cells, including their discovery, developmental origin, and functional roles in health and disease.
- We describe the concepts that have shaped our present-day views and understanding of microglia.
- We also present the key methods, technologies, and models that made possible the seminal discoveries across the various eras of microglia research.
- Lastly, we discuss the recent advancement of molecular, genetic, and imaging approaches, holding substantial promise for further discoveries.

2.1 Introduction

What relevance does the historical context have to today's research on microglia? With the pace of research and scientific discovery moving so rapidly in the present day, it is perhaps inevitable that those who are new to the field would focus on the latest outputs and deem work that has been published beyond the last few years as already 'dated'. Those with more than a passing interest in the neurosciences, however, find greater appreciation and deeper insight through a realisation of the concepts, the controversies, and breakthroughs that have shaped today's understanding of microglia. Indeed, it is remarkable to note the extent to which today's research is still directed at questions raised almost a century ago, and the evidence, in some areas, that merely confirms those pioneering observations (Rezaie and Male 2002a). Among the non-neural elements that constitute the nervous system, these enigmatic cells—the microglia—have arguably one of the most widely debated and contentious of historical perspectives (Rezaie and Male 2002a). In this chapter, we will examine the early history in more detail, beginning with pioneers of the late nineteenth and early twentieth centuries, and their contributions. We will thereafter journey through the renaissance and modern eras of research arriving at the new millennium. Throughout, we will consider major concepts and technical breakthroughs that have shaped present-day understanding of the biology (origin, form, function) of microglia and their responses to central nervous system (CNS) injury and disease. Figure 2.1 provides an overview of the history of research on microglia, from the pioneers, major discoveries, technical developments, and fundamental concepts across one and half centuries (up to 2013).

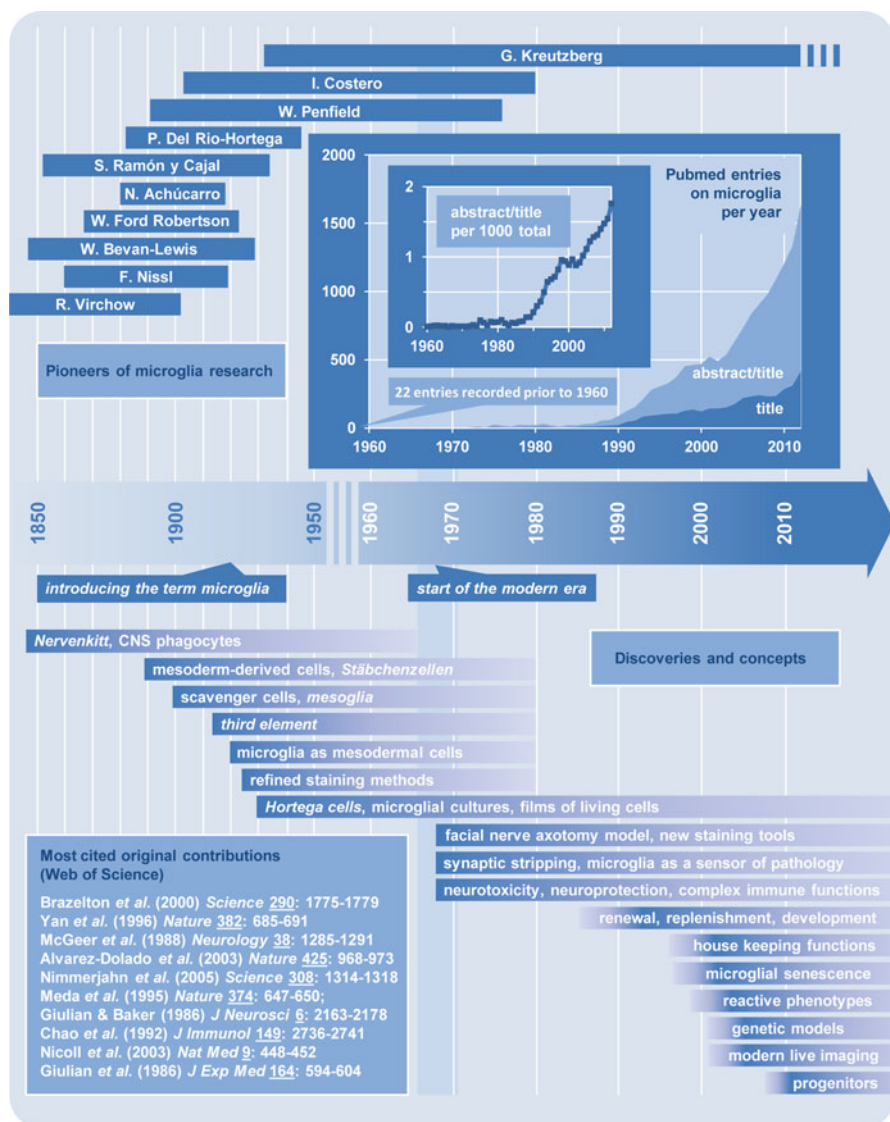


Fig. 2.1 Historical overview of research on microglia. Pioneers who contributed to the early discoveries are indicated with their biographic dates. Some major discoveries, technical developments, and fundamental concepts are shown below the timeline. The graphs (*inset*) illustrate the growth in research on microglia, according to the number of publications per year, carrying the term ‘microglia’ in the abstract and/or title (based on PubMed entries). The steady increase of microglia-related work is also reflected when normalised for the total record (number of microglial publications per 1000 total entries). The box lists the 10 most cited original contributions (by the topic microglia, based on the Web of Science)

2.2 The ‘Dawn’ and ‘Early’ Phases of Microglia Research

The discovery of ‘Nervenkitt’ (*‘neuroglia’*, literally translated as ‘nervous tissue glue’) by Rudolf Virchow in the mid-nineteenth century heralded a new era for the study of the nervous system (for historical reviews see: Ferrer 1973; Theele and Streit 1993; Barron 1995; Rezaie and Male 2002a). Virchow was among the first to recognise phagocytes filled with fatty substances in the CNS under pathological conditions (1851–1867). He referred to these cells as *‘Schaumzelle’* (foam cells). Gluge, before him (in 1841), had identified mesodermal phagocytic cells within injured brains as *‘Entzündungskugeln’* (inflammatory corpuscles). Wilhelm His (1888–1890) observed that mesodermal elements invade the developing human fetal spinal cord from the pia mater, towards the end of the second month of embryonic life, and distribute equally in white and grey areas. Between 1875 and 1900, Lachi, Duval, Gadow, Eichhorst, Schwalb, Friedman, Ranvier, Renaut, Lowe, and others (cited by Cajal 1925; del Río-Hortega 1920a, 1921a, 1932) were of the opinion that neuroglia in the white matter were mesoderm-derived. W. Lloyd Andriezen, however, proposed that protoplasmic neuroglia in the grey matter to be of mesoblastic origin, whereas the fibrous cells found in the white matter were considered ectodermal (Andriezen 1893). Cajal adopted this classification (protoplasmic and fibrous neuroglia) and referred to these cells as ‘astrocytes’, but he insisted that both populations were ectodermal in origin (Cajal 1913; see also Somjen 1988).

The principle of phagocytosis was first described by Ilja Metchnikoff in the late nineteenth century, already in the context of cell-based immunity and owing him in part the Nobel prize in 1908, shared with Paul Ehrlich. Even though phagocytes, namely macrophages, exhibit a much broader spectrum of activities, the importance of cells with the ability of clearing infectious agents and tissue debris in development, homeostasis, and defence was thus recognised as well as anticipated quite early.

Phagocytic cells found in damaged brains were on the whole considered to be mesodermal in origin, based on morphological and structural similarities with leukocytes. Franz Nissl suggested that glial cells in the brain adopt similar functions to macrophages in other tissues. He introduced the term “*Stäbchenzellen*” (rod cells) in 1899, associated with various stages of neurodegeneration, and related their functions to leukocytes in other tissues. In 1903, he proposed that phagocytes in the CNS originated from adventitial connective tissue of intracerebral vessels and mesenchyme, referring to these cells in 1904 as “*Gitterzellen*” (compound granular corpuscles). He considered them to be derived from mesoderm and not from blood cells (see Rezaie and Male 2002a). Like his contemporary Andriezen (1893), Bevan-Lewis (1899, 1906) was of the opinion that neuroglia were of different types, but unlike Andriezen (who had in fact been describing protoplasmic and fibrous astrocytes), Bevan-Lewis proposed a phagocytic ‘scavenging’ function for neuroglia in ‘*clearing out of fatigue products*’ within the CNS (Bevan-Lewis 1906). Marinesco, before him, had reported dying neurons being removed by glial cells through phagocytosis. Forster, Marchand, and Pick later concluded that phagocytes

could originate from neuroglia, and in 1910, Merzbacher was to include the term “*Abräumzelle*” (scavenger cell) in this group (see Rezaie and Male 2002a).

A significant breakthrough came from a somewhat unexpected and relatively inconspicuous source around the turn of twentieth century. Through developing a new technique to stain cellular elements within the CNS (a modification of the Golgi method using platinumous oxide) (Robertson 1899), the Scottish pathologist William Ford Robertson who had been studying the neuroglia in some depth (Robertson 1897, 1898) showed that ‘neuroglia’ were not in fact a homogeneous grouping of cells, but were actually composed of different sets of elements. In 1899, using this new ‘platinum method’, the 32-year old pathologist to the Scottish Asylums described a novel group of cells in the canine and human CNS, referring to these as ‘mesoglia’. He considered them to be derived from mesodermal elements (based on their staining properties), with morphologies that were quite distinct from other neuroglia (Robertson 1899, 1900a, b). Robertson later stated that the ‘mesoglia’ were able to act as phagocytes in certain pathological conditions (Robertson 1900a, b, see also Rezaie and Male 2002a). With the public announcement of his findings, Robertson became the first to demonstrate that the neuroglia were in fact composed of different cellular elements. John Turner was among the first to investigate Robertson’s mesoglia. He was able to verify these cells in tissue sections stained with methylene blue dye (Turner 1905).

It was around this time that Santiago Ramón y Cajal, the founding father of the Spanish School of Histology and Histopathology in Madrid (awarded the Nobel prize in 1906, joint with Camillo Golgi, for his contributions to the ‘neuron doctrine’) started to take a serious interest in the neuroglia. One of his young and talented students (and later colleague whom he held in great esteem), Nicolás Achúcarro, had studied with Emil Kraepelin and Alois Alzheimer in Munich and developed a keen interest in the neuroglia. Between 1908 and 1910, Achúcarro embarked on a series of investigations into these cells, focusing specifically on astrocytes and on *Stäbchenzellen* first reported by Nissl in 1898 in cerebral palsy. He began by employing modifications of the reduced silver nitrate method used by Cajal and others and went on to develop a more selective tannin and ammoniacal silver nitrate method to describe *Stäbchenzellen* (microglia ‘rod’ cells; see Graeber and Mehraein 1994). Achúcarro considered *Stäbchenzellen* to ‘adapt to degenerating pyramidal neurons’ and noted that their peculiar elongated configuration conformed to the structure of the nervous tissue, aligning with the dendrites (Achúcarro 1908). He studied experimental wounds and local inflammation within the hippocampus, showing morphological transformation of these ‘rod’ cells and their phagocytic activity, with high lipid content in the vicinity of necrotic foci. Achúcarro proposed that *Stäbchenzellen* were phagocytic cells of mesodermal origin (specialised leukocytes) that adapted mechanically to the form and direction of neuronal processes and were capable of engulfing the decay products of neurons in inflammatory processes. He considered these cells to belong to the category of phagocytic ‘*Abräumzellen*’ (Achúcarro 1908, 1909, 1910).

In 1911, Achúcarro presented his new technique (the method of tannin and ammoniacal silver) to the Spanish Society of Biology (Achúcarro 1911). Achúcarro

used this method in a number of investigations on the cerebral cortex in ‘general paralysis’ (syphilitic encephalopathy) and to describe changes in neuroglia in senile dementia. Cajal referred to Achúcarro’s work, as well as his own gold-sublimate method for neuroglia, extensively in his subsequent publications on the neuroglia (Cajal 1913, 1920a, b, 1925). 1911–1913 was a period of intense focus on neuroglia research (see García-Segura 2002; García-Marín et al. 2007). Using formol uranium nitrate and gold chloride sublimate methods, Santiago Ramón y Cajal described what he termed a ‘new class of cells that appeared to lack processes’ in 1913 (Cajal 1913). He referred to these ‘adendritic’, ‘apolar’, and ‘dwarf’ cells as the ‘third element’ within the CNS, distinguishing them from neurons and astrocytes (see García-Marín et al. 2007). Cajal described these cells within the white matter, and also occurring as perineuronal and perivascular satellites, considering them to be mesodermal in origin. He believed that the cells identified in the central white matter were analogous to Schwann cells in the peripheral nervous system (Cajal 1913). At the time, he was not aware of Robertson’s earlier work and only chanced across this later through a publication by Cerletti (lacking illustrative detail), which he considered in more detail in his 1920 and 1925 papers on the subject (Cajal 1920a, 1925). Achúcarro’s successes were tragically cut short, as the first symptoms of a fatal illness began to take hold of him just 3 years following this appointment, forcing him to abandon his research activities. Initially considered to be tuberculosis (a disease that had afflicted his brother), Achúcarro recognised the symptoms as a form of Hodgkin’s disease, which he self-diagnosed. Achúcarro’s death at the age of 37, on 23 April 1918, came as a tremendous blow to Ramón y Cajal (see Cajal et al. 1968; Bustamante 1982; Andres-Barquin 2002).

Pío del Río-Hortega is probably the most prominent figure of the ‘Spanish School’ after Cajal (Gonzalez 1971; Andres-Barquin 2002). A pupil and later friend of Achúcarro, del Río-Hortega, had studied Medicine at the University of Valladolid, obtaining his license in 1905 and his doctorate in histology from the University of Madrid in 1909. Del Río-Hortega applied to work with Cajal, and after a brief spell with Tello (Jorge Francisco Tello-Muñoz), whose work at this time focused on regeneration of the nervous system—see García-Segura 2002), in the newly established Laboratorio de Investigaciones Biológicas (Andres-Barquin 2002), joined Achúcarro’s laboratory in the Museum of Natural History. This was later merged with Cajal’s Institute and named the ‘Laboratorio de Histopatología (Histología Normal y Patológica) de la Junta para Ampliación de Estudios’. Here began his independent training, learning silver impregnation techniques and developing an interest in neuroglia. In 1916, his curiosity spurred by Cajal’s ‘third element’, del Río-Hortega began to seek more stable variations of Cajal and Achúcarro’s methods. Del Río-Hortega’s diligence in pursuit of the third element led to his first publications on neuroglia in 1916 and 1917. He had also begun to doubt the accuracy of Cajal’s concept that the ‘third element’ was composed solely of “corpuscles without processes” (see Cannon 1949, p. 239). Following closely in Achúcarro’s footsteps, del Río-Hortega developed a new modification of Achúcarro’s ammoniacal silver method (Achúcarro 1911), using silver carbonate (del Río-Hortega 1918), and identified distinct elements, initially the ‘microglia’, and what he called ‘interfascicular

cells' (del Río-Hortega 1919a, b, 1920a) which, after further modifications, he was able to define as 'oligodendroglia' (del Río-Hortega 1921b). He proposed that these cells were distinct populations; that the oligodendroglia belonged to the class of 'neuroglia' cells; and that microglia represented the true 'third element' on account of their mesodermal (as opposed to ectodermal) origin. He also alluded to the fact that Cajal had been unable to see the full extent of the morphology (fine cellular processes) of these cells in his 'third element' paper of 1913, due to the limitations of the technique he had used. Although Cajal eventually came to accept the existence of microglia as a separate class of cell (Cajal 1920a, 1925), he was not convinced regarding the oligodendroglia. Furthermore, Cajal considered Robertson's mesoglia and del Río-Hortega's microglia to be one and the same (Cajal 1920a, b). This contention was at the centre of a dispute that developed between Cajal and Hortega and led to tensions in their professional relationship.

Achúcarro, by this time, had become gravely ill (Jelliffe 1919). Consequently, del Río-Hortega's technical breakthrough and exemplary work were marred by the tragic illness and subsequent death of Nicolás Achúcarro in 1918. Del Río-Hortega succeeded Achúcarro in directing the Histopathology Laboratory and was later also appointed Histopathologist at the Provincial Hospital of Madrid. Going against Cajal's advice, del Río-Hortega proceeded to present his work to the Society of Biology. He published his work on microglia in four parts under the heading of 'the third element of the nervous system' (del Río-Hortega 1919a, b; see Fig. 2.2). Not only did this echo Cajal's own 1913 publication, but the work deigned to 'criticise and correct' it. Publishing his work without Cajal's approval had placed del Río-Hortega in a delicate position and tensions continued to grow (del Río-Hortega 1986), forcing his departure from the laboratory of Cajal in October 1919, and transfer to the Students' Residence (La Residencia de Estudiantes) located on the outskirts of Madrid in 1920, where he established a new laboratory.

Del Río-Hortega's follow-up paper on microglia (del Río-Hortega 1920a) related microglia with the different morphological forms of phagocytic cells found in the CNS and made the direct 'connection' with *Stäbchenzellen*. Cajal published a critique in the same journal volume (dated 15 December 1920), attributing del Río-Hortega's discovery to Robertson who, according to Cajal, had named these elements 'mesoglia' two decades prior to their 'rediscovery' as microglia by Hortega (Cajal 1920a). In a second (companion) paper directly following his critique, Cajal published his own method to detect mesoglia/microglia and their derivatives, using "a simple modification of the method of Bielschowsky" (Cajal 1920b, p. 136). He referred to mesoglia/microglia as the "cells of Robertson and of del Río". In his paper, Cajal compared the mesoglia of Robertson and microglia of del Río-Hortega to the interstitial corpuscles that Achúcarro had described earlier in 1910, referring also to the method he had published in 1911 (another variation of the Bielschowsky method) (Achúcarro 1910, 1911). Perhaps the problem also stemmed from inconsistencies and confusion surrounding published methods (and their citation), which were based on numerous modifications of existing methods, as well as a lack of clarity regarding the 'ownership' of these methods (see also Penfield 1924a, p. 437). For example, del Río-Hortega, having been inspired by Cajal and Achúcarro's

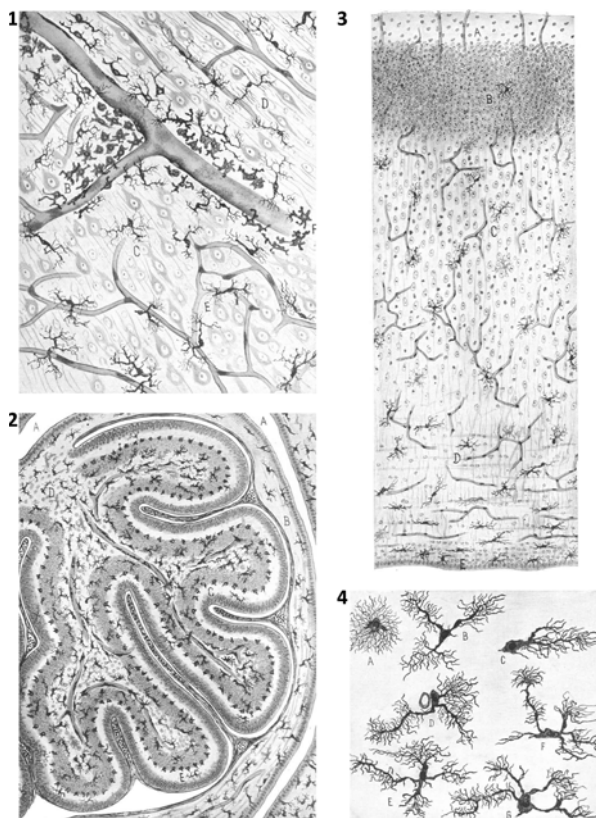


Fig. 2.2 Original microglia drawings from Pío del Río Hortega. Reprinted with permission from *Memorias de la Real Sociedad Española de Historia Natural (del Río-Hortega 1921a)* [In Spanish]. Original figure legends are transcribed. (1) Vertical section of the 4-day old rabbit brain protuberance. (A) blood vessel; (B, F) perivascular microglia with tuberosities and pseudopods; (C) ramified microglia; (D) cell with branches that follow the direction of the nervous fibres; (E) vascular satellites. (2) Cerebella microglia in the 4-day-old rabbit. (A) fourth ventricle; (B) medullary velum; (C) cerebellar folium; (D) microglia migrating through the white matter; (E) microglial cell reaching the grey substance. (3) Cerebral circumvolution of the 1-day-old rabbit. (A) molecular layer; (B, C), cortical layers; (D) white matter, from which microglia ascend to the inferior and medial layers of the cortex; (E) ependyma. (4) Simple ramified forms of the cerebral white substance in the 9-day-old rabbit. (A) hairy microglia; (B, C, D) mono- and tripolar cells, with penniform appendices; (E, F, G) multipolar cells with dendritic tufts

approaches, had undertaken a number of trials using the Bielschowsky ammoniacal silver method (the reagent used by Achúcarro), and by substituting carbonate for nitrate (Cajal had used a reduced silver nitrate method), had obtained finer micelles that were capable of impregnating neuroglia and revealing these cells in much greater detail (see Ortíz Picón 1971). Del Río-Hortega published this method in February 1918, and in November of that year presented his initial findings to the Spanish Society of Biology “On the true significance of neuroglial ‘ameboid’ cells”.

This paper noted the appearance of ameboid cells in inflammatory and destructive processes. It provided evidence that astrocytes did not participate in the formation of rod cells, or granuloadipose corpuscles (Ortíz Picón 1971). In fact, del Río-Hortega published a number of variants of his method (see for example del Río-Hortega 1919c), which he reviewed shortly before his death, in a four-part publication entitled “The method of silver carbonate. General review of the technique and its application in Normal and Pathological Histology”. This collection was published in the Archives of Normal and Pathological Histology, Buenos Aires (del Río-Hortega 1943a, b, 1944a, b).

In his 1921 paper on the histogenesis, normal development, exodus, and distribution of microglia (del Río-Hortega 1921a; see Fig. 2.2), del Río-Hortega provided a full translation (in Spanish) of Robertson’s works (Robertson 1898, 1900b), emphasising that what Robertson had described as ‘mesoglia’ in fact corresponded to the type of neuroglia del Río-Hortega had himself described in some detail and had referred to as ‘oligodendroglia’ (see del Río-Hortega 1921b). In the footnote on the last page, he invited Cajal to examine the original preparations for himself (del Río-Hortega 1921a). It appears from this tail note that del Río-Hortega had secured some of Robertson’s original samples (see also Ortíz Picón 1983). It is unlikely that Cajal ever took del Río-Hortega up on this offer, but Wilder Penfield who visited his laboratory 3 years later did review “an original preparation of Robertson” and referred to this in his 1924 paper (Penfield 1924a, p. 433). Del Río-Hortega renamed the new laboratory in La Residencia de Estudiantes the ‘Laboratory of Normal and Pathological Histology’ to provide greater scope to the work that was undertaken there (Palmero and del Río-Hortega 2004).

The impact of the work conducted in that laboratory was extraordinary, attracting international scientists to learn the new methods for detecting the neuroglia. Among those who visited del Río-Hortega was Wilder Penfield. Having closely followed the protocols of the Spanish School, including del Río-Hortega’s published methods, Penfield had been attempting to stain microglia and oligodendroglia in brain tissue sections between 1921 and 1923. He wanted, in particular, to investigate these cells in epilepsy. He had started this work at Columbia College of Physicians and Surgeons, and continued with his attempts by looking at brain tissue scars later at the Presbyterian Hospital Laboratories in New York (Gill and Binder 2007), convinced that glial cells were central to understanding the causes of epilepsy, as well as tissue repair processes. For that purpose, Penfield had already made numerous attempts using Cajal’s and del Río-Hortega’s published methods for the neuroglia, but these had been disappointing and he had been unable to interpret the results. In his eagerness to set off, Penfield had dashed a letter to del Río-Hortega and set sail for Madrid before having received a reply. This finally arrived, forwarded by telegram from New York, while Penfield was halfway across the Atlantic Ocean. It was a single word ‘venga’ (come) (Penfield 1977). He arrived in April 1924 and remained in the lab until July. For independent accounts of Penfield’s stay at La Residencia de Estudiantes, see Obrador (1975), García-Albea (2004), and Gill and Binder (2007).

In his 1924 paper, Penfield noted that the method of Robertson “was specific when successful but it was so capricious” and that it had not been possible to

reproduce the descriptions provided by the Scottish pathologist. Likewise, del Río-Hortega's method had also given variable results in different hands, typically staining one or the other cell type more prominently—rather than providing uniform and selective labelling. Penfield went on to devise further modifications (Penfield 1924a, b, 1925) and a more 'reliable' method for combined staining of these distinct classes of cells, which he published in 1928. This was itself a further modification of Globus's method, trialled at length for consistency of staining by Penfield's assistant, Fulstow, over a period of 1 year whilst working within Penfield's laboratory in New York (Penfield 1928). Penfield confirmed del Río-Hortega's findings and disseminated these across Europe, North America, and Canada. During his visit to La Residencia in 1924, he submitted two influential papers: the first published in *Brain* (1924), the other in the first edition of the *American Journal of Pathology* (1925), in which he confirmed the existence of microglia, and after a further modification of del Río-Hortega's stain, the oligodendroglia. In his 1924 paper 'Oligodendroglia and its relation to classical neuroglia', he agreed with del Río-Hortega's initial assumptions that the apolar and adendritic cellular elements described by Cajal in 1913 were microglia and oligodendrocytes which appeared to be devoid of cellular processes because of the limitations of the technique employed, i.e. an imperfect technique producing incomplete staining of the cells. He also pointed out that microglia represented a small proportion of perineuronal and perivascular satellite cells (Penfield 1924a), although perivascular cells and parenchymal microglia were not yet recognised as distinct populations at this time (see Graeber and Streit 1990b; Streit and Graeber 1993). Penfield also believed that Robertson's mesoglia cells corresponded to del Río-Hortega's oligodendrocytes (Penfield 1924a). The paper he submitted to the *American Journal of Pathology* (1925) on phagocytosis of microglia in gliomas was also published in French in Cajal's own laboratory journal the previous year (Penfield 1924b). In this paper Penfield provides a rather general summary of del Río-Hortega's earlier work along with his own (in human material and in cats, rabbits, and mice). He referred also to the work by Collado who had been studying microglia in cases of rabies, and to Metz and Spatz (1924) who had confirmed the morphological characteristics of these cells, among other investigators (Penfield 1925). He further described del Río-Hortega's 'fountains' of microglia within the developing nervous system, areas where these cells were encountered in greatest densities and thought to be centres for their genesis. Moreover, he stressed the migratory and phagocytic activities of microglia following experimental injury, before looking at two cases of brain tumours that he termed 'gliomas'—the first study of its kind. He proposed that microglia acted as scavenger cells to clear away the products of degeneration in the area surrounding the glioma and performed 'dendrophagocytosis' within the tumour itself. His impression was that microglia transported the ingested substances to the outer surface of blood vessels and discharged these into the vessel lumen or perivascular space, based on the assumption that *"the presence of scavenger cells about vessels indicates transfer and delivery of ingested substance rather than new formation of these cells..."* (Penfield 1925). During his visit, he moved from normal histology to neuropathology, examining these glial cells in experimentally induced

tissue injury, specifically in response to the formation of scars (del Río-Hortega and Penfield 1927). The 1927 paper ‘Cerebral cicatrix—the reaction of neuroglia and microglia to brain wounds’ described morphological changes (in microglia and astrocytes) that accompanied scar formation in response to puncture wounds induced in rabbit brains, and more extensive wounds generated in canine cerebral hemispheres after craniotomy, followed over a period of several months (del Río-Hortega and Penfield 1927). The paper describes stages in glial scar formation clearly, with initial microglial activation and phagocytosis, later reorganisation of astrocytes surrounding the wound, and the laying down of connective tissue over time (del Río-Hortega and Penfield 1927).

Cajal however, while coming to accept microglia as a distinct class of cells, was not convinced that interfascicular glia or ‘oligodendroglia’ were a component of his ‘third element’, but that adendritic cells, including perivascular and satellite cells and dwarf cells of the white matter, were the ‘real third element’ (Cajal 1920a). More than two decades later, some authors still clung to the idea that microglia and oligodendroglia did not represent the complete picture, and that Cajal’s ‘adendritic’ cells should be classed separately as ‘adendroglia’ (Andrew and Ashworth 1945). In turn, Cajal had considered that perhaps some of the mesoglia cells described by Robertson were in fact incompletely stained microglia (Cajal 1925). Having read the influential work of the German neurohistologists Metz and Spatz (1924), and that of Penfield (1924a, b), who had independently reproduced del Río-Hortega’s findings and confirmed his theories using his silver carbonate method, as well as other investigators at the time (e.g. see for example Gans 1923; Bailey and Hiller 1924; Rezza 1925), Cajal later publically acknowledged microglia as a distinct entity, in his extended treatise on the neuroglia, published in 1925. This work was also published in French in order to reach a wider audience (Cajal 1925). Through his ‘official recognition’, Cajal had given del Río-Hortega merit for the discovery of ‘normal’ microglia, and Robertson the credit for discovery of the cells del Río-Hortega referred to as oligodendroglia. Returning to the Presbyterian Hospital in New York in September 1924, Penfield inaugurated the Laboratory of Neurocytology and met his research associate William Cone, who subsequently wrote a paper on ‘Acute Pathological Changes in Neuroglia and Microglia’ published in 1928. Penfield was to influence a number of other colleagues and associates who worked with him in New York and later on at the Montreal Neurological Institute, instilling in them his own enthusiasm and keen interest in pursuing research into the microglia (and oligodendroglia). Among these were Dorothy Russell, John Kershman, and Webb Haymaker who had worked with Alpers in Pennsylvania, and Carmichael at the London National Hospital. It was around this time that Penfield founded the idea to write a textbook on the general principles of neuropathology (without describing specific diseases); he was looking for a ‘mechanistic description of disease pathophysiology’ (Gill and Binder 2007). He wrote to 27 eminent investigators around the world, del Río-Hortega among them, and managed to secure their contribution to his three-volume collection: ‘Cytology and Cellular Pathology of the Nervous System’ (see Penfield 1932).

Dorothy Russell's contribution to research on microglia was made through investigating the functions of these cells by means of intravital dyes (trypan blue) administered intraperitoneally and examining cellular responses to aseptic cerebral puncture wounds in rabbits (Russell 1929). Russell showed that trypan blue could be taken up by all transitional forms of microglia from typical (normal) forms to *Gitterzellen* (compound granular corpuscles) and by ameboid or spindle-shaped cells lying in the adventitia of cerebral vessels. She proposed that such intravital staining was related to phagocytic function. She also identified these cells with the rest of the reticulo-endothelial system, which supported Hortege's contention that microglia was a mesodermal element. There was no evidence for uptake of dye by the neuroglia (Russell 1929).

In Germany, Creutzfeldt and Metz (1926), following up on the earlier work by Metz and Spatz (1924), charted the involvement of microglia ('Hortegazellen') in acute as well as chronic degenerative processes, progressive paralysis, in tuberculous meningitis, in presenile dementia, and the specific association of these cells with senile plaques (Creutzfeldt and Metz 1926; see also Timmer 1925 for an early discussion on the relationship between microglia and senile plaques). By 1930, the concepts of microglia and of oligodendroglia were firmly established, as can be read in the Society Transactions of the 11th Annual International Neurologic Assembly, held in Paris (Winkler et al. 1931). The mesodermal nature of microglia, however, was not universally accepted. Others (e.g. Polderman 1926; Schaffer 1926; Puijs 1927), while accepting that microglia were a class apart from astrocytes (the 'macroglia'), questioned whether these cells could in fact be derived from the neuroepithelium (see Winkler et al. 1931).

Meanwhile, a series of influential studies were emerging from North American laboratories. Key among these were papers by William Cone (Penfield's coworker from The Department of Surgery and Pathology at the Presbyterian Hospital in New York), Bernard Alpers from Philadelphia (and the Laboratorio de Histologia Normal y Patologica, Madrid), and Carl Rand and Cyril Courville from the Neurological Service and Neuropathological Laboratory of the Los Angeles County General Hospital. Cone described acute changes in neuroglia and the transformation of microglia (rod cells and compound granular corpuscles) in routine human autopsy material (poliomyelitis and cerebrospinal meningitis), including responses to 10-day-old surgical needle tracks (ventricular punctures) (Cone 1928). Alpers, who had studied with del Río-Hortega, described reactions of cells within the CNS to experimental intoxication with urea in rabbits (Alpers 1930). He described profound changes within the cerebral white matter affecting fibrous and perivascular astrocytes (transforming to 'ameboid cells' with degeneration of vascular end-feet), with only minor changes affecting the microglia—typically fusiform or nodular swellings scattered at intervals along microglial processes in more severe cases. These changes varied with the degree of toxicity (Alpers 1930). Rand and Courville undertook a detailed histological examination of cellular changes in the human brain in 24 cases of fatal head injury (with survival ranging from a few hours to several months following injury). They used Penfield's 'combined method' for detecting microglia and oligodendroglia in tissue fixed with formaldehyde (Penfield 1928).

These authors reported that changes in the microglia depend directly on the severity of the contusion, its age, and proximity of individual cells to the point of greatest injury. They noted that the degree and timing of morphological changes in the microglia depended on the severity of the injury. Transitional forms could be found within the first 24 h following injury and fully developed compound granular corpuscles (foamy macrophages) around 4 days' survival time, with the development of vacuoles and swelling within these cells corresponding to their phagocytic activity (Rand and Courville 1932). They did not agree with the views of Penfield and Cone that rod cells were a typical 'stage' in the transformation between normal microglia and formation of compound granular corpuscles (rod cells were only noted in one case with 'dementia paralytica') (Rand and Courville 1932). They concluded that microglia react to local destruction and disintegration of tissue and persist as compound granular corpuscles as long as the products of disintegration were present.

Del Río-Hortega published the first of two definitive synopses on microglia in the English language, in 1932, in Penfield's 'Cytology and Cellular Pathology of the Nervous system' (del Río-Hortega 1932). The second publication was in *The Lancet*, in 1939, while he was at Oxford (del Río-Hortega 1939). In a four-part publication, he provided a thorough review of his silver carbonate method and its various modifications (del Río-Hortega 1943a, b, 1944a, b). Del Río-Hortega died in Buenos Aires, from a malignant cancer in 1945. His work, however, continued to live on through researchers whom he had influenced and inspired and was integrated within systematic investigations which took place later on.

The earliest accounts of microglia in tissue culture were provided by Marinesco and Minea (Winkler et al. 1931, pp. 663–664), Wells and Carmichael (1930) as well as Costero (1930a, b, c, 1931). Of these, the most definitive studies were those reported by Isaac Costero, who was the first to culture human microglia and to record their activities using time-lapse cinemicroscopy. AQ Wells and Edward Arnold Carmichael's work published in 1930 formed part of a long-standing collaboration between the Strangeways Research Laboratory in Cambridge and St Bartholomew's Hospital in London (see Wilson 2005). Carmichael, who had already started researching microglia and published a paper in 1929 looking at the microglial response to intracerebral injection of blood in rabbits (Carmichael 1929), joined up with Wells to examine cultured cells derived from the embryonic fowl nervous system (pons and medulla) and the retina in solid and fluid media, using the 'coverslip technique' that had been established at the time, along with standard 'static' staining and photographic methods (Wells and Carmichael 1930). Wells and Carmichael showed that cells 'resembling normal microglia' derived from nervous system tissues displayed selective affinity for silver impregnation methods, took up vital dyes (trypan blue), and were similar in their properties to 'wandering cells' or 'histiocytes' (i.e. macrophages; see Maximow 1928) cultured from periosteum and limb buds that belonged to the reticulo-endothelial system. On this basis, they considered that microglia also belonged to this system and were therefore of mesoblastic origin (Wells and Carmichael 1930). They described 'remarkable activity' in these cells: "*Protoplasmic protrusions were constantly thrown out and then withdrawn. Mitochondria and cell granules were in constant violent movement. Highly refractile*

fat droplets were present in varying amounts.” (Wells and Carmichael 1930). Webb Haymaker later adapted del Río-Hortega’s silver impregnation technique to the staining of tissue cultures, in a paper read at the meeting of the International Association of Medical Museums, New York, April 1935, and published in the scientific apparatus and laboratory methods section of *Science* (Haymaker and Sánchez-Pérez 1935; see also von Mihálik 1935). Costero, having spent a number of years studying under del Río-Hortega (1922–1931), had developed quite a deep interest in microglia, writing his own paper in support of del Río-Hortega’s findings concerning the ‘third element’ when he was just 22 years old (Costero 1925). A scholarship in 1929 enabled him to travel to Germany, to the Paul Ehrlich Institute in Frankfurt, where he trained in tissue culture techniques (1929–1930) under Drs Caspari and Vollmar (Palmero 2005). There, he made the first cinefilm in the world documenting the experimental behaviour of microglia isolated in culture, using time-lapse cinematography (Fernández-Guardiola 1997). This material formed the basis of Costero’s first manuscript demonstrating the experimental behaviour of microglia under normal and pathological conditions (Costero 1930a), accepted for publication by Dr. Wilhelm Kolle, Director of the Paul Ehrlich Institute (Fernández-Guardiola 1997). Costero became the first to thoroughly investigate the *dynamic* nature of these enigmatic cells ‘live’ under cell culture conditions. His cinefilm was apparently shown by del Río-Hortega on his visits to various universities and institutions around Europe to much acclaim and played an important part in establishing del Río-Hortega’s concepts concerning the microglia (Fernández-Guardiola 1997). Costero returned to Spain, where he wrote two further papers on the cultivation and study of microglia ‘in vitro’ (Costero 1930b, c). Costero’s findings at this time are particularly noteworthy, since they reflect a conscious anticipation of the motile behaviour of microglia that was to be reported 75 years later using in vivo imaging techniques in transgenic mice with fluorescent protein-expressing microglia (Davalos et al. 2005; Nimmerjahn et al. 2005).

Del Río-Hortega referred to Costero’s work in his treatise on microglia, published in the second volume of Penfield’s ‘Cytology and Cellular Pathology of the Nervous System’ in 1932: “*Recent investigations by Costero (1930a, b, c) clearly demonstrate the existence of ameboid movements and the capacity of migration of the microglia. This investigator has succeeded in obtaining cultures of microglia in vitro and he shows that the motile elements seen by other investigators in explants of nervous tissue are microgliocytes. In cultures, the microgliocytes show shapes ranging from the globose types characteristic of the fat granule cells to the bipolar and branched forms. The former occur near the cultivated tissue, while the bipolar and branched cells are found in the plasma. The observations of Costero on the activity of the microglia are conclusive since all stages, from the moment the microgliocytes enter into motion and send off prolongations with pseudopodia (lateral spines) to phagocytosis of erythrocytes, tissue detritus and carmine granules, are present in the cultures. In explants of nervous tissue and in pure cultures of microglia we find a strong argument in favour of its inclusion among the elements of the reticulo-endothelial system.*” (del Río-Hortega 1932, p. 518).

The concept that microglia belonged to the ‘reticulo-endothelial system’ (of Aschoff 1924), later renamed the ‘mononuclear phagocyte system’ (see Rezaie and Male 2002a), was established by the 1940s. Del Río-Hortega had referred to his own work and that of his students Jiménez de Asúa (del Río-Hortega and Jiménez de Asúa 1921; Jiménez de Asúa 1927) and Isaac Costero (1930a, b, 1931) who made significant advances to his own studies on two fronts: (1), the concept that microglia belonged to the reticulo-endothelial system proposed by Aschoff (Jiménez de Asúa 1927; Aschoff 1924; Russell 1929; Visintini 1931; Belezky 1931, 1932; Bolsi 1936; and others); and (2) the morphological characteristics, motile behaviour, and phagocytic functions of microglia cultivated *in vitro* (Costero 1930a, b, 1931). Henry Dunning and his colleagues Lewis Stevenson and Jacob Furth gave a clear account of the evidence supporting Jiménez de Asúa’s proposal. These were based on properties (similarities in morphology, uptake of colloidal dyes, behaviour and functions *in vitro*) that microglia shared with mononuclear phagocytes resident within other tissues, for example the liver, spleen and kidney (which were called ‘histiocytes’ at the time; see Maximow 1928; Dunning and Stevenson 1934; Dunning and Furth 1935). These investigators also referred to a study carried out by Lebowich (1934), who observed that microglia were capable of phagocytosing bacteria. Von Mihálik had concluded that there were no differences between macrophages cultured from the brain, liver, and subcutaneous tissue (von Mihálik 1935). Further studies on the ontogeny of microglia and their relationship with the reticulo-endothelial system were carried out by Belezky (1931, 1932), von Sántha (1932), Juba (1933, 1934), von Sántha and Juba (1933), and by Dougherty (1944) (see also Cardona 1937).

John Kershman, Assistant Professor of Neurology at McGill University, was among the first to chart the development and differentiation of microglia within the human fetal nervous system, significantly extending the initial studies presented by Rydberg (1932), von Sántha (1932), and Juba (1933), in his exemplary study published in 1939 (Kershman 1939). The detail and accuracy of the descriptions provided make this one of the key landmark papers on microglia. Penfield’s influence is acknowledged by Kershman therein. He separately confirmed observations in mammals by a number of previous researchers, showing that microglia appear within the human brain, with the first evidence of vascularisation (Kershman 1939).

From 1941 to 1960, only 14 articles appear listed on NCBI PubMed (using ‘microglia’ as the search term entered under ‘all fields’). These are studies by Vazquez-Lopez (1942) on microglia in the neurohypophysis, Kurobane (1950) on microglia in gliomas, Meo (1950) on cerebral changes in leukemia, Costero (1951, 1952) on microglia in rheumatism, Mazzi (1952) who looked at the brain in teleosts, Herrera (1953) who examined microglial genesis, Imamura (1954) focusing on gliomas, Field (1955) who focused on the development of microglia and the influence of cortisone, Pickering and Vogel (1956) examining demyelinating lesions, Jufe (1957) on rabies, Koenig (1958) addressing nucleic acid and protein turnover, Wolman (1958) who examined the mechanism of selective impregnation, and Tsyarkin (1959) examining microglia in senile dementia and their role in the structural genesis of senile plaques. There are other articles that are not currently listed

on PubMed, for example Dougherty (1944) and Bullo (1945). So it is clear that the above list does not represent the complete picture, but it does provide an indication of the relative paucity of research on microglia that followed the death of del Río-Hortega in 1945, and for more than a decade after the end of the second world war. Glees' publication in 1953 provides a good point of reference on the neuroglia up to that period (Glees 1953).

We end this section with the words of del Río-Hortega. Summing up two decades of work on microglia in a lecture delivered at Oxford University in 1938, del Río-Hortega provided a concise account of the state of knowledge with respect to the origins, forms, and functions of the microglia up to that period. This was published in *The Lancet* the following year (del Río-Hortega 1939). In his conclusion he noted: *“Nerve tissue contains a type of cell with properties corresponding to those of the reticulo-endothelial system. The chief function of these cells (the microglia) is the phagocytosis of waste products, and, if need be, of red blood-cells..... Even when they are in apparent rest, microglial cells remain capable of migration, and under pathological conditions they are mobilised to undertake phagocytic activity. Phagocytosis in nerve centres is a specific function of microglia; despite opinions to the contrary, neither the astrocytes nor the oligodendrocytes take part in it. Microglia cells take up the products of disintegration of the nerve tissue, digesting and elaborating them, and making them finally disappear. These ideas have been developed in my papers from 1919 to 1921, and though they have been discussed in many subsequent papers by other workers, almost nothing new has been added to our knowledge of this subject. Today these ideas are widely accepted.”* (del Río-Hortega 1939, p. 1026).

At the time this statement was made, it was perhaps unlikely to envisage that it would be decades before research on microglia would ‘take off’ once again. Yet the modern era is indebted to these pioneering achievements, and progress has been made through building on these earlier discoveries.

2.3 The ‘Renaissance’ Period

Much of the credit for the revival of interest in microglia and our present-day knowledge can be attributed to Georg Kreutzberg who was studying the mechanisms of axonal regeneration in the 1960s (Kreutzberg 1963, 1967, 1968, 1972, 1996; Kreutzberg and Barron 1978), and those whom he inspired and influenced, including Manuel Graeber, Wolfgang Streit, Helmut Kettenmann, and Richard Banati, among others. Kreutzberg began investigating the facial nerve transection model through a series of light and electron microscopic and autoradiographic studies (see Moran and Graeber 2004 for a review on this model). He observed that excision of the facial nerve (distal to the facial motor nucleus within the brainstem) produced characteristic responses in astrocytes and microglia associated with motor neuronal cell bodies, which enabled the peripheral nerve to regenerate (Blinzinger and Kreutzberg 1968; see also the work of Cammermeyer 1965a, b). Blinzinger and Kreutzberg first

described the phenomenon of ‘synaptic stripping’ (Blinzinger and Kreutzberg 1968), whereby microglia in the facial motor nucleus initially displaced synapses from traumatised neuronal somata, followed by ensheathment of these cell bodies by astrocytes, preventing further synaptic input, allowing the injured neurons to conserve energy, recuperate, and the peripheral nerve to regenerate over time. Until then, it had been generally assumed that the sole function of microglia was to carry out phagocytosis and ‘clearance’ (e.g. of red blood corpuscles, dead cells, or pathogens like bacteria), and the expectation was that these cells would simply engulf, assimilate, and thus remove the damaged neuronal cell bodies. The novel findings therefore came both as a surprise and a revelation—this was the first evidence for a ‘neuroprotective’ function of microglia; their involvement in neuronal regeneration. While the discovery of synaptic stripping was just one of the seminal contributions of this school, it was a fundamental addition to the known spectrum of microglia functions. Synaptic ‘nursing’ and ‘pruning’ are essential elements of a portfolio which critically involves microglia in processes of CNS development, maintenance, and recovery (Kettenmann et al. 2013) (for further reading, see Chap. 9).

Later (in the 1980s and 1990s), Kreutzberg and his colleagues showed, using this model, that the microglial response within the CNS was tailored to the extent of consequent ‘damage’ inflicted to the neuron (Kreutzberg and Barron 1978; Graeber and Streit 1990a; Graeber et al. 1988, 1993, 1998; Streit et al. 1989; Haas et al. 1993). Two key insights were gleaned from these studies: (1), the blood–brain barrier within the brainstem nucleus remained intact in this model, so that both ‘synaptic stripping’ and phagocytosis were functions carried out by microglia alone, without recruitment of mononuclear phagocytes from the periphery, and (2), microglia were shown to be capable of proliferating following axotomy, and therefore had the capacity to ‘self-renew’ their population without recruitment of progenitors from the blood (Graeber et al. 1988, 1993, 1998; Graeber 1993; Haas et al. 1993; Moran and Graeber 2004; Ajami et al. 2007). More recently, it has been argued that ‘synaptic stripping’ cannot be extended more generally to models of chronic neurodegeneration, and that degeneration of synapses and envelopment of a degenerating terminal are neuron autonomous events in which microglial involvement is merely ‘guilt by association’ (Perry and O’Connor 2010). Nevertheless, there is growing evidence that microglia are indeed able to remove synaptic terminals, and in this respect contribute to remodelling and plasticity of the nervous system in development, with implications for neurodevelopmental disorders, even including autism and Rett syndrome (Boggio et al. 2010; Tremblay et al. 2010; Paolicelli et al. 2011; Paolicelli and Gross 2011; Derecki et al. 2012; Hughes 2012; Schafer et al. 2012).

2.4 The Period of ‘Awakening’

The 1980s saw an ‘awakening’ in the understanding of microglial ‘immune’ functions, the processes of neuroinflammation, and the concept of ‘innate immunity’ inspiring large-scale research on microglia *in vitro* and *in vivo*, with a particular

focus on Alzheimer's disease. Neuroglial cells moved into the spotlight, and immunological concepts and approaches fuelled research in the neurosciences. It was at this time also that the myeloid nature of microglia gained broader attention, supported and influenced by immunological work on mononuclear phagocytes, and a distinction between perivascular macrophages and perivascular microglia was clarified.

The morphological transformation and phagocytic function of microglia, and their role in glial scar formation (in response to stab injury, for example) had already gained widespread recognition following del Río-Hortega's publication in Penfield's 'Cytology and Cellular Pathology of the Nervous System' (reprinted by Hafner in 1965) (del Río-Hortega 1932), and in his Lancet paper (del Río-Hortega 1939). However, evidence for the concept of microglia as 'immunocompetent' cells that were capable, like their macrophage counterparts within other tissues, of presenting antigen to T lymphocytes (through expression of Major Histocompatibility Complex (MHC) class II antigen), of secreting pro- and anti-inflammatory cytokines, as well as cytotoxic factors, of signalling to cerebral endothelium, and initiating an immune response within the CNS, was first drawn together in the 1980s by Streit, Graeber, and Kreutzberg (Streit et al. 1988). These authors went on to propose that microglia represent 'networks' of immune surveillance within the CNS (Graeber and Streit 1990a; Gehrmann et al. 1993, 1995). In 1996, Kreutzberg coined the term 'pathological sensor' to describe the inherent activity of the microglia, as occurring in a 'heightened' or 'alert' state, monitoring the environment, and ready to initiate a rapid response to insult or injury. The article is still one of the most cited in the field. The view of microglia as the 'innate immune system' of the CNS later led to the concept of 'neuroinflammation' (Streit et al. 2004a; Mrak 2009) in which microglia were the key cellular mediators in acute and chronic inflammatory responses associated with infectious (e.g. human immunodeficiency virus (HIV)) and chronic neurodegenerative diseases such as multiple sclerosis, Alzheimer's disease, and prion diseases (see McGeer and McGeer 2011 for a history of innate immunity in neurodegenerative disorders). Microglia were found to play roles in both innate (or 'cell-mediated') and adaptive immunity within the CNS.

The dichotomy in the nature of microglial functions (neurotoxicity versus neuroprotection) is now well-recognised (Czeh et al. 2011; Zhang et al. 2011), and reference to microglia functioning in this regard as 'a double-edged sword' and a 'pathological sensor' can be traced back to Georg Kreutzberg (Kreutzberg 1995, 1996). The concept of 'neuroinflammation', however, is being re-examined in the light of the continuum of microglial activation and multi-faceted responses in both healthy and pathological states (Hanisch and Kettenmann 2007; Graeber 2010; Graeber et al. 2011).

A separate focus of work that began during this period was a revival in research into the origin and cell lineage of microglia. Eng Ang Ling and co-workers, pursuing the autoradiographic work of Imamoto and Leblond (1978) and that of Kitamura (1969, 1973), were particularly interested in defining the origins of microglia, which had remained contentious for decades (Fujita and Kitamura 1975; Ling 1976a, b;

Boya et al. 1979; Fujita et al. 1981). Ling and colleagues described two forms of microglia in the neonatal and postnatal rodent brain—ameboid cells within white matter tracts, with special reference to the corpus callosum, and ramified cells in the gray matter (Ling and Tan 1974; Ling 1979; Leong and Ling 1992). They observed that ameboid cells were taking up carbon particles injected intravenously, and that a subpopulation of these cells could transform into the resident (ramified) microglial population in adult animals (Ling 1979; Kaur et al. 1985; Wu et al. 1992). The literature on the origin of microglia, namely their relationship with the mononuclear phagocyte system and the initial view that microglia were derived from circulating blood monocytes, is summarised in the review by Kaur et al. published in 2001 (for related reviews, see also Ling and Wong 1993; Theele and Streit 1993; Cuadros and Navascués 1998, 2001; Rezaie 2003, 2007; Rezaie et al. 1999; Rezaie and Male 2002a, b).

Bone marrow (BM) reconstitution experiments by Hickey and Kimura in the late 1980s, and later by a number of other investigators, showed that donor BM-derived stem cells transferred to irradiated hosts (rodents) could subsequently be traced as ‘microglia’ within the brain parenchyma (Hickey and Kimura 1988; Lassmann et al. 1993; Lassmann and Hickey 1993; Flügel et al. 2001; Priller et al. 2001; Simard and Rivest 2004). Likewise gender-mismatched BM-transplant recipients were found to have a small number of donor-derived cells located within the human brain, mainly around vasculature (perivascular positions), at postmortem (Unger et al. 1993). However, the fact that BM-derived elements primarily took up residence within the brain at perivascular positions and were largely absent in the parenchyma proper (Hickey and Kimura 1988; Unger et al. 1993) was intriguing. Observations that these cells were able to differentiate into ‘microglia-like cells’ with immunological properties resurfaced questions concerning the relationship between perivascular macrophages and parenchymal microglia that had been posed two decades earlier (see Kitamura and Hattori 1972). Manuel Graeber, Wolfgang Streit and colleagues re-examined this relationship, offered clarification for differences between perivascular cells (perivascular macrophages) and perivascular microglia, and demonstrated that perivascular cells were preferentially located within the basement membrane (glia limitans) surrounding cerebral vessels (Graeber et al. 1992; Graeber and Streit 1990b; Streit and Graeber 1993). While perivascular cells appeared to be repopulated by BM-derived progenitors, with a fairly consistent high turnover, and served as immunoregulatory ‘go-betweens’ connecting the nervous system and the peripheral immune system (Williams et al. 2001), microglia in contrast were capable of self-renewal with little need for recruitment from the BM, under non-pathological conditions (see Ajami et al. 2007; Mildner et al. 2007). In fact, a criticism levied against irradiation experiments was that the consequent damage to (opening of) the blood–brain barrier and additional impact on CNS cells, facilitated ‘engraftment’ of donor BM progenitors, whereas an intact ‘healthy’ barrier and unchallenged tissue would restrict this (see Ajami et al. 2007; Mildner et al. 2007; Ginhoux et al. 2010).

2.5 The Turn of the Twenty-first Century and the New Millennium

Around the turn of the century, genetic models in combination with imaging techniques offered new options and precipitated research activity on a massive scale, focusing on responses, reactive phenotype diversity, origin, turnover and replenishment, and interactions with both resident CNS and infiltrating immune cells. Microglia were no longer considered solely as ‘destructive’ phagocytes. The first house-keeping functions were revealed, ending the concept of a ‘resting’ or ‘dormant’ cell. Contributions to normal development (e.g. synaptic ‘pruning’/remodeling) as well as to neurological diseases were further unraveled, venturing into neuropsychiatric disorders (Bilbo and Schwarz 2009; Chen et al. 2010). Neuron–glial interactions, the roles of microglia in autoimmunity, chronic pain, in brain tumours and aging were demonstrated. Regional specifications of microglia throughout the CNS and long-term outcomes of microglial challenges began to be examined.

Dana Giulian and colleagues were among the first to culture microglia from rodent brains. They investigated the properties of these cells during the mid-1980s (Giulian 1987; Giulian and Baker 1985, 1986; Giulian and Ingeman 1988; Giulian et al. 1988). Studies soon focused on the cultivation and characterisation of adult and fetal human microglia (Hayes et al. 1988; Grenier et al. 1989; Lee et al. 1992; Williams et al. 1992; Lauro et al. 1995). Cell cultures paved the way for extensive characterisation of microglial morphology and phenotype (Grenier et al. 1989; Rieske et al. 1989; Sedgwick et al. 1991; Giulian et al. 1995), identification of their secretory profiles, cellular responses, and interactions with other CNS cell types (e.g. Giulian 1987; Suzumura et al. 1987; Bocchini et al. 1988; Raivich et al. 1993; Sawada et al. 1993; Hanisch 2002; Pocock and Kettenmann 2007). This led to a greater understanding of the neurotoxic and neurotrophic repertoire of these cells (Banati et al. 1993; Banati and Graeber 1994; Nakajima and Kohsaka 1993a, Nakajima and Kohsaka 1993b, 1998, 2001, 2004; Giulian et al. 1994; Hanisch 2002), among these their expression of growth factors and mitogen receptors (Raivich et al. 1993), neurotransmitter receptors (Pocock and Kettenmann 2007), and cytokine and receptor profiles (Hanisch 2002). Microglia were found to ramify in response to a number of factors, most prominently trophic factors released by astrocytes in cocultures (Suzumura et al. 1990, 1991). The proteomic and genomic expression patterns of microglia have been profiled (Duke et al. 2004; Moran et al. 2004; Glanzer et al. 2007; Parakalan et al. 2012; Veremeyko et al. 2012) and their electrophysiological properties examined (Kettenmann et al. 1993; Draheim et al. 1999; Prinz et al. 1999), including those of the ramified variety maintained *in vitro* (Eder et al. 1999) and in tissue slices (Boucsein et al. 2000). Helmut Kettenmann and colleagues first drew attention to the fact that cultured microglia possess a distinct pattern of membrane ion channels (including an inward-rectifying potassium channel) that distinguished these cells from other mononuclear phagocytes, including peritoneal macrophages, a unique property that was shared with a small subpopulation of progenitors located within the bone marrow (Kettenmann et al. 1990; Banati et al. 1991; Kettenmann 1994).

Attention soon turned towards deciphering the motile nature of these cells, much as it had done earlier with Costero's studies (Costero 1930a, b, 1931). This time, however, with more sophisticated (light, confocal, and time-lapse) microscopic imaging techniques. Microglial cell dynamics and morphological 'plasticity' were investigated in slice cultures (Hailer et al. 1996; Dailey and Waite 1999; Stence et al. 2001; Grossmann et al. 2002; Petersen and Dailey 2004; Kurpius et al. 2006; Grinberg et al. 2011), in isolated cells as well as co-cultures (Ward et al. 1991; Glenn et al. 1992; Rezaie et al. 2002; Ohsawa and Kohsaka 2011), and 'real-time' microglial activation could now be examined (e.g. see Rangroo Thrane et al. 2012). A number of studies also began to examine the behaviour of ramified cells (Ward et al. 1991; Booth and Thomas 1991; Kloss et al. 1997; Eder et al. 1999; Rosenstiel et al. 2001; Rezaie et al. 2002). The morphological plasticity of microglia was demonstrated even in their fully ramified forms and, with the later confirmation of these findings *in vivo* (Davalos et al. 2005; Nimmerjahn et al. 2005), it was clear that microglia were highly dynamic cells capable of motility, constantly on the move even at 'rest'. In this respect, microglia could no longer be regarded as 'resting' in their ramified forms in the normal, healthy brain (see Rezaie 2007). These observations impacted also long-held definitions of the 'activation' of these cells (Hanisch and Kettenmann 2007), which were in fact not 'step-wise' but occurring along a dynamic 'continuum', and offered further support for their rapid morphological and phenotypic response to insult or injury. These features—dynamic cell motility and plasticity—remain unique to this cell type within the CNS.

Areas that had until recently remained relatively less well-explored included the functional role(s) played by microglia in the normal, healthy adult brain, and during development. It was clear that the functional status of microglia depended on their immediate environment (Neumann 2001). Signalling from, and direct contact with, neurons and astrocytes are essential to their maintenance within the healthy nervous system (e.g. see Biber et al. 2007). Neuronal fractalkine (Harrison et al. 1998; Maciejewski-Lenoir et al. 1999) and CD200 as well as their corresponding receptors present on microglia (Hoek et al. 2000), and normal electrical activity of neurons, were discovered to maintain microglia in their 'resting' states—the number of ligand-receptor pairs and conditions with activity control still being incomplete (Kettenmann et al. 2011, 2013; Hanisch 2013a, b, c) (for further reading, see Chaps. 3 and 9). Diffusible factors (including the trophic factors MCSF (macrophage colony stimulating factor) and GMCSF (granulocyte-macrophage colony stimulating factor)), released from astrocytes, and direct contact with these cells were likewise found to be important for maintaining microglia in their ramified (presumed 'non-activated') states (Suzumura et al. 1990, 1991; Liu et al. 1994; Sievers et al. 1994; Fujita et al. 1996; Tanaka and Maeda 1996; Eder et al. 1999; Schilling et al. 2001; see Rezaie and Male 2002b for review). Discussion of potential roles of these cells in the healthy brain, including in development, was the subject of a recent symposium (Tremblay et al. 2011).

The normal 'housekeeping' activities of microglia in the healthy nervous system (see Kettenmann 2007; Raivich 2005), with roles in surveillance (policing/patrolling) and phagocytosis (scavenging/clearance), are now firmly established

(Nimmerjahn et al. 2005; Davalos et al. 2005). Key insight into their function in maintaining a healthy nervous system was derived through their expression of functional TREM-2 (triggering receptor expressed on myeloid cells-2) (Schmid et al. 2002), required for the regular clearance of ‘debris’ within the nervous system (including apoptotic neurons) without inducing an inflammatory response, via a process termed ‘controlled phagocytosis’ (Neumann and Takahashi 2007; Takahashi et al. 2005, 2007). More recent evidence supports the existence of subsets of microglial cells, with distinct phenotypes (and potential immunological diversity), whose functions may be more diverse than originally thought, and further specialised to support the brain regions they subserve (de Haas et al. 2008; Olah et al. 2011, 2012; Scheffel et al. 2012; Hanisch 2013b, c). For in-depth reviews on our progressive understanding of the physiological functions of microglial cells, see Färber and Kettenmann (2005), Kettenmann (2006), Hanisch and Kettenmann (2007), Ransohoff and Perry (2009), and Kettenmann et al. (2011) (for further reading, see Chap. 3).

Views on the origins and lineage of fetal and adult mononuclear phagocytes were also changing at the turn of the twenty-first century, with emerging concepts for differences in lineage, form, and functions between fetal and adult macrophages (see Chan et al. 2007). Tissue-resident mononuclear phagocytes in particular were now considered to belong to the fetal macrophage lineage which developed independent of monocyte-derived macrophages (Shepard and Zon 2000). A number of studies had shown that microglia were already present during embryonic and fetal stages in the rodent and human CNS (see Dalmau et al. 2003; Rezaie et al. 1997, 1999; Rezaie 2003; Chan et al. 2007), which precluded the BM as the main source of microglial progenitors. The yolk sac was proposed as the ‘developmental’ source of microglial progenitors (Alliot et al. 1999; see Chan et al. 2007), later confirmed by Miriam Merad’s group using fate-mapping experiments (Ginhoux et al. 2010). The fact that microglia were specifically of myeloid lineage had already been indicated, given their expression of PU.1 (Walton et al. 2000), a transcription factor essential for the development of all myeloid cells including monocyte-derived macrophages (Greaves and Gordon 2002; see Chan et al. 2007). Frederic Geissmann’s group (Geissmann et al. 2010; Gomez Perdiguero et al. 2013; Schulz et al. 2012) confirmed that microglia, Langerhans cells of the skin and Kupffer cells of the liver develop from Myb-independent, FLT3-independent, but PU.1-dependent precursors that express CSF-1 receptors. Wang et al. (2012) proposed interleukin 34 (IL-34) as the tissue-restricted ligand of CSF-1 receptors, required for the development of both Langerhans cells and of microglia. Most recently, additional factors and steps for microgliogenesis have been added, confirming PU.1 and introducing also interferon regulatory factor 8 (Irf8) (Kierdorf et al. 2013). For recent, relevant reviews on the origin and lineage of microglia see Saijo and Glass (2011), Greter and Merad (2013), and Ginhoux et al. (2013).

2.6 The Technical Bases

Del Rio-Hortega's ammoniacal silver method and the 'improved' methods of Penfield (1928), Penfield and Cone (1937), and others (e.g. McCarter 1940; Fain 1963; Gallyas 1963; Naoumenko and Feigin 1963; Malik 1964) were still being used, albeit selectively within histopathology laboratories in Europe and elsewhere around the world in the 1950s, 1960s, and 1970s, to detect microglia and to examine their responses in various pathological conditions. The techniques proved to be too capricious for routine use, however, with varying results obtained in different laboratories (and even in the same labs, on different samples). Electron microscopy was in its heyday during the 1960s and 1970s, as a tool to investigate the cellular cytoarchitecture of the brain, and a number of investigators turned their attention to examining the ultrastructural morphology of microglia in more detail (e.g. Blinzinger and Hager 1962; Yasuzumi et al. 1964; Hager 1968; Mori and Leblond 1969; Blakemore 1975; Boya 1975). There had been some who questioned the existence of these cells, given difficulties in identifying them (see Vaughn and Peters 1968; Kitamura 1969; Cammermeyer 1970). Cammermeyer (1970) reviewed this debate in greater depth and provided a good coverage of the state of knowledge (and controversies) during this period.

Silver impregnation methods (Gallyas 1963; Naoumenko and Feigin 1963) and morphological studies were replaced by histochemical methods (Mladenov and Gerebtzoff 1967; Kraśnicka and Renhawek 1969, 1970; Ibrahim et al. 1974) as a new tool for investigating mononuclear phagocytes (including lysosomal enzymes found in macrophages) and microglia (see Rezaie and Male 2002a). Enzyme histochemical methods, however, much like the prior silver impregnation techniques, also proved to lack specificity for microglia. They were technically demanding procedures, requiring tissues to be prepared in a specific way, combined with skilled training and a 'good eye' to be able to reliably discriminate cells (Esiri and Booss 1984).

Histochemical staining with plant lectins introduced in the late 1970s and 1980s (Streit and Kreutzberg 1987) was soon replaced by the use of antibodies and immunohistochemical methods to detect surface and intracellular antigens. These enabled the cellular phenotype and distribution of microglia and other populations of mononuclear phagocytes associated with the CNS (e.g. perivascular cells, supraependymal macrophages, and Kolmer cells) to be examined for the first time by Hugh Perry, Siamon Gordon, and others (Oehmichen et al. 1979; Hume et al. 1983; Perry et al. 1985; Perry and Gordon 1988; Imamura et al. 1990; Lawson et al. 1990, 1992). Factors that modulated the microglial phenotype could be examined (Perry 1994). Complex immunophenotypic characterisation was proposed as a way to differentiate between so-called 'resting' and 'activated' cells, based on constitutive 'high' or 'low' levels of expression of CD45 and CD11b/c antigens by microglia (Sedgwick et al. 1991; Ford et al. 1995). Subsets of activated microglia continue to be selectively discriminated by their cellular phenotype, e.g. differential expression of MHC class II, CD68, CD11b/c and CD45 using light microscopy and more sophisticated

fluorescence and confocal microscopic imaging techniques. A more reliable method for detecting all forms of microglia *in situ* and *in vitro* was developed in Shinichi Kohsaka's lab by Imai and colleagues raising a polyclonal antibody against a novel protein called 'Iba1' (ionised calcium adapter-binding molecule-1) (Imai et al. 1996; Ito et al. 1998). Iba1 is encoded by a gene located within a segment of the MHC class III region, between the Bat2 and tumour necrosis factor (TNF) alpha genes (Imai et al. 1996), and expressed by mononuclear phagocytes, including microglia. Immunohistochemistry with Iba1 antibody detected all morphological and functional forms of microglia in both rodent and human preparations allowing direct comparisons to be made in translational studies, and between species (Ito et al. 1998; Hirasawa et al. 2005). Although a considerable step forward, this tool still does not allow recently recruited/infiltrating macrophages to be discriminated from CNS-resident microglia (see Kettenmann et al. 2011 for an overview of methods for microglia detection).

Over the last two decades advances in imaging techniques (light, fluorescence, confocal, time-lapse, and electron microscopy), combined with immunoassays (e.g. ELISA and Elispot), electrophysiological methods, proteomic and molecular techniques (e.g. 2D gel electrophoresis, *in situ* hybridisation, RT-PCR, Western blotting), laser capture microdissection, and gene profiling methods, have rapidly become powerful 'everyday' tools to examine the physiological functions of microglia under varied conditions, from living cells (single isolated cells, microglial cocultures, slice culture preparations) to histological preparations (frozen and fixed tissue sections) obtained at postmortem. Each and every study has had its limitations, the greatest of which has always been the unanswered query: To what extent can the multitude of observations and experimental findings *in vitro*, *ex-vivo*, and *in situ* (in tissue slices for example) be extrapolated to microglial functions *in vivo*? This has been the ultimate drive—understanding microglial functioning within the intact CNS environment, and perhaps the most exciting period in the history of these cells lies ahead of us, but there have already been fundamental advances on several fronts over the last decade; each allowing us a glimpse of the 'real world' of the microglia (inside the living and intact nervous system).

The first is in a clinical setting: imaging 'activated' microglia using positron emission tomographic (PET) scanning of the human brain—a methodological breakthrough developed by Richard Banati and coworkers, which relies on intravenous injection of a radiolabelled ligand (PK11195) that selectively binds to peripheral benzodiazepine-binding sites, found on all mononuclear phagocytes, including microglia (Banati et al. 1997), and subsequent PET imaging of the brain to visualise the bound ligand (Banati 2002, 2003; Banati et al. 2000). Using this procedure, the *in vivo* imaging of microglia was first demonstrated, and quantitated, in patients with multiple sclerosis (in which microglial activation is associated with lesions or 'plaques' located within the white matter), as a measure of disease severity (Banati et al. 2000). Microglial activation has subsequently been visualised in a number of neurodegenerative conditions (Cagnin et al. 2001, 2002, 2007). Although the selectivity and specificity of the technique have been questioned (peripheral

benzodiazepine receptor/18KDa translocator protein ligands may not bind exclusively to microglia, but may additionally detect activated astrocytes—see Lavis et al. 2012), use of the radioactive ligand may modify microglial functions and induce bystander effects on microglial activity (see Choi et al. 2011), and resident ‘non-activated’ microglia which have limited (or lack such) binding sites cannot be detected using this method, it is still a significant step forward in being able not only to identify, but to pinpoint and quantitate activated microglial involvement in situ in pathological disease progression. Newer and more selective ligands are being developed (see for example, Imaizumi et al. 2008; Politis et al. 2012). These imaging techniques have important value not only for charting the clinical progression and anatomical localisation of disease (which could act as an aid to diagnosis), but also in determining the clinical efficacy and/or responses to drug treatments aimed at slowing down or arresting disease progression, including those that target and modify microglial functions directly (Aldskogius 2001; Skaper 2011; Harry and Kraft 2012).

The second is within an experimental domain: ‘real-time’ in vivo imaging of microglia in transgenic and knock-in mice engineered to express fluorescent mononuclear phagocytes (for example, by tagging a fluorophore such as enhanced Green Fluorescent Protein (e-GFP) to the fractalkine receptor gene or *Iba1* gene), and two-photon fluorescence microscopy (Davalos et al. 2005; Nimmerjahn 2012; Nimmerjahn et al. 2005) (for further reading, see Chap. 4). The combination of genetic engineering and fluorescence microscopy has allowed microglia within the upper 100–200 μm of the mouse cerebral cortex (cortical layers 1, 2 and upper layer 3) to be visualised in vivo and in 3D, through an ‘optical window’ or thinned skull, and therefore in their intact and undisturbed environment, for the first time (Nimmerjahn et al. 2005). These studies have confirmed the resting motility of microglia and charted and confirmed the characteristic microglial responses to local brain injury in vivo (chemotaxis, morphological transformation, phagocytosis), including localised responses to laser-induced damage to cerebral microvessels (Davalos et al. 2005). This combined technology (genetic engineering and in vivo imaging) promises to offer further insights into the behaviour of microglia, the dynamic interaction between microglia and synapses being one such contemporary focus, in the developing and aged brain, and in a variety of disease models. For example, crossing mice that express green fluorescent protein tagged to the fractalkine receptor (CX3CR1-GFP mice) with Thy1-YFP mice (expressing yellow fluorescent protein in neurons) has already enabled a more discrete visualisation of microglia interactions with dendritic spines directly in vivo (see Tremblay 2011; Tremblay et al. 2010). With new animal models becoming available for conditional targeting of microglia, more and more sophisticated options are available for visualisation, tracing, and manipulation of these cells (Goldmann et al. 2013; Yona et al. 2013). The combined application of modern technologies and genetic approaches is now a significant driver pushing research forward (Prinz et al. 2011).

2.7 Looking to the Future

Where will the field move from here? While much has been learned concerning the form and functions of microglia from cell culture and tissue slice work, the physiology of these cells (their cellular interactions with neurons, macroglia, and endothelium) in their natural environment *in vivo* still needs to be investigated. Understanding the versatility of these cells and dynamic control over their phenotype in health and in disease, facilitating their phagocytic capabilities to clear senile plaques (for example in brain tumour progression and in chronic pain), their roles in development, potential for engraftment, conditional gene expression, and as therapeutic and pharmacological targets within the CNS, all remain to be explored. Looking back over more than a century of work (see Fig. 2.1), it is clear that these are exciting times to be involved in research on microglia. The next epoch in the history of microglia research has just begun.

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Microglia in Health and Disease

Tremblay, M.-È.; Sierra, A. (Eds.)

2014, XIV, 486 p. 32 illus., 20 illus. in color., Hardcover

ISBN: 978-1-4939-1428-9