BRAIN COMMUNICATIONS

An update on the rod microglia variant in experimental and clinical brain injury and disease

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Contemporary microglia morphologies include ramified, activated and amoeboid, with the morphology of microglia considered highly coupled to the cellular function. Rod microglia are an additional activated microglia variant observed in the ageing, injured and diseased brain. Rod microglia were reported frequently in the early 1900s by neuropathologists in post-mortem cases of general paresis, Alzheimer's disease and encephalitis, and then remained largely ignored for almost 100 years. Recent reports have renewed interest in rod microglia, most notably after experimental traumatic brain injury. Rod microglia are formed by the narrowing of the soma and retraction of planar processes, which results in the appearance of an elongated, rod-shaped cell. Rod microglia are most commonly observed in the cortex, aligned perpendicular to the dural surface and adjacent to neuronal processes; in the hippocampus, they are aligned perpendicular to hippocampal layers. Furthermore, rod microglia form trains with one another, apical end to basal end. By replicating the process of sketching microscopic observation, rod microglia are re-defined by circumnutation around the long axis. In this update, we summarize the rod microglia variant in clinical and experimental literature and advocate for investigation into mechanisms of rod microglia origin and function.

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Abbreviations: CDB = corticobasal degeneration; CD68 = cluster of differentiation 68; DPI = day post-injury; FTLD = frontotemporal lobar degeneration; Iba1 = ionized calcium-binding adapter protein; MAPT = microtuble-associated protein tau; P2RY12 = purinergic receptor; TBI = traumatic brain injury; TDP = TAR DNA-binding protein 43; TMEM119 = transmembrane protein 119

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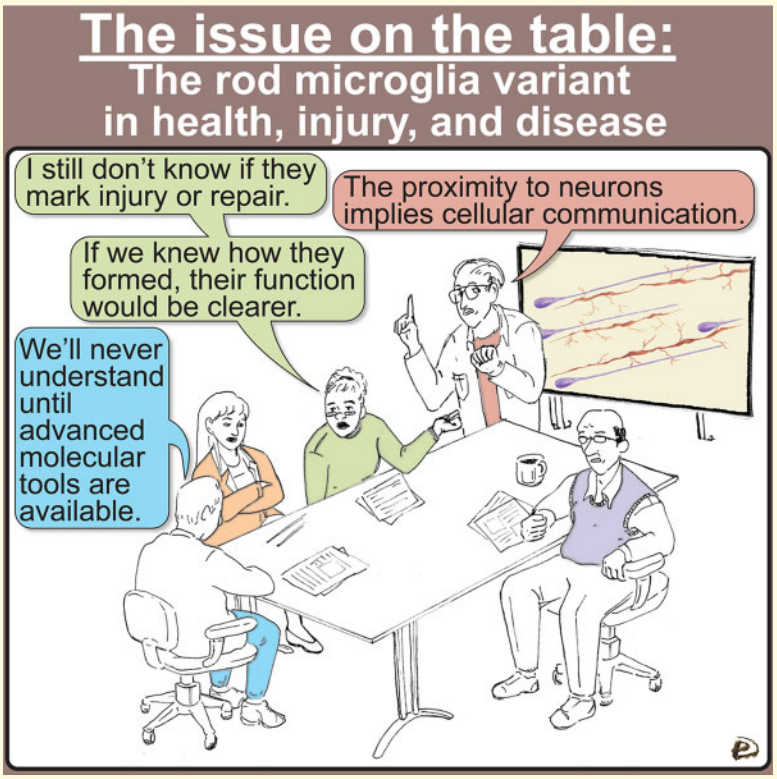
Graphical Abstract



Introduction

For biological organisms, cell classification is under constant refinement to incorporate new observations that challenge existing cell taxonomy. Heterogeneity and diversity among microglia, the innate immune cell of the brain, force reclassifications based on structure, expression and function. Among the array of morphological variants, a recent review detailed the tissues and condition in which the rod microglia variant has been reported (Holloway *et al.*, 2019). In contrast to classical activation and phagocytic variants, rod microglia have been observed but not incorporated into unified theories of neuroinflammation. Here, we provide an update on rod microglia, an overlooked morphological variant of activated microglia identified in injured and diseased brains, in order to propose modern approaches to isolate, identify and investigate the formation and function of rod microglia.

Microglia, the resident immune cells of the central nervous system, make up 5–10% of all cells in the brain (Aguzzi *et al.*, 2013) and microglia morphology and function are linked (Shemer *et al.*, 2015; Wolf *et al.*, 2017). Functionally, microglia engage in surveillance and subsequent maintenance of healthy brain to ensure normal brain development and homeostatic function through adulthood. Maintenance includes phagocytosis during axonal and synaptic pruning to execute synaptic plasticity of neural circuitry (Schafer *et al.*, 2013), promotion of neurogenesis through cytokine secretion (Butovsky and Weiner, 2018) and neuronal support through the secretion of neurotrophic factors such as nerve growth factor and brain-derived neurotrophic factor (Elkabes *et al.*, 1996). In the surveillance/maintenance role, microglia predominantly hold a ramified (inactivated) morphology. Ramified microglia are associated with small cell bodies and highly branched processes extend radially from the cell body. Processes blanket the extracellular environment in the healthy brain and create a dynamic network of active process extension and retraction (Nimmerjahn *et al.*, 2005; Wake *et al.*, 2009; Tremblay *et al.*, 2011). In particular, microglial cell and process motility has been associated with the anaesthetized state, whereas microglia hold their position during wake activity, indicating that microglia functions depend on systemic and local signals (Liu *et al.*, 2019; Stowell *et al.*, 2019).

While microglia play a critical role in the healthy brain, they are most recognized for a role in the suppression or exacerbation of neuroinflammation through the innate immune response. In response to signals in the extracellular environment, microglia morphology changes with functional implications to follow. During pathological events, such as ageing, injury and disease, signalling molecules from neurons and other glial cells bind microglial receptors (Fields and Stevens-Graham, 2002). Upon ligand binding, ramified microglia can transition to a

deramified or activated morphology (Ziebell *et al.*, 2015). Activation (or deramification) involves the retraction and thickening of processes, swelling of the soma and expression of inflammatory surface antigens. This morphological change can be observed in single microglia or quantified in photomicrographs by skeleton analysis, which measures the number of endpoints and process length of skeletonized microglia (Morrison and Filosa, 2013; Morrison *et al.*, 2017). Activated microglia produce their own trophic factors, inflammatory cytokines and chemokines to propagate inflammatory signalling through the microglial network, and recruit additional microglia and peripheral macrophages to the local area (Raivich *et al.*, 1999; Neumann *et al.*, 2009; Kierdorf and Prinz, 2013; Ziebell *et al.*, 2015). Functionally, activated microglia phagocytose cellular debris and migrate to the signal source, likely through chemotaxis (Raivich *et al.*, 1999; Stence *et al.*, 2001; Neumann *et al.*, 2009). To migrate, activated microglia extend their remaining processes forward and maintain a tail of processes opposite to the direction of travel (Carbonell *et al.*, 2005). Activation can lead to an amoeboid morphology, the absence of processes, which is mostly non-migratory and scavenging.

At one time, microglia were believed to be one of the two phenotypes: ramified or activated. Now, microglial activation is considered to be part of a spectrum and microglia variants continue to be further defined using next-generation single-cell RNA sequencing (Keren-Shaul *et al.*, 2017; Mathys *et al.*, 2017; Tay *et al.*, 2018; Hammond *et al.*, 2019). Single-cell RNA sequencing has revealed diverse sex-, time- and region-dependent microglia populations during development (Hammond *et al.*, 2019; Li *et al.*, 2019; Masuda *et al.*, 2019), in neurodegenerative disease (Keren-Shaul *et al.*, 2017; Mathys *et al.*, 2017; Tay *et al.*, 2018; Hammond *et al.*, 2019; Masuda *et al.*, 2019; Sala Frigerio *et al.*, 2019) and other inflammatory states (Sousa *et al.*, 2018; Tay *et al.*, 2018; Hammond *et al.*, 2019; Masuda *et al.*, 2019; Sankowski et al., 2019). The complexity of microglia morphology, expression and associated function compels continuous refinement and classification of microglia variants as new information, in the context of historical observation, guides our understanding of microglia in healthy and diseased conditions of the central nervous system. From here on out, this article focuses on the rod microglia variant.

Rod microglia variant

The rod microglia variant is a distinct morphology of activated microglia for which phenotypic expression and function remain unknown. As a caveat, the presence of rod microglia in brain injury, disease and ageing currently ascribes them as a variant of activated microglia; however, new information may encourage further reclassification. Fully formed rod microglia are characterized by elongated cell bodies with processes that project primarily from the apical and basal ends (Fig. 1) (Taylor *et al.*, 2014). Rod microglia may be distinct from those microglia condensed into a cylindrical shape due to compact spaces of white matter tracts. In pathological grey matter, rod microglia have been identified by microscopic visualization in the ageing, injured and diseased brain, typically found aligned with neuronal elements that may be damaged or vulnerable to damage. Due to non-specific antigen staining (e.g., Iba1, CX3CR1 and CD68), we are unable to differentiate rod-shaped microglia from rod microglia in degenerating white matter tracts and pathological grey matter, respectively. Furthermore, rod microglia may emerge as a separate phenotype than rod-shaped microglia in cases of optic nerve injury and disease (Holloway *et al.*, 2019).

After neuropathological reports in the early 20th century that included rod microglia, little has been published on rod microglia in neurological diseases and disorders. In 2012, we rediscovered the rod microglia morphology following experimental diffuse traumatic brain injury (TBI) using midline fluid percussion injury in the adult rat (Ziebell *et al.*, 2012). Ionized calcium-binding adapter protein (Iba1, general microglia and macrophage marker) revealed the elongated cell bodies of rod microglia aligned apical to basal end in trains perpendicular to the dural surface and adjacent to apical dendrites (Fig. 1B) (Ziebell *et al.*, 2012; Taylor *et al.*, 2014; Witcher *et al.*, 2018). In reverence to the early handdrawn depictions of rod microglia from neuropathological brain, we feature contemporary light and fluorescent photomicrographs of immunolabelled rod microglia alongside modern artistic renditions in this review (Fig. 1C).

Morphologically, the length of a rod microglia is no longer than ramified microglia, but the soma is elongated and narrower with processes projecting in a cylindrical, rather than spherical, space (Taylor *et al.*, 2014). Over time post-TBI, planar processes that extended off of the lateral surface of the microglia soma retract [1 day post-injury (DPI)] to the extent where planar processes are all but absent by 7DPI (Taylor *et al.*, 2014). Processes that extend off the apical and basal ends of the soma do not necessarily reach beyond the initial space occupied by ramified microglia (Taylor *et al.*, 2014). Secondary branching of rod microglia processes is decreased compared to ramified microglia, indicating that rod microglia express only primary apical and basal processes. The largest increase in cell length: cell width ratio (overall area occupied by the soma and all processes) is observed at 7DPI when we considered the rod microglia morphology fully formed (Taylor *et al.*, 2014; Morrison *et al.*, 2017). While deramification analyses, such as skeleton analysis, are not sensitive to rod microglia detection, fractal analyses that detect cell complexity and elongation do identify rod microglia presence over time after diffuse TBI (Morrison *et al.*, 2017). Our approach to replicate the methods by last century's neuropathologists to draw rod microglia led to the observation that rod microglia and their processes twist or spiral in the cortex of the

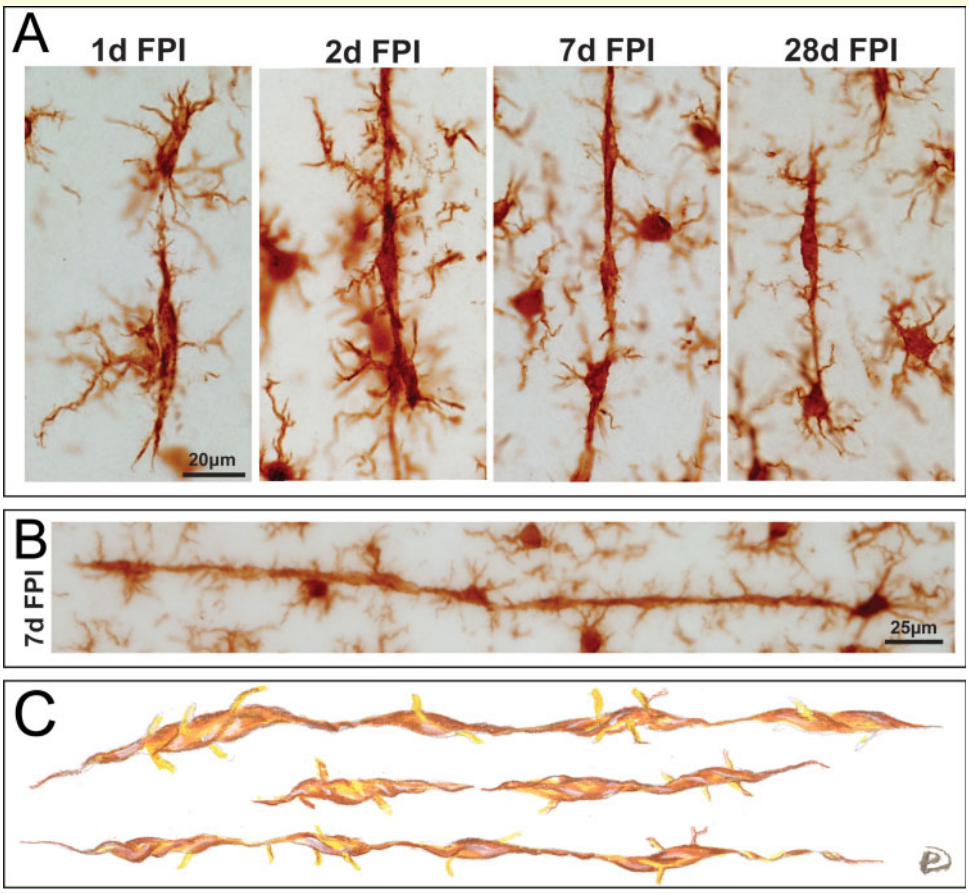


Figure 1 Rod microglia present in the somatosensory cortex after traumatic brain injury. (**A**) Photomicrographs of Iba1+ rod microglia in the somatosensory rat cortex at 1, 2, 7 and 28 days after diffuse traumatic brain injury by midline fluid percussion injury. Apical and basal processes remain, as planar processes retract with the progression of brain injury pathology. (**B**) Rod microglia align from apical to basal end to form trains perpendicular to the dural surface after fluid percussion injury. Trains are most abundant at 7 days post-injury. (**C**) Rod microglia and rod microglia trains are shown with acrylic gauche and coloured pencil.

injured rat brain (Fig. 2). Rod microglia processes resemble oscillatory movements or circumnutations of climbing plants as they grow in a spiral or twisting motion (Stolarz, 2009). We propose that the twisting permits microglia to form apical and basal processes with the structural strength necessary to penetrate through the parenchyma. We conclude that the elongation of microglia to form rod microglia is due to the retraction of planar processes, narrowing of the soma and refinement of apical and basal processes to twisted primary branches. Future investigations can observe the function of rod microglia under time-lapse microscopy.

Historical depictions of rod microglia

Franz Nissl first described rod microglia (or Stäbchenzellen) in 1899 in brains of patients diagnosed as general paresis of the insane (Nissl, 1899). Santiago Ramón y Cajal, Pío del Río Hortega, Nicolás Achúcarro and Alois Alzheimer later sketched cortical rod-like glial cells in their work on post-mortem patients with general paresis. Rod microglia became a neuropathological marker of general paresis in the early 1900s (Noda, 1921; Cajal *et al.*, 1991; Sierra *et al.*, 2016). Nissl (1899) observed and sketched rod microglia aligned with the dendrites of neighbouring neurons, with extended processes into cortical neuronal layers. Alzheimer noted an abundance of rod microglia in the outer cortical layers, aligned perpendicular to the cortical surface in clinical cases of paresis (Alzheimer, 1904; Noda, 1921). Ugo Cerletti proposed the unique shape of rod microglia to be a result of an adaption to neurons (Cerletti, 1905; Noda, 1921). Additional studies in the early 20th century reported rod microglia in the cerebral cortex in clinical general paresis, malaria, multiple sclerosis, epilepsy, Alzheimer's disease and encephalitis; however, their density and distribution were not described completely (Speilmeyer, 1906; Noda, 1921). Extending beyond the cortex, rod microglia have been reported in the hippocampus in experimental cases of rabies and cerebellar

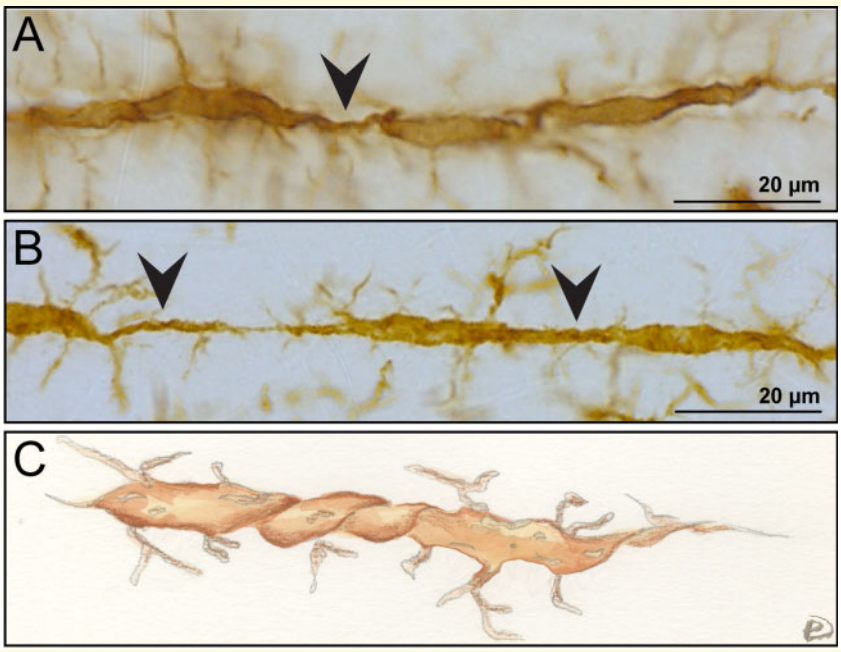


Figure 2 Rod microglia appear twisted along the primary axis of the cell. (**A**, **B**) Photomicrographs of rat somatosensory cortex after diffuse traumatic brain injury stained with Iba1 for microglia. Along the primary axis of rod microglia, the soma and processes appear twisted along the long axis of the cell (black arrow head). This observation is known as circumnutation in climbing plants. (**C**) Rod microglia circumnutation is shown with watercolour. For description, refer text.

cortex in clinical cases of paresis, cerebral atrophy and multiple sclerosis (Straeussler, 1906; Achucarro, 1909; Noda, 1921; Cajal *et al.*, 1991). Ramón y Cajal's *Degeneration and Regeneration of the Nervous System* contains descriptions of rod microglia present in the dog and cat cerebrum after fatal cerebral wounds; however, methods on the wounds, operations and brain regions containing rod microglia are not described in detail (Cajal *et al.*, 1991). The historical descriptions remain observational, under various clinical conditions, without validation of function or phenotype.

Many of the early findings described rod microglia as thin and delicate in the cerebral cortex, whereas rod microglia described in the cerebellum were noted to have wider, shorter nuclei, while still maintaining a rod-like shape (Ulrich, 1910; Noda, 1921). Perhaps, one spatio-pathological feature of rod microglia is the volume constraint placed on cell body dimensions by local cytoarchitecture. More so, activated microglia with a rod morphology in different pathological brain regions may represent the same or different phenotypes. As it was accepted practice for the time, camera lucida drawings from simple microscopes were a synthesis of many observations to illustrate the investigator's inference on rod microglia, as demonstrated with a contemporary charcoal sketch (Fig. 3). The early work reinforced rod microglia as critical to the pathological and diseased brain.

Rod microglia cell-surface expression

Contemporary studies have relied on known microglia and macrophage-specific histological markers to visualize microglia, and within those observations, rod microglia are among the other microglia morphologies evident in health, injury and disease. After experimental single diffuse TBI and colocalization of Iba1+ and CD68 (cluster of differentiation 68; upregulated with inflammation) were observed in rod microglia; however, colocalization of Iba1+ rod microglia and OX-6 (major histocompatibility complex class II marker) was intermittent (not all cells in a train of rod microglia were stained) (Ziebell *et al.*, 2012). As such, rod microglia may overlap with the tissue macrophage immunophenotype. Few other markers have delineated the rod microglia from microglia and macrophages.

As originally concluded by Nissl and Cerletti from their histological preparations, contemporary immunohistochemical labelling confirmed that trains of rod microglia align with neuronal processes (microtubule-associated protein 2, neurofilament and pan-neuronal marker) rather than astrocyte and oligodendrocyte glial cells (glial fibrillary acidic protein, 2′,3′-cyclic-nucleotide 3′-phosphodiesterase) after experimental diffuse TBI (Ziebell *et al.*,

Figure 3 Rod microglia were commonly reported in pathological tissue in the early 1900s. Rod microglia in the cerebral cortex after experimental diffuse traumatic brain injury drawn with charcoal similar to historical sketches of rod microglia by pathologists at the turn of the 20th century.

2012). Witcher *et al.* (2018) further showed Iba1+ rod microglia aligned specifically to the apical dendrites of pyramidal neurons (Thy1-YFP-H mice) and in close proximity to axotomized neurons in the somatosensory cortex after diffuse TBI, which suggested, but not concluded, that rod microglia align with unmyelinated processes after injury (Fig. 4) (Nissl, 1899). To date, no conclusive evidence exists as to whether myelin promotes or deters the formation of rod microglia. Similar findings have been observed in cases of neurodegeneration. In post-mortem cases of amyotrophic lateral sclerosis, rod microglia were aligned with degenerating apical dendrites in the motor cortex of amyotrophic lateral sclerosis cases with TDP-43 pathology (common pathology in both sporadic and familial amyotrophic lateral sclerosis cases), and in an experimental mouse model of amyotrophic lateral sclerosis with TDP-43 pathology (Jara *et al.*, 2019). In a post-mortem case of Alzheimer's disease, rod microglia were described proximal to PHF1+ (marker of hyper-phosphorylated tau aggregations) neurons, and did not colocalize with the neurons (Bachstetter *et al.*, 2015). While the exact functions for rod microglia remain unknown, these data suggest that rod microglia may repair or further breakdown damaged neurons, from a position adjacent to the dendrite.

Histological studies have added insight to the origin of rod microglia. Accumulating, but not conclusive, evidence suggests that rod microglia originate from resident CNS microglia. For example, after diffuse TBI, there is intermittent alignment between Iba1+ rod microglia and P2RY12+ (purinergic receptor, resident microglia marker) (Witcher *et al.*, 2018). However, sparse bromodeoxyuridine labelling in the formations of rod microglia train was inconclusive to conclude a proliferation origin of rod microglia (Witcher *et al.*, 2018). In post-mortem multiple sclerosis cases, subpial grey matter lesions contained Iba1+, TMEM119+ (transmembrane protein 119, resident microglia marker) and P2RY12+ rod microglia (van Wageningen *et al.*, 2019). These phenotypic markers support rod microglia as resident microglia cells, unlikely to infiltrate from the periphery, and may form by differentiation or proliferation of existing microglia.

Temporal presence of rod microglia in experimental brain injury and disease

The time course of rod microglia emergence and resolution in most diseases is poorly understood, as clinical cases afford a single histological observation at the conclusion of the disease process. Experimental diffuse TBI is a synchronous pathological event that damages, rather than destroys, tissue, and hence affording the greatest probability to observe fields of rod microglia and trains (Ziebell *et al.*, 2012; Lifshitz *et al.*, 2016). After experimental diffuse TBI, rod microglia were represented by a bimodal distribution of the total microglia population over the first week post-injury. Rod microglia accumulated at 2 and 6 h post-injury and again at 7DPI, with a decrease in the percentage population at 1DPI and 2DPI (Ziebell *et al.*, 2017). As such, rod microglia, like activated microglia, emerge and resolve dynamically with the damage and repair cascades that evolve over the first week post-injury. While rod microglia remained present at 21DPI and 28DPI, population percentages were lower than at 7DPI (Taylor *et al.*, 2014; Ziebell *et al.*, 2017). In addition to midline fluid percussion injury, rod microglia have been reported at 1- and 3DPI in lateral fluid percussion injury in cortical regions of the mouse (Mukherjee *et al.*, 2013; Harrison *et al.*, 2015; Witcher *et al.*, 2018) and in the entorhinal cortex of adult, male rats after repeated closed-head injury (projectile concussive impact model) (Madathil *et al.*, 2018). Questions remain regarding the phenotypic expression of rod microglia as activated microglia over the time course of health, injury and disease. The rod microglia may execute different functional roles based on time, location and intensity of pathophysiological signalling. Presently, investigations into

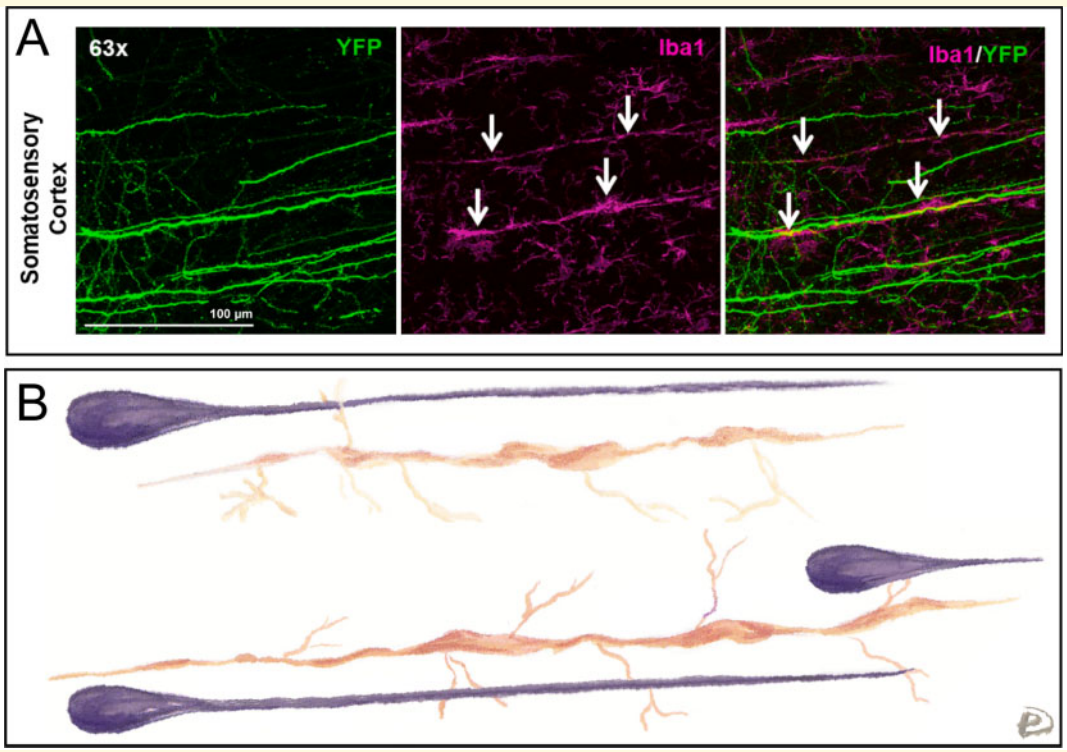


Figure 4 Rod microglia align with neuronal dendrites after traumatic brain injury. (**A**) Rod microglia (Iba1) align with apical dendrites of cortical pyramidal neurons (YFP) in Thy1-YFP-H mice at 7 days after diffuse traumatic brain injury in somatosensory cortex (Witcher *et al.* 2018). (**B**) Rod microglia (brown) are shown with their processes interacting with neuronal processes (purple) using acrylic gauche and coloured pencil.

rod microglia remain observational with quantification limited to cell number.

Rod microglia have been reported across a wide variety of preclinical models of neurological disease and injury. Rod microglia were observed in the CA1 region of the hippocampus in adult, male rats 2-week post-status epilepticus (prolonged, continuous seizures) (Wyatt-Johnson et al., 2017) and at 5-month post-status epilepticus (Upadhya *et al.*, 2019). Additionally, rod microglia were observed in the hippocampus of aged chimpanzees with Alzheimer's disease-like pathology and in an APP/PS1 mouse model of Alzheimer's disease (Edler *et al.*, 2018; Holloway *et al.*, 2020). However, rod microglia were excluded from quantitative analysis in the APP/PS1 mouse model study due to infrequent observation in histological sections (Holloway *et al.*, 2020). In an experimental model of transient ischaemic attack, rod microglia were reported in the penumbral region of the ipsilateral cortex of rats after 10 min of middle cerebral artery occlusion and 24 or 48 h of reperfusion (Zhan *et al.*, 2008). Preclinical investigations control injury severity and timing to continue to evaluate the dynamic temporal emergence of rod microglia; however, most reports indicate the presence of rod microglia with limited spatiotemporal quantification, primarily limited to the most vulnerable brain regions in the span of peak neuropathology. In 2010, Graeber (2010) concluded that rod microglia had been reported only in damaged, but structurally preserved tissue rather than necrotic regions with extensive cell death. This conclusion drives investigation away from injury foci to the penumbra and beyond. While the functions of rod microglia are unknown, their presence in preserved tissue could suggest a supportive role for damaged neurons rather than a cytotoxic role in dead tissue (Graeber, 2010). Furthermore, their temporal expression at acute and subacute time points and resolution at chronic time points after injury suggests a role in the resolution of inflammation, which would position rod microglia as a critical therapeutic target.

Rod microglia in post-mortem tissue

Prior to our findings in 2012, rod microglia were reported in the cortex of post-mortem cases of Alzheimer's disease, subacute sclerosing panencephalitis, epilepsy/encephalitis and human immunodeficiency virus (Kure *et al.*, 1990; Graeber and Mehraein, 1994; Streit and Sparks, 1997; Wierzba-Bobrowicz *et al.*, 2002; Wirenfeldt *et al.*, 2009). The emergence of rod microglia is unknown in the course of most diseases, and likely

responsive to local pathophysiology. The density and prevalence of rod microglia throughout the human brain may depend on a complex combination of factors including genetics, age, disease and life experience, which has yet to be investigated. However, there is renewed neuroimmunology interest in this morphological variant and recent post-mortem clinical work has identified rod microglia in the cortex and hippocampus of multiple diseases.

Bachstetter *et al.* (2015) described rod microglia in the hippocampus of ~60% of a case series that included cases of Alzheimer's disease, hippocampal sclerosis of ageing, Alzheimer's disease with hippocampal sclerosis of ageing, dementia with Lewy bodies and cognitively intact controls. Microglia morphology was quantified in five CA1 images and one or more rod microglia was considered a positive case. The average number of rod microglia/case ranged from 2.1 to 3.9 cells; a few cases had profuse rod microglia populations, but no other shared pathology among them. A follow-up study looked at rod microglia in two different autopsy series (Bachstetter *et al.*, 2017). Despite the common occurrence of rod microglia after experimental TBI at acute post-injury time points, no association was found between the presence of rod microglia and a self-reported history of TBI or the presence of rod microglia and Alzheimer's disease-related pathology, years to decades after injury or the onset of disease (Bachstetter *et al.*, 2017). Rod microglia were observed in both the frontal cortex and the hippocampus in almost 20% of cases aged 70+ years (Bachstetter *et al.*, 2017). These observational studies concluded that rod microglia are associated with ageing at the time of autopsy, regardless of clinical neurodegenerative diagnosis (Bachstetter *et al.*, 2015; Bachstetter *et al.*, 2017). In contrast, Woollacott *et al.* (2020) reported rod microglia in cases of Alzheimer's disease and frontotemporal lobar degeneration (FTLD), but rarely reported rod microglia in age-matched controls. Specifically, rod microglia have been reported in FTLD subtypes that include FTLD–TDP (TAR DNA-binding protein 43) with *C9orf72* mutations, FTLD–CDB (corticobasal degeneration) and FTLD–MAPT (microtuble-associated protein tau) (Mao *et al.*, 2019; Sakae *et al.*, 2019; Woollacott *et al.*, 2020). Further support for augmented pathology between diseases arrives from a report of higher rod microglia numbers in grey matter of Down syndrome Alzheimer's disease cases compared to control Alzheimer's disease cases (Martini *et al.*, 2020). Hence, rod microglia are present in the aged, injured and diseased brain, but not necessarily associated with the pathophysiology unique to each condition. Thus, development of molecular detection tools for rod microglia is critical to determine the function, presence and contribution of rod microglia to the onset, diagnosis, progression and recovery of neurodegenerative conditions.

Lee *et al.* (2017) reported rod microglia in post-mortem cases of autism spectrum disorder. Rod microglia were present in the dorsal anterior cingulate cortex of toddlers aged 2–3 years with autism spectrum disorder and controls with no autism spectrum disorder diagnosis. While the presence of rod microglia was reported in a higher percentage of autism spectrum disorder cases, no significant differences between groups were found (Lee *et al.*, 2017). One interpretation lies with the fact that the causes of death in the control group include disease or injury relevant pathology. As this study was the first to identify rod microglia in the developing toddler brain, many questions emerge about the morphologic, phenotypic and functional similarities and differences between rod microglia in the ageing versus developing brain. Since rod microglia are present in development, disease, pathology, degeneration, repair and possibly regeneration, an avenue for research opens to identify their functional role and develop tools to monitor change.

Reports of rod microglia in clinical and preclinical disease models continue to be published, and yet little is known about this microglia variant. Studies thus far have been limited, if not restricted, to histological measures. Most often, photomicrographs are acquired after immunohistochemistry, microglia morphologies are counted, or the presence of rod microglia is simply reported; the field lacks molecular and genetic tools to advance the understanding of rod microglia in the context of neuroinflammation. In the extant literature, discrepancies exist between the presence of rod microglia in experimental models of injury or disease and post-mortem cases. For example, rod microglia are abundant in the brain-injured rodent and rarely reported in Alzheimer's disease mouse models. In contrast, rod microglia have not been reported commonly in post-mortem cases of TBI, whereas repeated reports in post-mortem cases of Alzheimer's disease. To reconcile these observations, first, rod microglia observed across species may be similar, because equivalent histological markers (e.g., Iba1, CD68 and CX3CR1) identify epitopes on rod microglia. Next, the majority (if not all) of experimental studies involved young rodents, for whom there is no age-related pathophysiology to induce rod microglia. For the majority of clinical cases, the individuals are aged, in which pre-existing conditions, tissue procurement or natural ageing may contribute to the presence of rod microglia. Even in conditions of sudden death, an underlying pathology may induce rod microglia, which is dissimilar from the naïve rodent brain. Finally, accumulated evidence indicates that rod microglia recede, perhaps with the resolution of inflammation, and hence evading detection at the time of autopsy. Therefore, the comparisons of rod microglia between human and animal are important, and yet remain unequivocal; future studies can better align preclinical and human studies of rod microglia.

Future studies of rod microglia

Mechanisms of the formation and function of rod microglia need further investigation. The contemporary

histological methods only identify rod microglia with elongated cell bodies among fields of other microglial morphologies. This literature has applied light, confocal and multi-photon microscopy to observe, measure and count rod microglia. At the electron microscopic level, rod microglia could be interrogated with regard to cytoskeletal structure, association with neurons, proximity to myelin, coupling between consecutive cells in a train and intracellular organelles. This observation may require 3D-reconstruction with serial block face-scanning electron microscopy. However, in most experimental and clinical cases, the rod microglia intermittent presentation, small size and inability to uniquely mark the particular variant make electron microscopy dependent on new cellular markers.

Post-mortem histology cannot address the origin of rod microglia, where initial bromodeoxyuridine studies were inconclusive (Witcher *et al.*, 2018). Existing origin and fate-mapping technologies, however, could give insight to the formation of rod microglia from ramified or activated microglia upon injury or disease progression (Ginhoux *et al.*, 2010). The $Cx3cr1^{creER}R26R^{Confetti}$ ('Microfetti') mouse model allows multi-colour fluorescent fate mapping and could provide additional information about the formation of rod microglia train (Tay *et al.*, 2017). The formation of trains of rod microglia entices investigations beyond photomicrographs, where time-lapse *in vivo* imaging techniques (multi-photon microscopy, miniscopes) of transgenic and reporter mouse models can track the formation of rod microglia by migration, proliferation or differentiation (Taylor *et al.*, 2014). Multi-photon imaging can discriminate microglia, their morphology and local cytoarchitecture, albeit under anaesthesia or restraint. The advances in three-photon imaging allow increased depth of imaging through a thinned skull in awake mice, which is less invasive than a traditional cranial window implant (Wang *et al.*, 2018). Miniscopes, miniature fluorescent microscopes, are a new and exciting way to study rod microglia *in vivo* without the use of anaesthesia or restraint although a cranial implant is required (Ghosh *et al.*, 2011). The combination of multi-photon or miniscope imaging with implanted microprisms would allow *in vivo* imaging of multiple cortical layers at once and could provide additional insight on the formation and clearance of rod microglia (Andermann *et al.*, 2013). The invasive procedure of cranial window and micro-prism implants, however, are likely to cause neuroinflammation and subsequent microglia activation, possibly even the induction of rod microglia.

Histological and imaging methods still fall short of defining the function of rod microglia in health and disease. New molecular detection tools are necessary to distinguish and isolate the rod morphology variant from other microglial morphologies, and then elucidate the function of this cell type in the aged, injured and diseased brain. Genomic, proteomic, metabolomic and lipidomic profiles of rod microglia would define the variant comprehensively. Pathway analyses may identify cytoskeleton (circumnutation) and growth factors that distinguish rod microglia structure and cell signalling pathways from other variants in the heterogeneity among microglia. Single-cell sequencing of microglia has revealed the diversity of microglia in the developing, healthy and injured or diseased brain. However, recent single-cell sequencing studies have used whole-brain homogenates or known myeloid cell markers to pre-sort cell populations (Masuda *et al.*, 2019). These approaches do not account for spatial or temporal changes in microglia and are unable to incorporate rod microglia into the interpretation, because molecular expression profiles for rod microglia are non-existent. Yet, the recent development of spatial transcriptomics could be applied to the punctate and sporadic observations of rod microglia to uncover their gene expression. Spatial transcriptomics allows gene expression to be mapped onto visualized intact histological sections (Ståhl *et al.*, 2016; Chen *et al.*, 2020). Additionally, laser capture microdissection affords the opportunity to harvest cells based on visualized morphology. Downstream applications of laser capture microdissection include multiomic analyses of isolated rod microglia and as a substrate to develop molecular detection tools (e.g. antibodies, oligonucleotides and receptor agonists/antagonists) of rod microglia. Single-cell approaches could suggest rod microglia function and validate whether rod microglia include subtypes within this variant across the time courses of various diseases.

Biomarker discovery techniques beyond omics-based approaches can identify unique rod microglia cell-surface markers and develop rod microglia-specific antibodies. Rod microglia antibodies would expand investigational techniques beyond immunohistochemistry to include Western blot, ELISA, immunoprecipitation, flow cytometry and FACS. Once cell-surface markers unique to rod microglia are identified, translational potential lies in non-invasive imaging (DTI, MRI and PET), where fields of rod microglia in the cortex may reduce the anisotropy by transforming ramified microglia to rod microglia or trains of rod microglia. Unique receptor targets on rod microglia could be leveraged to activate or inhibit rod microglia appearance, clearance or activation. With a rod microglia molecular profile, we can pursue individual or sets of biomarkers to track disease state based on the presence of rod microglia, develop rod microglia-specific antibodies, transgenic mice, as well as develop pharmacological agents to promote or deter their formation.

The discussion thus far has focussed on *in vivo* studies of rod microglia, yet *in vitro* investigations must be considered. A full review of *in vitro* evidence for rod microglia is presented by Au and Ma (2017). In brief, cultured microglia took on a rod microglia morphology after migrating to the site of a physical scratch in poly-D-lysine and laminin-coated surface (Tam and Ma, 2014; Tam *et al.*, 2016). The formation of rod microglia train began at 1 day *in vitro* and remained until six 1 day *in vitro*

and rod microglia were highly proliferative compared to amoeboid microglia (Tam and Ma, 2014; Tam *et al.*, 2016). On a laminin-free scratched surface, however, rod microglia trains receded by three 1 day *in vitro*, suggesting laminin to play a role in the formation of rod microglia trains (Tam *et al.*, 2016). Cultured rod microglia showed immunoreactivity to proliferating cell nuclear antigen and lower levels of pro-inflammatory cytokines than cultured amoeboid microglia (Tam and Ma, 2014). However, after exposure to lipopolysaccharide, rod microglia took on an amoeboid morphology and upregulated pro-inflammatory markers (Tam and Ma, 2014), which indicated that rod microglia remain highly adaptable and rapidly respond to stimuli in their microenvironment. *In vitro* approaches provide insight to signals that could promote/deter the formation of rod microglia; however, it remains unclear whether rod microglia are a single manifestation or represent an array of phenotypic expression.

Conclusion

In the end, rod microglia are positioned at critical spatial and temporal junctions in disease progression, by which their detection, inhibition and/or activation could improve disease understanding and outcome. Until molecular profiles and detection tools can distinguish rod microglia from other morphologies, their role in the natural course of disease and impact on recovery remains a topic of conjecture. Further investigation is at a standstill until new molecular tools can interrogate rod microglia. As we have shown, diffuse TBI provides an experimental model to reproducibly and synchronously induce vast fields of rod microglia to conduct investigations on molecular, structural and functional analysis of rod microglia in the presence of neurological injury. The near-term future holds promise to crack the code on the contribution of rod microglia to the neuroinflammation framework.

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Competing interests

The authors report no competing interests.

Data availability

As this is a review article, no primary data were generated. Interested parties are encouraged to email the authors directly for additional information.

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