Review

American Society for Neurochemistry

ASN Neuro
Volume 12: 1–10

DOI: 10.1177/1759091420925335
journals.sagepub.com/home/asn
SAGE

Shedding Light on the Dark Side of the Microglia

Marie-Kim St-Pierre[1], Eva Šimončičová[1,2,3], Eszter Bögi[2], and Marie-Ève Tremblay[1]

Abstract

Microglia, the resident immune cells of the central nervous system, are not a homogeneous population; their morphology, molecular profile, and even their ultrastructure greatly vary from one cell to another. Recent advances in the field of neuroimmunology have helped to demystify the enigma that currently surrounds microglial heterogeneity. Indeed, numerous microglial subtypes have been discovered such as the disease-associated microglia, neurodegenerative phenotype, and Cd11c-positive developmental population. Another subtype is the dark microglia (DM), a population defined by its ultrastructural changes associated with cellular stress. Since their first characterization using transmission electron microscopy, they have been identified in numerous disease conditions, from mouse models of Alzheimer's disease, schizophrenia, fractalkine signaling deficiency to chronic stress, just to name a few. A recent study also identified the presence of cells with a similar ultrastructure to the DM in *postmortem* brain samples from schizophrenic patients, underlining the importance of understanding the function of these cells. In this minireview, we aim to summarize the current knowledge on the DM, from their initial ultrastructural characterization to their documentation in various pathological contexts across multiple species. We will also highlight the current limitations surrounding the study of these cells and the future that awaits the DM.

Keywords

dark microglia, heterogeneity, nonhomeostatic conditions, postnatal development, electron microscopy, mouse models, human brain samples

Received March 19, 2020; Revised April 16, 2020; Accepted for publication April 17, 2020

Since the first description of microglia by Pío del Río Hortega, major advances in understanding their origin, morphological, functional, and genetic aspects were uncovered by pioneering research (Sierra et al., 2019). Stemming from the predominant focus of the past two decades on the role of microglia in pathological conditions, a passionate debate began to revolve around their function in the homeostatic maintenance of the healthy brain and their phenotypic diversity (Salter and Stevens, 2017; Stratoulias et al., 2019).

In spite of comprising approximately only 10% of the total central nervous system (CNS) cell population, microglia are found to be extensively, albeit unevenly, distributed throughout the white and gray matter (Lawson et al., 1990) and are essential for the regulation of complex developmental, homeostatic, and pathological processes (Salter and Stevens, 2017). Aside from acting as the CNS innate immune defense system in response to environmental stressors, microglia are known to regulate neuronal development and synaptic plasticity most notably during learning and the adaptation to the environment (Tremblay et al., 2010; Schafer et al., 2012; Parkhurst et al., 2013). Not only are they

[1]Axe Neurosciences, Centre de Recherche du CHU de Québec-Université Laval
[2]Department of Pharmacology, Jessenius Faculty of Medicine, Comenius University, Martin, Slovak Republic
[3]Institute of Experimental Pharmacology and Toxicology, Centre of Experimental Medicine, Slovak Academy of Sciences, Bratislava, Slovak Republic

Corresponding Author:
Marie-Ève Tremblay, Division of Medical Sciences, University of Victoria, Victoria, BC V8W 2Y2, Canada.
Email: evetremblay@uvic.ca

able to facilitate genesis and maturation of new synapses (Parkhurst et al., 2013), considerable data have highlighted several pathways by which microglia mediate synaptic pruning, that is, the elimination of exuberant or dysfunctional synapses. Microglia are able to affect synaptic plasticity through phagocytosis of whole synaptic connections (Tremblay et al., 2010; Paolicelli et al., 2011), synaptic "nibbling" (trogocytosis; Weinhard et al., 2018), or physical separation of pre- and postsynaptic elements with the help of their dynamic processes known as synaptic stripping (Moran and Graeber, 2004).

Efficient remodeling and refinement of synaptic connections are imperative for adequate brain wiring (Schafer et al., 2012; Kettenmann et al., 2013). Abnormal microglial activity resulting from aging or pathological insults was associated with connectivity issues underlying cognitive impairments and behavioral deviations across models of numerous neurodevelopmental disorders, such as schizophrenia (Sellgren et al., 2019) and autistic spectrum disorders (Zhan et al., 2014; Kleinhans et al., 2016), as well as neurodegenerative diseases such as Alzheimer's disease (AD; Hong et al., 2016) and Parkinson's disease (PD; Lecours et al., 2018).

Expanding literature strongly supports the notion that microglia comprise a heterogenous population with region- and state-specific differences in distribution, morphology, molecular signature, and function across the lifespan (Hammond et al., 2019; Stratoulias et al., 2019). Moreover, in response to specific stimuli and local microenvironment cues, multiple adaptive morphological phenotypes have been described— from steady-state ramified microglia to amoeboid, hypertrophic, rod-like, and dystrophic/senescent microglia (Savage et al., 2019a). The concept of microglial heterogeneity has grown over the past years, and several distinctive subpopulations/phenotypes were brought to light by means of state-of-the-art imaging techniques, genetic approaches, or transcriptomic single-cell/bulk RNA analyses. A transient surge of a CD11c+ microglial subpopulation was detected in early postnatal development, supporting neurogenesis and myelination primarily in white matter regions (Wlodarczyk et al., 2017). Comparable on spatiotemporal and functional levels, the proliferative-region-associated microglia subset was described as amoeboid-like shaped and possessing high phagocytic and metabolic activity (Hagemeyer et al., 2017; Li et al., 2019; Staszewski and Hagemeyer, 2019). It was also shown transcriptionally that proliferative-region-associated microglia significantly overlap with the disease-associated microglia (DAM) subpopulation (Keren-Shaul et al., 2017), whose putative neuroprotective function has been suggested in the early stages of AD (Deczkowska et al., 2018). Microglia are no longer considered a homogenous population, and a greater understanding of the mechanisms behind the genesis of their subpopulations and susceptibility toward different phenotypes could hold the key to improve the treatment of neurological disorders.

Discovering the Dark Microglia

Microglial heterogeneity is currently experiencing an intellectual boom as novel microglial subpopulations/phenotypes (subtypes) are emerging. One of these are the dark microglia (DM), whose identification, using high-spatial resolution transmission electron microscopy, was based on their unique dark appearance and distinctive ultrastructural features (Bisht et al., 2016b). Although DM research has markedly progressed since their initial discovery (see Table 1 for overview), a veil of mystery still covers several key features of these cells, for example, cell-specific markers, origin, and their functionality across disease contexts. For this reason, ultrastructural analysis is presently the most appropriate tool to characterize and study the DM.

At first glance, the most obvious ultrastructural feature distinguishing DM from typical microglia is their altered heterochromatin pattern (Bisht et al., 2016b). Compared with typical microglia, whose nucleoplasm consists of a lighter, electron-lucent euchromatin, accompanied by a dark, electron-dense heterochromatin lining the inner part of the nuclear membrane and small dark patches randomly distributed throughout the nucleoplasm (Peters et al., 1991), DM display a condensed and electron-dense nucleoplasm resulting in a near loss of the classical microglial pattern (Bisht et al., 2016b). Previous studies have suggested that considerable chromatin remodeling is associated with active phenotypic changes driven by altered gene expression (Lardenoije et al., 2015). This association, however, has not yet been investigated in the DM. It was also suggested that the DM appearance emanates from well-preserved proteins exhibiting high affinity toward osmium tetroxide, a fixative used for the preparation of electron microscopy samples to contrast lipids (Bisht et al., 2016a; for a more detailed explanation of electron microscopy sample preparation for DM analyses, see St-Pierre et al., 2019). Furthermore, DM display several oxidative and metabolic stress-induced alterations including disrupted mitochondria, dilation of the Golgi apparatus and endoplasmic reticulum, as well as cell shrinkage (Bisht et al., 2016b). It is important to note that while DM show ultrastructural alterations, evidence from electron microscopy analyses suggests that DM are not undergoing apoptosis or necrosis considering their lack of various nuclear alterations associated with these modes of cell death (e.g., blebbing, rounding, fragmentation; Bisht et al., 2016a) (Figure 1). However, thorough molecular

Table 1. Summary of the Studies Describing Dark Microglial Cells.

	Species	Model	Age at sacrifice	Region	Ultrastructural description
(Bisht et al., 2016b)	Mouse	C57BL/6 APP^{Swe}-PS1ΔE9 CX3CR1 KO (with/without stress) Social defeat stress	14 months 6, 14, and 21 months 16–20 weeks 8–9 weeks	Hippocampal CA1 Subgranular layer of the cerebral cortex Basolateral amygdala Median eminence of the hypothalamus	Electron-dense cyto- and nucleoplasm ER and Golgi dilation Altered mitochondria Loss of heterochromatin pattern Processes with acute angles Interaction with synapses Near vasculature elements Highly phagocytic
(Hui et al., 2018)	Mouse	C57BL/6, Poly I:C injection at E9.5	P80-P90	Dentate gyrus polymorphic layer	Dark microglia defined by Bisht et al.
(Elgayar et al., 2018)	Rat	Albino, rotenone injection	Adult	*Substantia nigra*	Dark microglia defined by Bisht et al. Associated with blood capillaries
(Uranova et al., 2018)	Human	Schizophrenia patients($n = 21$)	56.4 ± 17.2 years old	White matter near the Layer VI of prefrontal cortex	Described as dystrophic microglia (dark and electron-dense nucleus, cytoplasm with vacuoles)
(El Hajj et al., 2019)	Mouse	APP^{Swe}-PS1ΔE9	14 months	Hippocampal CA1 *strata radiatum* and *lacunosum-moleculare*	Dark microglia defined by Bisht et al., (associated with plaques and TREM2+) Intermediate stages of dark microglia (typical microglial heterochromatin pattern, darker cytoplasm, ER dilation)
(Kavetsky et al., 2019)	Mouse	C57BL/6-*Npc1*^{nmf164}, Western diet	8 weeks	Molecular layer of the cerebellum	Dark activated microglial processes interacting with synapses
(Calì et al., 2019)	Rat	Unknown	P14	Layer VI of somatosensory cortex	Darker cytosol
(Pedicone et al., 2020)	Mouse	3-month-old C57BL/6, injected biweekly for 3 weeks with K161	4 months	Cerebral cortex	Not determined

Note. ER = endoplasmic reticulum; KO = knockout; TREM2 = triggering receptor expressed on myeloid cells 2.

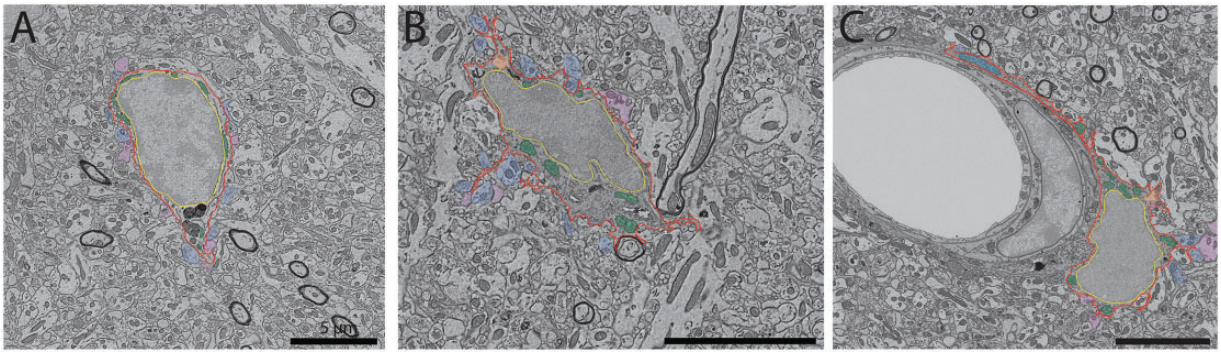

Figure 1. Electron micrographs of typical (A), intermediate (B) and dark microglia (C) in the hippocampus CA1, *strata radiatum* and *lacunosum-moleculare*, of mice given a high fat diet, taken at a resolution of 5 nm using a scanning electron microscope. A: Typical microglia are shown with an electron-lucent cytoplasm and a clear heterochromatin pattern. B and C: Intermediate and dark microglia display an electron-dense cyto- and nucleoplasm that possesses either a chromatin pattern similar to typical microglia (intermediate microglia, B) or a complete loss of chromatin pattern (dark microglia, C). Signs of oxidative stress (dilation of the endoplasmic reticulum, elongated mitochondria) as well as phagocytic inclusions are shown in B and C. Yellow contour = nucleus, red contour = cytoplasm and nucleus, green = mitochondrion, pink = postsynaptic element, light purple = presynaptic element, orange = phagocytic inclusion, * = dilation of endoplasmic reticulum, and blue = elongated mitochondrion. Scale bars = 5 μm.

analyses would be required to confirm that these cells are not dying.

DM also possess numerous thin and long processes forming acute angles in contrast to the typical microglia that display thicker and shorter processes with more obtuse angles (Bisht et al., 2016b; Figure 1). Previous transmission electron microscopy observations have shown that DM extensively interact with their microenvironment, making contacts with neurons, other glial cells, and components of the neuropil (axon terminals, dendritic spines, synapses, and other cellular elements). Their abundant endosomes also suggested their highly phagocytic nature (Bisht et al., 2016b). DM tend to form clusters and are found in close proximity to capillaries. Indeed, around two thirds of the DM observed by transmission electron microscopy ensheathed the brain vasculature, with approximately one eighth of them contacting two blood vessels (Bisht et al., 2016b).

Reports from recently published studies point toward a microglial role in the formation of the blood–brain barrier and maintenance of blood vessels (Dudvarski Stankovic et al., 2016; Halder and Milner, 2019; Koizumi et al., 2019). In this context, the decline of vascular integrity and microvascular density (Xu et al., 2017) accompanied by elevated permeability of blood–brain barrier seen over the course of aging and during pathological conditions such as chronic inflammation (da Fonseca et al., 2014; Haruwaka et al., 2019) hints toward a possible vascular-related role for the DM.

DM are not always found: their near-total absence in normal adult brain (Bisht et al., 2016b) but prevalence in pathology suggests that their appearance is either a cause or consequence (e.g., functional need for the DM) of parenchymal changes. Mouse models of long-term stress exposure (model of social defeat or chronic unpredictable stress), aging (14-month-old mice), AD pathology (amyloid precursor protein-presenilin 1 [APP-PS1] mouse model where APP possesses a double Swedish mutation and PSEN1 has an exon-9-deleted mutation; Jankowsky et al., 2004), and fractalkine signaling deficiency (CX3CR1 knockout mice) were all reported to possess an increased number of DM in several brain areas such as the hippocampal CA1 region (*strata lacunosum-moleculare* and *radiatum*), subgranular layers of cerebral cortex, basolateral nucleus of the amygdala, and hypothalamic median eminence (Bisht et al., 2016b). Because neuronal circuit remodeling is a shared feature of various pathological conditions, it is likely that DM play a crucial role in the plasticity of synapses seen in these contexts. This role, however, has yet to be fully uncovered.

A difference in immunoreactivity to various proteins associated with myeloid cells can be observed between the DM and the typical microglia. Contrary to the latter, DM display a low immunoreactivity for homeostatic myeloid cell markers such as ionized calcium-binding protein (IBA1) and green fluorescent protein (GFP) in the case of CX3CR1-GFP reporter mice (Bisht et al., 2016b). Their ramified processes encircling various elements of the synaptic neuropil strongly express phagocytic microglia-specific 4D4 (Bisht et al., 2016b) and the integrin alpha M subunit (CD11b), a complement receptor 3 element involved in microglia-dependent synaptic pruning (Schafer et al., 2012). In addition, in specific conditions such as AD pathology, the DM were found to express triggering receptor expressed on myeloid cells 2 (TREM2), particularly

when associated with amyloid β plaques (Bisht et al., 2016b). These cells also displayed increased phagocytosis of amyloid β as well as interactions with dystrophic synaptic elements (Bisht et al., 2016b). Further immunohistochemical analyses revealed negative reactivity of DM toward other cell type markers such as ALDH1L1 (astrocytes), OLIG2 (oligodendrocytes), or CD11c (dendritic cells; expressed also by the DAM and neurodegenerative phenotype [MGnD]; Keren-Shaul et al., 2017; Krasemann et al., 2017) or major histocompatibility complex (MHC)II (antigen-presenting cells; Bisht et al., 2016b). No sensitivity was detected also for some markers of cells with a myeloid origin such as CD206 (perivascular macrophages) and 4C12 (inflammatory monocytes), or the microglial homeostatic marker P2RY12 (purinergic receptor; Bisht et al., 2016b). Although their origin remains unclear with the evidence currently available, their immunoreactivity to the phagocytic microglial marker 4D4 and lack of immunoreactivity to 4C12 suggest an origin from the embryonic yolk sac, similar to the typical microglia. Supporting this hypothesis, their presence was observed in the absence of C-C chemokine receptor type 2 (Bisht et al., 2016b), using a knockout mouse model where infiltrating monocytes do not migrate in the CNS (Mildner et al., 2007; Gómez-Nicola et al., 2014).

Advances in the Dark Microglial Field

Development

Although much remains to be discovered, a strong foundation was built with the ultrastructural description of the DM, therefore stimulating further studies to elucidate the role of DM in various species and models. Indeed, since the initial description of the DM by Bisht et al., numerous studies have provided insightful findings in regard to this microglial subtype. Because one hypothesis is that DM might contribute to excessive remodeling of synaptic connections leading to abnormal neuronal circuitry formation, it is crucial to focus on early periods of brain development where extensive and dynamic microglia-dependent synaptic remodeling occurs (Paolicelli et al., 2011; Schafer et al., 2012). Interestingly, a recent study hinted to the presence of DM during development (postnatal day 14), a period undergoing pronounced synaptic plasticity (Chandrasekaran et al., 2015), in the rat somatosensory cortex (Calì et al., 2019). This elegant study reconstructed in three-dimensions numerous cell types, including microglia, with the help of serial block-face scanning electron microscopy. From their reconstruction, the authors discovered that approximately one third of microglia imaged in Layer VI had a dark cytosol, a characteristic of the intermediate state (i.e., microglia showing both typical and DM features) and the fully formed DM defined by Bisht et al. (2016b). It is unclear, however, if these cells possess other ultrastructural characteristics commonly seen in DM, such as dilation of the endoplasmic reticulum/Golgi apparatus and altered mitochondria. The presence of DM in the postnatal developmental stage could be somehow related to the overabundance of cellular debris and active pruning processes resulting from dynamic tissue remodeling.

Psychiatric Disorders

The presence of the DM was also characterized in a mouse model of maternal immune activation, with the help of transmission electron microscopy (Hui et al., 2018). This environmental stressor was previously associated with microglial priming, which causes their exacerbated inflammatory and nonphysiological response to secondary challenges, thus promoting a wide range of psychiatric disorder-like behaviors (Mattei et al., 2017; Tay et al., 2017). Prenatal administration (at embryonic day 9.5) of polyinosinic:polycytidylic acid (poly I:C), a molecule mimicking viral double-stranded RNA infection (Smith et al., 2007), resulted in a significant increase of DM density in the hippocampal dentate gyrus (DG) polymorphic layer of male offspring, without significant differences seen in females at adulthood (Hui et al., 2018). Combined with the noticeable schizophrenia-like phenotype of male offspring, this finding hints to possible sex-related differences in the pathophysiological mechanisms that would affect the genesis of DM or vice versa. The DG plays a key role in learning, memory formation, and spatial orientation functioning as a niche of neurogenesis throughout life (Spalding et al., 2013; Gonçalves et al., 2016). A possibility exists that extensive interactions between the DM and synapses in the DG could contribute to abnormal functional connectivity, though additional insights are necessary.

Moreover, a recent *postmortem* study has shown microglial cells that are ultrastructurally similar to the DM subtype in the prefrontal cortex of schizophrenia patients (Uranova et al., 2018). Indeed, these microglia, described as "dystrophic," possessed an electron-dense nucleus and cytoplasm filled with vacuoles (Uranova et al., 2018). Interestingly, these cells were located in the white matter, in close proximity to oligodendrocytes. Considering that these DM were found in the white matter next to oligodendrocytes (Uranova et al., 2018), it is likely that they could have a direct or indirect effect on oligodendrocytic functions such as myelin formation.

Neurodegenerative Diseases and Neuroinflammation

Consistent with results of the first article describing DM in AD pathology (Bisht et al., 2016b), plaque-associated

DM-like cells were distinguished in the hippocampus CA1 of adult APP-PS1 mice using correlative light, transmission and scanning electron microscopy (El Hajj et al., 2019). AD is a progressive neurodegenerative disease that is characterized by the extracellular accumulation of plaques containing fibrillar amyloid β and intracellular accumulation of hyperphosphorylated tau protein into the shape of neurofibrillary tangles (Spires-Jones and Hyman, 2014). Resulting neuronal and synaptic loss strongly correlate with the cognitive impairment and dementia described in AD patients (Spires-Jones and Hyman, 2014). In the APP-PS1 mouse model, El Hajj et al. (2019) also observed microglia with a preserved, distinct heterochromatin pattern and a less-dark appearance that suggested the existence of an intermediate phenotype between the typical and DM (El Hajj et al., 2019), thus further supporting the idea that DM might be part of a heterogenous microglial spectrum.

DM were seen for the first time in PD pathology by examining the *substantia nigra* of male albino rats that received subcutaneous injections of rotenone, a broad-spectrum insecticide, piscicide, and pesticide that inhibits the Complex I of the mitochondrial electron transport chain (Elgayar et al., 2018). PD is a neurodegenerative pathology characterized by, but not limited to, synaptopathy, presence of misfolded α-synuclein aggregates (Lewy bodies), and loss of dopaminergic neurons in the *substantia nigra pars compacta* that result in cognitive and motor deficits (Schirinzi et al., 2016; Lecours et al., 2018). In this study, Elgayar et al. (2018) found, using transmission electron microscopy, the presence of DM near the vasculature, a feature similar to what was seen previously (Bisht et al., 2016b). The authors identified ultrastructural features normally associated with the DM such as condensation of the cyto- and nucleoplasm, highly phagocytic processes and altered mitochondria (Bisht et al., 2016b). However, the study also revealed some apparent divergences from the DM characterized by Bisht et al. Indeed, the DM described in this study possessed a heterochromatin pattern similar to what can be seen in typical microglia (Elgayar et al., 2018). Therefore, the DM in the rotenone rat model could represent an intermediate stage, an analogous phenomenon to what was identified in an AD mouse model (El Hajj et al., 2019). Although their function has not been investigated, the intermediate and dark microglia could play a role in the synaptic loss seen in AD and PD, as these cells were previously shown to interact extensively with synaptic elements.

Following up on these findings, the presence of DM was investigated in the same brain area as El Hajj et al., in a lipopolysaccharide (LPS)-induced mouse model of sickness behavior (Savage et al., 2019b). The connection between systemic inflammation and microglial response in sickness behavior has been extensively covered (Dantzer et al., 2008; Hoogland et al., 2015). While the acute systemic injection of LPS (1 mg/kg) was able to generate profound alterations of the inflammatory profile and microglial ultrastructure, no cell harboring ultrastructural features of DM was recognized at a 24-hour time point. Another condition, a mouse model of Niemann-Pick type C, where neurodegeneration and inflammation are found, revealed the presence of the DM. Indeed, Kavetsky et al. (2019) investigated NPC^{nmf164} mice given for 5 weeks either a Western diet or a regular diet. Niemann-Pick type C is a genetic condition with mutations in the NPC genes (*Npc1/2*), which most notably lead to dysfunctional lysosomes (Wheeler and Sillence, 2019). While looking at microglia in the molecular layer of the cerebellum of both males and females with transmission electron microscopy, the authors identified DM processes interacting with synapses, a phenomenon especially seen in the mice given a Western diet (Kavetsky et al., 2019). This finding indicates that Western diet exacerbates the inflammation found in this mouse model and causes alterations sufficient to generate DM. Although DM are frequently found in an inflammatory brain parenchyma like that of NPC^{nmf164} mice, it is not always the case (e.g., in acute inflammation induced by LPS injection), suggesting that the genesis of the DM might depend on a specific mechanism or pathway related to inflammation, or require chronic challenges.

Beyond Electron Microscopy

Electron microscopy is not the only tool that was used to characterize the DM. Indeed, Pedicone et al. (2020) performed flow cytometry experiments, gating with CD11b and TREM2, to determine if DM were present in the cerebral cortex of 4-month-old C56BL/6 mice injected biweekly for 3 weeks with a pan-SH-2 containing inositol 5' polyphosphatase (SHIP)1/2 inhibitor, K161. The protein SHIP-1 is able to bind to the adaptor protein DAP12 resulting in the inhibition of the TREM2 signaling pathway (Nizami et al., 2019). Therefore, modulating SHIP1/2 pathway to prevent this inhibition could be useful for the treatment of neurodegenerative diseases where TREM2 was shown to have a beneficial effect (Melchior et al., 2010; Wang et al., 2015). TREM2 was previously identified as a key protein for the genesis of various microglial subtypes including the DAM and MGnD (Keren-Shaul et al., 2017; Krasemann et al., 2017). CD11b, an essential component of the complement receptor 3 involved in synaptic pruning, is a protein expressed by both dark and typical microglial cells (Bisht et al., 2016b; Hopperton et al., 2018). The study showed no difference of CD11b and TREM2 mean fluorescence intensity between controls and K161-treated animals and therefore concludes that DM do not emerge as a result of

the K161 treatment (Pedicone et al., 2020). However, in this case, the interpretation of these results does not necessarily take into account all information concerning the DM. TREM2 and CD11b are not exclusively expressed by the DM and therefore cannot be used as specific markers for this subtype (Bisht et al., 2016b). Moreover, while it is possible to determine the immunoreactivity of a cell for a particular protein with electron microscopy, it is difficult, albeit close to impossible, to quantify with exactitude immunoreactivity levels. Therefore, the levels of proteins such as CD11b and TREM2 that are expressed by the DM versus the typical microglia or other subtypes are yet unknown, and their varying expression cannot be currently used as a tool to distinguish other populations from the DM. The expression of TREM2 by the DM was identified in an APP-PS1 model but not characterized yet in other models. It would be crucial to determine, using electron microscopy, if this subtype expresses CD11b and TREM2 in the K161-treated mice for a full interpretation of the results as well as assess the specificity of the protein to DM (see Table 1 for a full summary of the published dark microglial studies to the authors knowledge).

Conclusion

Microglial heterogeneity has been a topic of interest in the past few years, and one of these subtypes, the DM, has been no exception. These unique microglial cells first described using transmission electron microscopy are found in numerous pathological conditions where synaptic plasticity is heightened, such as in mouse models of AD, chronic stress, fractalkine signaling deficiency, and aging (Bisht et al., 2016b). Recently, they have also been identified in rats and humans (Elgayar et al., 2018; Uranova et al., 2018; Calì et al., 2019), suggesting that this subtype is conserved across species. Studying these cells and learning more about their function could, in the future, be applied to the human condition. Indeed, these cells that are associated with ultrastructural features of oxidative stress seem to interact excessively with synaptic elements, suggesting that their presence could exacerbate the synaptic loss seen in pathology. Modulating these cells could hence be a potential new therapeutic target for neurodegenerative disease such as AD and PD.

While electron microscopy allows to provide an in-depth ultrastructural analysis of the DM, many questions remain to be answered due to technical limitation. Indeed, their unique ultrastructural feature, most notably their dark cyto- and nucleoplasm as well as the lack of chromatin pattern, is the only way to properly identify these cells currently. Therefore, electron microscopy is the only tool that can be used to characterize these cells.

Fortunately, new electron microscopy techniques, such as serial-block face and focused ion beam scanning electron microscopy, that can reconstruct taken images in three dimensions, have been developed and can help answer some of the remaining questions regarding the DM. Identifying a specific marker for this subtype, which could be used to perform high-throughput molecular analyses and create transgenic models, will be crucial for the future of the DM field. With these transgenic models, it would be possible for researchers to isolate these cells and further process them using state-of-the-art RNA sequencing for instance to learn more about their function, especially in regard to inflammation, the vasculature and synapses, and their altered transcriptome compared with the typical microglia (e.g., determine if they possess a more pro- or anti-inflammatory profile). Moreover, although some clues have already been provided regarding the DM origin (e.g., immunopositivity for 4D4 and immunonegativity for 4C12, as well as presence in C-C chemokine receptor type 2 knockout mice), determining their gene expression could provide more certitude as to whether they come from the periphery or the embryonic yolk sac. In addition, dynamic analysis of DM process motility using *in vivo* two-photon microscopy could be useful to determine how their hyperramifications seen in electron microscopy interact with neurons and synapses.

Authors' Note

Marie-Ève Tremblay is also affiliated with Division of Medical Sciences, University of Victoria.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: M.-K. S.-P. holds a Canadian Institute of Health Research and a Fonds de recherche du Québec-Santé doctoral scholarship. E. S. holds a national scholarship program of the Slovak Republic provided by Slovak Academic Information Agency. M.-E. T. holds a Tier II Canada Research Chair in Neurobiology of Aging and Cognition.

ORCID iD

Marie-Ève Tremblay https://orcid.org/0000-0003-2863-9626

References

Bisht, K., Sharma, K., Lacoste, B., & Tremblay, M.-È. (2016a). Dark microglia: Why are they dark? *Commun Integr Biol*, *9*, e1230575. https://doi.org/10.1080/19420889.2016.1230575

Bisht, K., Sharma, K. P., Lecours, C., Gabriela Sánchez, M., El Hajj, H., Milior, G., et al. (2016b). Dark microglia: A new

phenotype predominantly associated with pathological states. *Glia*, *64*, 826–839. https://doi.org/10.1002/glia.22966

Calì, C., Agus, M., Kare, K., Boges, D. J., Lehväslaiho, H., Hadwiger, M., et al. (2019). 3D cellular reconstruction of cortical glia and parenchymal morphometric analysis from serial block-face electron microscopy of juvenile rat. *Prog Neurobiol*, *183*, 101696. https://doi.org/10.1016/j.pneurobio.2019.101696

Chandrasekaran, S., Navlakha, S., Audette, N. J., McCreary, D. D., Suhan, J., Bar-Joseph, Z., et al. (2015). Unbiased, high-throughput electron microscopy analysis of experience-dependent synaptic changes in the neocortex. *J Neurosci*, *35*, 16450–16462. https://doi.org/10.1523/JNEUROSCI.1573-15.2015

da Fonseca, A. C. C., Matias, D., Garcia, C., Amaral, R., Geraldo, L. H., Freitas, C., et al. (2014). The impact of microglial activation on blood-brain barrier in brain diseases. *Front Cell Neurosci*, *8*, 362. https://doi.org/10.3389/fncel.2014.00362

Dantzer, R., O'Connor, J. C., Freund, G. G., Johnson, R. W., & Kelley, K. W. (2008). From inflammation to sickness and depression: When the immune system subjugates the brain. *Nat Rev Neurosci*, *9*, 46–56. https://doi.org/10.1038/nrn2297

Deczkowska, A., Keren-Shaul, H., Weiner, A., Colonna, M., Schwartz, M., & Amit, I. (2018). Disease-associated microglia: A universal immune sensor of neurodegeneration. *Cell*, *173*, 1073–1081. https://doi.org/10.1016/j.cell.2018.05.003

Dudvarski Stankovic, N., Teodorczyk, M., Ploen, R., Zipp, F., & Schmidt, M. H. H. (2016). Microglia-blood vessel interactions: A double-edged sword in brain pathologies. *Acta Neuropathol*, *131*, 347–363. https://doi.org/10.1007/s00401-015-1524-y

El Hajj, H., Savage, J. C., Bisht, K., Parent, M., Vallières, L., Rivest, S., et al. (2019). Ultrastructural evidence of microglial heterogeneity in Alzheimer's disease amyloid pathology. *J Neuroinflammation*, *16*, 87. https://doi.org/10.1186/s12974-019-1473-9

Elgayar, S. A. M., Abdel-Hafez, A. A. M., Gomaa, A. M. S., & Elsherif, R. (2018). Vulnerability of glia and vessels of rat substantia nigra in rotenone Parkinson model. *Ultrastruct Pathol*, *42*, 181–192. https://doi.org/10.1080/01913123.2017.1422066

Gómez-Nicola, D., Schetters, S. T., & Hugh Perry, V. (2014). Differential role of CCR2 in the dynamics of microglia and perivascular macrophages during prion disease. *Glia*, *62*, 1041–1052. https://doi.org/10.1002/glia.22660

Gonçalves, J. T., Schafer, S. T., & Gage, F. H. (2016). Adult neurogenesis in the hippocampus: From stem cells to behavior. *Cell*, *167*, 897–914. https://doi.org/10.1016/j.cell.2016.10.021

Hagemeyer, N., Hanft, K.-M., Akriditou, M.-A., Unger, N., Park, E. S., Stanley, E. R., et al. (2017). Microglia contribute to normal myelinogenesis and to oligodendrocyte progenitor maintenance during adulthood. *Acta Neuropathol*, *134*, 441–458. https://doi.org/10.1007/s00401-017-1747-1

Halder, S. K., & Milner, R. (2019). A critical role for microglia in maintaining vascular integrity in the hypoxic spinal cord. *PNAS*, *116*, 26029–26037. https://doi.org/10.1073/pnas.1912178116

Hammond, T. R., Dufort, C., Dissing-Olesen, L., Giera, S., Young, A., Wysoker, A., et al. (2019). Single-cell RNA sequencing of microglia throughout the mouse lifespan and in the injured brain reveals complex cell-state changes. *Immunity*, *50*, 253–271.e6. https://doi.org/10.1016/j.immuni.2018.11.004

Haruwaka, K., Ikegami, A., Tachibana, Y., Ohno, N., Konishi, H., Hashimoto, A., et al. (2019). Dual microglia effects on blood brain barrier permeability induced by systemic inflammation. *Nat Commun*, *10*, 1–17. https://doi.org/10.1038/s41467-019-13812-z

Hong, S., Beja-Glasser, V. F., Nfonoyim, B. M., Frouin, A., Li, S., Ramakrishnan, S., et al. (2016). Complement and microglia mediate early synapse loss in Alzheimer mouse models. *Science*, *352*, 712–716. https://doi.org/10.1126/science.aad8373

Hoogland, I. C. M., Houbolt, C., van Westerloo, D. J., van Gool, W. A., & van de Beek, D. (2015). Systemic inflammation and microglial activation: Systematic review of animal experiments. *J Neuroinflammation*, *12*, 114. https://doi.org/10.1186/s12974-015-0332-6

Hopperton, K. E., Mohammad, D., Trépanier, M. O., Giuliano, V., & Bazinet, R. P. (2018). Markers of microglia in post-mortem brain samples from patients with Alzheimer's disease: A systematic review. *Mol Psychiatry*, *23*, 177–198. https://doi.org/10.1038/mp.2017.246

Hui, C. W., St-Pierre, A., El Hajj, H., Remy, Y., Hébert, S. S., Luheshi, G. N., et al. (2018). Prenatal immune challenge in mice leads to partly sex-dependent behavioral, microglial, and molecular abnormalities associated with schizophrenia. *Front Mol Neurosci*, *11*, 13. https://doi.org/10.3389/fnmol.2018.00013

Jankowsky, J. L., Fadale, D. J., Anderson, J., Xu, G. M., Gonzales, V., Jenkins, N. A., et al. (2004). Mutant presenilins specifically elevate the levels of the 42 residue beta-amyloid peptide in vivo: Evidence for augmentation of a 42-specific gamma secretase. *Hum Mol Genet*, *13*, 159–170. https://doi.org/10.1093/hmg/ddh019

Kavetsky, L., Green, K. K., Boyle, B. R., Yousufzai, F. A. K., Padron, Z. M., Melli, S. E., et al. (2019). Increased interactions and engulfment of dendrites by microglia precede Purkinje cell degeneration in a mouse model of Niemann Pick type-C. *Sci Rep*, *9*. https://doi.org/10.1038/s41598-019-51246-1

Keren-Shaul, H., Spinrad, A., Weiner, A., Matcovitch-Natan, O., Dvir-Szternfeld, R., Ulland, T. K., et al. (2017). A unique microglia type associated with restricting development of Alzheimer's disease. *Cell*, *169*, 1276–1290.e17. https://doi.org/10.1016/j.cell.2017.05.018

Kettenmann, H., Kirchhoff, F., & Verkhratsky, A. (2013). Microglia: New roles for the synaptic stripper. *Neuron*, *77*, 10–18. https://doi.org/10.1016/j.neuron.2012.12.023

Kleinhans, N. M., Reiter, M. A., Neuhaus, E., Pauley, G., Martin, N., Dager, S., et al. (2016). Subregional differences in intrinsic amygdala hyperconnectivity and hypoconnectivity in autism spectrum disorder. *Autism Res*, *9*, 760–772. https://doi.org/10.1002/aur.1589

Koizumi, T., Kerkhofs, D., Mizuno, T., Steinbusch, H. W. M., & Foulquier, S. (2019). Vessel-associated immune cells in

cerebrovascular diseases: From perivascular macrophages to vessel-associated microglia. *Front Neurosci*, *13*, 1291. https://doi.org/10.3389/fnins.2019.01291

Krasemann, S., Madore, C., Cialic, R., Baufeld, C., Calcagno, N., El Fatimy, R., et al. (2017). The TREM2-APOE pathway drives the transcriptional phenotype of dysfunctional microglia in neurodegenerative diseases. *Immunity*, *47*, 566–581.e9. https://doi.org/10.1016/j.immuni.2017.08.008

Lardenoije, R., Iatrou, A., Kenis, G., Kompotis, K., Steinbusch, H. W. M., Mastroeni, D., et al. (2015). The epigenetics of aging and neurodegeneration. *Prog Neurobiol*, *131*, 21–64. https://doi.org/10.1016/j.pneurobio.2015.05.002

Lawson, L. J., Perry, V. H., Dri, P., & Gordon, S. (1990). Heterogeneity in the distribution and morphology of microglia in the normal adult mouse brain. *Neuroscience*, *39*, 151–170. https://doi.org/10.1016/0306-4522(90)90229-w

Lecours, C., Bordeleau, M., Cantin, L., Parent, M., Paolo, T. D., & Tremblay, M.-È. (2018). Microglial implication in Parkinson's disease: Loss of beneficial physiological roles or gain of inflammatory functions? *Front Cell Neurosci*, *12*, 282. https://doi.org/10.3389/fncel.2018.00282

Li, Q., Cheng, Z., Zhou, L., Darmanis, S., Neff, N. F., Okamoto, J., et al. (2019). Developmental heterogeneity of microglia and brain myeloid cells revealed by deep single-cell RNA sequencing. *Neuron*, *101*, 207–223.e10. https://doi.org/10.1016/j.neuron.2018.12.006

Mattei, D., Ivanov, A., Ferrai, C., Jordan, P., Guneykaya, D., Buonfiglioli, A., et al. (2017). Maternal immune activation results in complex microglial transcriptome signature in the adult offspring that is reversed by minocycline treatment. *Transl Psychiatry*, *7*, e1120–e1120. https://doi.org/10.1038/tp.2017.80.

Melchior, B., Garcia, A. E., Hsiung, B.-K., Lo, K. M., Doose, J. M., Thrash, J. C., et al. (2010). Dual induction of TREM2 and tolerance-related transcript, Tmem176b, in amyloid transgenic mice: Implications for vaccine-based therapies for Alzheimer's disease. *ASN Neuro*, *2*, e00037. https://doi.org/10.1042/AN20100010

Mildner, A., Schmidt, H., Nitsche, M., Merkler, D., Hanisch, U.-K., Mack, M., et al. (2007). Microglia in the adult brain arise from Ly-6ChiCCR2+ monocytes only under defined host conditions. *Nat Neurosci*, *10*, 1544–1553. https://doi.org/10.1038/nn2015

Moran, L. B., & Graeber, M. B. (2004). The facial nerve axotomy model. *Brain Res Rev*, *44*, 154–178. https://doi.org/10.1016/j.brainresrev.2003.11.004

Nizami, S., Hall-Roberts, H., Warrier, S., Cowley, S. A., & Di Daniel, E. (2019). Microglial inflammation and phagocytosis in Alzheimer's disease: Potential therapeutic targets. *Br J Pharmacol*, *176*, 3515–3532. https://doi.org/10.1111/bph.14618

Paolicelli, R. C., Bolasco, G., Pagani, F., Maggi, L., Scianni, M., Panzanelli, P., et al. (2011). Synaptic pruning by microglia is necessary for normal brain development. *Science*, *333*, 1456–1458. https://doi.org/10.1126/science.1202529

Parkhurst, C. N., Yang, G., Ninan, I., Savas, J. N., Yates, J. R., Lafaille, J. J., et al. (2013). Microglia promote learning-dependent synapse formation through BDNF. *Cell*, *155*, 1596–1609. https://doi.org/10.1016/j.cell.2013.11.030

Pedicone, C., Fernandes, S., Dungan, O. M., Dormann, S. M., Viernes, D. R., Adhikari, A. A., et al. (2020). Pan-SHIP1/2 inhibitors promote microglia effector functions essential for CNS homeostasis. *J Cell Sci*, *133*. https://doi.org/10.1242/jcs.238030

Peters, A., Palay, S. L., & deF Webster, H. (1991). *The fine structure of the nervous system: Neurons and their supporting cells*. Oxford University Press.

Salter, M. W., & Stevens, B. (2017). Microglia emerge as central players in brain disease. *Nat Med*, *23*, 1018–1027. https://doi.org/10.1038/nm.4397

Savage, J. C., Carrier, M., & Tremblay, M.-È. (2019a). Morphology of microglia across contexts of health and disease. *Methods Mol Biol*, *2034*, 13–26. https://doi.org/10.1007/978-1-4939-9658-2_2

Savage, J. C., St-Pierre, M.-K., Hui, C. W., & Tremblay, M.-E. (2019b). Microglial ultrastructure in the hippocampus of a lipopolysaccharide-induced sickness mouse model. *Front Neurosci*, *13*, 1340. https://doi.org/10.3389/fnins.2019.01340

Schafer, D. P., Lehrman, E. K., Kautzman, A. G., Koyama, R., Mardinly, A. R., Yamasaki, R., et al. (2012). Microglia sculpt postnatal neural circuits in an activity and complement-dependent manner. *Neuron*, *74*, 691–705. https://doi.org/10.1016/j.neuron.2012.03.026

Schirinzi, T., Madeo, G., Martella, G., Maltese, M., Picconi, B., Calabresi, P., et al. (2016). Early synaptic dysfunction in Parkinson's disease: Insights from animal models. *Mov Disord*, *31*, 802–813. https://doi.org/10.1002/mds.26620

Sellgren, C. M., Gracias, J., Watmuff, B., Biag, J. D., Thanos, J. M., Whittredge, P. B., et al. (2019). Increased synapse elimination by microglia in schizophrenia patient-derived models of synaptic pruning. *Nat Neurosci*, *22*, 374–385. https://doi.org/10.1038/s41593-018-0334-7

Sierra, A., Paolicelli, R. C., & Kettenmann, H. (2019). Cien Años de Microglía: Milestones in a century of microglial research. *Trends Neurosci*, *42*, 778–792. https://doi.org/10.1016/j.tins.2019.09.004

Smith, S. E. P., Li, J., Garbett, K., Mirnics, K., & Patterson, P. H. (2007). Maternal immune activation alters fetal brain development through interleukin-6. *J Neurosci*, *27*, 10695–10702. https://doi.org/10.1523/JNEUROSCI.2178-07.2007

Spalding, K. L., Bergmann, O., Alkass, K., Bernard, S., Salehpour, M., Huttner, H. B., et al. (2013). Dynamics of hippocampal neurogenesis in adult humans. *Cell*, *153*, 1219–1227. https://doi.org/10.1016/j.cell.2013.05.002

Spires-Jones, T. L., & Hyman, B. T. (2014). The intersection of amyloid beta and tau at synapses in Alzheimer's disease. *Neuron*, *82*, 756–771. https://doi.org/10.1016/j.neuron.2014.05.004

Staszewski, O., & Hagemeyer, N. (2019). Unique microglia expression profile in developing white matter. *BMC Res Notes*, *12*, 367. https://doi.org/10.1186/s13104-019-4410-1

St-Pierre, M.-K., Bordeleau, M., & Tremblay, M.-È. (2019). Visualizing dark microglia. *Methods Mol Biol*, *2034*, 97–110. https://doi.org/10.1007/978-1-4939-9658-2_8

Stratoulias, V., Venero, J. L., Tremblay, M., & Joseph, B. (2019). Microglial subtypes: Diversity within the microglial community. *EMBO J*, *38*, e101997. https://doi.org/10.15252/embj.2019101997

Tay, T. L., Béchade, C., D'Andrea, I., St-Pierre, M.-K., Henry, M. S., Roumier, A., et al. (2017). Microglia gone rogue: Impacts on psychiatric disorders across the lifespan. *Front Mol Neurosci*, *10*, 421. https://doi.org/10.3389/fnmol.2017.00421

Tremblay, M.-È., Lowery, R. L., & Majewska, A. K. (2010). Microglial interactions with synapses are modulated by visual experience. *PLoS Biol*, *8*, e1000527. https://doi.org/10.1371/journal.pbio.1000527

Uranova, N. A., Vikhreva, O. V., Rakhmanova, V. I., & Orlovskaya, D. D. (2018). Ultrastructural pathology of oligodendrocytes adjacent to microglia in prefrontal white matter in schizophrenia. *NPJ Schizophr*, *4*, 26. https://doi.org/10.1038/s41537-018-0068-2

Wang, Y., Cella, M., Mallinson, K., Ulrich, J. D., Young, K. L., Robinette, M. L., et al. (2015). TREM2 lipid sensing sustains the microglial response in an Alzheimer's disease model. *Cell*, *160*, 1061–1071. https://doi.org/10.1016/j.cell.2015.01.049

Weinhard, L., di Bartolomei, G., Bolasco, G., Machado, P., Schieber, N. L., Neniskyte, U., et al. (2018). Microglia remodel synapses by presynaptic trogocytosis and spine head filopodia induction. *Nat Commun*, *9*, 1228. https://doi.org/10.1038/s41467-018-03566-5

Wheeler, S., & Sillence, D. J. (2019). Niemann-Pick type C disease: Cellular pathology and pharmacotherapy. *J Neurochem*. Advance online publication. https://doi.org/10.1111/jnc.14895

Wlodarczyk, A., Holtman, I. R., Krueger, M., Yogev, N., Bruttger, J., Khorooshi, R., et al. (2017). A novel microglial subset plays a key role in myelinogenesis in developing brain. *EMBO J*, *36*, 3292–3308. https://doi.org/10.15252/embj.201696056

Xu, X., Wang, B., Ren, C., Hu, J., Greenberg, D. A., Chen, T., et al. (2017). Age-related impairment of vascular structure and functions. *Aging Dis*, *8*, 590–610. https://doi.org/10.14336/AD.2017.0430

Zhan, Y., Paolicelli, R. C., Sforazzini, F., Weinhard, L., Bolasco, G., Pagani, F., et al. (2014). Deficient neuron-microglia signaling results in impaired functional brain connectivity and social behavior. *Nat Neurosci*, *17*, 400–406. https://doi.org/10.1038/nn.3641