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Abstract

Microglia are the primary immune cells of the central nervous system. However, recent data indicate that microglia also contribute to diverse physiological and pathophysiological processes that extend beyond immune-related functions and there is a growing interest to understand the mechanisms through which microglia interact with other cells in the brain. In particular, the molecular processes that contribute to microglia-neuron communication in the healthy brain and their role in common brain diseases have been intensively studied during the last decade. In line with this, fate-mapping studies, genetic models and novel pharmacological approaches have revealed the origin of microglial progenitors, demonstrated the role of self-maintaining microglial populations during brain development or in adulthood, and identified the unexpectedly long lifespan of microglia that may profoundly change our view about senescence and age-related human diseases. Despite the exponentially increasing knowledge about microglia, the role of these cells in health and disease is still extremely controversial and the precise molecular targets for intervention are not well defined. This is in part due to the lack of microglia-specific manipulation approaches until very recently and to the high level of complexity of the interactions between microglia and other cells in the brain that occur at different temporal and spatial scales. In this review, we briefly summarize the known physiological roles of microglia-neuron interactions in brain homeostasis and attempt to outline some major directions and challenges of future microglia research.

Introduction

To understand the physiology and diseases of the nervous system, the great majority of studies have focused on neuronal functions and interactions for several decades. However, recent research has highlighted the crucial role for glial cells in the maintenance of neuronal integrity from development to aging, whilst the contribution of glial function (or dysfunction) to common diseases of the brain has become increasingly accepted. The recognition that microglia are not only the main immunocompetent cells of the brain but represent a multifunctional and essential element of the mammalian nervous system with tempting opportunities for cell-specific manipulation has opened new avenues in neuroscience research. Given the emerging role of microglia in diverse physiological and pathophysiological processes, understanding microglial function is now considered as one of the most interesting questions for the upcoming years in neuroscience (Südhof, 2017). Recent technological advances now allow some forms of microglia-specific manipulation, which used to be extremely challenging due to the lack of microglia-specific markers that discriminate them from other tissue macrophages and because self-maintaining microglia populations are mostly protected by the blood brain barrier, making targeted pharmacological manipulation difficult. Single-cell transcriptomic- and fate-mapping studies shed light on the origin of microglia from primitive yolk sac macrophages and on their remarkable adaptation to the nervous tissue during early brain development (Ginhoux et al., 2010; Kierdorf et al., 2013). These studies have also revealed that microglial phenotypes appear to be largely influenced by the brain microenvironment, including the region-specific expression of microglial genes as well as the marked changes that occur in microglial transcriptomes by age in adults (De Biase et al., 2017; Olah et al., 2018). Nevertheless, in spite of the recent revolution in transcriptomics, genomics and modern microscopy technologies allowing the investigation of microglial function at different temporal and spatial scales in health and disease, several important questions remained unanswered. In particular, the mechanisms through which microglial actions may influence the maintenance, functioning and dysfunction of neurons from

individual cells to large networks and how exactly these processes could contribute to common human diseases, are far from being understood.

Evolutionary role of glia – insights into the possible origin of microglia-neuron interactions

Due to the high degree of specialization, complexity and their substantial isolation from the immune system and the systemic circulation, neuronal circuits in the central nervous system require continuous support from neuroglial cells. These cells maintain homeostasis by aiding the growth and re-structuring of neuronal processes, myelinate axons, maintain metabolic balance, adjust brain perfusion to local demand, and eliminate cell debris and non-required synapses to support the normal functioning of complex neuronal networks (Araque and Navarrete, 2010; Attwell et al., 2010; Jäkel and Dimou, 2017). The evolution of glial cells has been associated with their specialization and an increasing glia to neuron ratio, which coincided with the growing complexity of the nervous system, particularly, the brain (Reichenbach, 1989). Primordial glial cells appeared early in evolution. Proto-astrocytes provided metabolic support to neurons (Bacaj et al., 2008), whilst the formation of the blood-brain barrier (BBB) by astrocytes isolated the nervous tissue from the rest of the body (Obermeier et al., 2016). At a later stage of evolution, formation of the BBB emerged as a major function of endothelial cells (Abbott, 2005), whereas astroglial BBB is present in most invertebrates including crustaceans, insects and cephalopods (Abbott and Pichon, 1987). While vertebrates possess a well-developed tight BBB, some vestiges of primitive glial barriers remain in the mammalian CNS in regions lacking vascular BBB such as at the areas of the choroid plexus, pituicytes in the hypothalamic–hypophyseal system or the circumventricular organs (Obermeier et al., 2016), which are also sites known for intense neuroendocrine and neuroimmune interactions between the periphery and the brain. The increase in the size of the organisms required a faster communication between the periphery and the central nervous system. The appearance of oligodendrocytes and Schwann cells provided higher velocity of impulse conduction through myelination in ancestral

invertebrates. Evolutionary emergence of microglia was linked with the appearance of compact neuronal masses and the increased demand of specialized phagocytic and immune functions, including mechanisms of neural protection (Sherwood et al., 2006; Mota and Herculano-Houzel, 2014). In fact, microglia-like cells, which can be labelled by weak silver carbonate, a classical stain for vertebrate microglia, contribute to the repair of nerve cells in invertebrates (Morgese et al., 1983). In the injured leech nervous system, small amoeboid microglial cells migrate to the lesion site and display phagocytic activity (Morgese et al., 1983), produce antimicrobial peptides in response to infectious attack (Schikorski et al., 2008) and control regenerative processes (Napoli and Neumann, 2010). Microglial cells with migratory activity have also been described in snails and insects (Sonetti et al., 1994). Thus, the open circulatory system of invertebrates lent itself to the origin of neuroimmune cooperative events, whereby macrophage-like immune cells gained the capabilities for penetration and residence within "privileged" neuronal compartments (Stefano and Krearn, 2015).

From the neuro-immune interactions' point of view, a large set of evolutionary conserved molecules are shared by the immune and the nervous systems including complex signalling systems with overlapping ligands and receptors (Kioussis and Pachnis, 2009). In particular, various macrophage/microglial-derived secretory products are capable of altering neuronal activity, which is associated with the release of neuron-derived mediators that can shape immune cell function. For example, interleukin-1β (IL-1β) released by microglia/macrophages alters neuronal activity and the release of dopamine and norepinephrine, whilst the functional involvement of opioid and monoaminergic mechanisms in regulation of cytokine production has been widely documented (Elenkov et al., 2000; Finley et al., 2008). Both IL-1β and monoamines are produced in response to neural trauma and circulating IL-1β and norepinephrine exert multiple effects on different cells and organs in the body, including the vasculature, inflammatory cells and neurons. Many of these shared signalling mechanisms are functionally linked with diverse forms of injury or neuronal death in mammals or the development of neurodegenerative- and psychiatric diseases in humans (Elenkov et

al., 2000; Allan et al., 2005; Denes et al., 2011; Mélik Parsadaniantz et al., 2015; Stefano and Krearn, 2015). Some of these signalling pathways, have been observed in invertebrates and vertebrates during 500 million years of evolution, such as iNOS (Peruzzi et al., 2004), anandamide (Stefano et al., 1996) or 17β-estradiol (Stefano et al., 2003). Throughout this evolutionary process, the complexity of neuronal networks grew in an astonishing amount. Knowing this, it is not surprising, that along this shared journey microglia stepped up from the "silent guardian" bystander position of early parenchymal macrophages, and became highly complex homeostatic cells of the brain.

Evidence for the role of microglia-neuron interactions during brain development

Microglial cells are of myeloid origin and derive from progenitors generated in the yolk sac. Unlike ectodermal macroglial cells that populate the CNS after the appearance of neurons, microglial progenitors are already present around the neural tube as early as E9 in mice, and from the 5th gestational week in humans (Ginhoux et al., 2010; Verney et al., 2010). Thus, microglia are in ideal position to regulate neuro-, glio- and angiogenesis. After their entry, microglia migrate tangentially and then radially in the developing nerve tissue and undergo intense proliferation before reaching a close to the final density in the early postnatal period in mice (Navascués et al., 2000; Monier et al., 2007; Verney et al., 2010; Nikodemova et al., 2015). Then, the microglial population is maintained by balanced proliferation and apoptosis throughout life, without the contribution of circulating immune cells (Askew et al., 2017; Huang et al., 2018). Parenchymal microglia reside in the CNS together with self-maintaining populations of perivascular and meningeal macrophages, as well as choroid plexus macrophages that are replaced by blood-borne cells to some extent (Goldmann et al., 2016; Mrdjen et al., 2018). Due to the partially common origin of microglia and some other tissue macrophages, it is difficult to define the precise contribution of microglia to CNS development. Accumulating evidence from fate-mapping and genomic studies indicates deficient brain development to various degrees when microglial functionality is impaired. In mice lacking Pu.1, a regulator of myeloid

differentiation, monocytes and macrophages are absent throughout the body, including all CNS macrophages and microglia (Schulz et al., 2012; Kierdorf et al., 2013; Goldmann et al., 2016). Pu.1-null mice are born with severe immune deficits and die within a short period of time (McKercher et al., 1996). However, they show decreased neuronal proliferation rate and lower macrogliogenesis, both of which can be rescued with exogenous microglia (Antony et al., 2011). CSF-1 receptor (CSF1R) is solely expressed by microglia in the CNS (Erblich et al., 2011) and CSF1R-mediated signalling is required for the maintenance of microglial populations (Gómez-Nicola et al., 2013; Olmos-Alonso et al., 2016). Models targeting CSF1 or CSF1R impact on numbers and functionality of microglia, circulating- or tissue macrophages to different extent. CSF1R null mutations completely eliminate microglia, whereas CSF1 elimination causes only a reduction in microglial numbers (Ginhoux et al., 2010; Erblich et al., 2011). Mice with disrupted CSF1-CSF1R axis suffer from multiple morbidities, with pronounced alterations in the CNS: CSF1R deficient mice have smaller brains, altered density of neurons and macroglial cells in the cortex, atrophy in the olfactory bulb, smaller ventricles and disturbed connection of hemispheres (Dai et al., 2002; Erblich et al., 2011; Nandi et al., 2012). Another ligand of CSF1R is IL-34, the absence of which also results in decreased microglial population in a region-specific manner (Greter et al., 2012; Wang et al., 2012). Except for microglia and Langerhans cells, IL-34 deletion seems to have a minor effect on the number of other tissue macrophages and does not result in as severe phenotypes as seen after CSF1R deletion.

Importantly, the developmental deficits associated with the absence or dysfunction of microglia are probably due to complex interactions between microglia, endothelial cells, astrocytes, neurons and tentatively other cell types. Deficiency of the TGFβ1/TGFbR2 pathway in microglia or disrupted PU.1 or SDF-1 signalling in microglia/macrophages is associated with impaired embryonic angiogenesis and BBB formation (Fantin et al., 2010; Ginhoux et al., 2010; Butovsky et al., 2014), which also have a major impact on the formation of neuronal networks in the brain and profoundly alter outcome after brain injury. Microglia also contribute to embryonic neurogenesis (Walton et al., 2006; Cunningham et al., 2013), to the migration and differentiation of neural precursor cells (Aarum et al.,

2003; Antony et al., 2011), promote neuronal survival (Morgan et al., 2004; Ueno et al., 2013), axon growth (Pont-Lezica et al., 2014; Squarzoni et al., 2014), synapse formation (Lim et al., 2013; Parkhurst et al., 2013; Miyamoto et al., 2016), activity-dependent synapse elimination (Schafer et al., 2012), synapse and spine remodelling (Lim et al., 2013; Parkhurst et al., 2013; Weinhard et al., 2018), positioning of interneurons in the forebrain (Squarzoni et al., 2014) and the formation of cortical layers (Ueno et al., 2013). Developmental synapse elimination by microglia is critical for proper brain maturation (Paolicelli et al., 2011). The absence or malfunction of microglia during development results in dysfunctional neuronal networks, defasciculation of axons, altered spine density and synapse numbers (Erblich et al., 2011; Paolicelli et al., 2011; Nandi et al., 2012; Pont-Lezica et al., 2014; Zhan et al., 2014; Miyamoto et al., 2016). The role of microglia during brain development and after neonatal brain injury is discussed in detail in a recent review article (Mallard et al., 2018).

Microglia-neuron interactions from synapses to neuronal networks

In spite of the large number of studies investigating basic cellular and molecular mechanisms of microglia-neuron interactions, knowledge about the precise sites of action and the integration of signals generated at the level of individual synapses or complex neuronal networks is limited. However, evidence from different studies indicates that although microglia are seemingly dispensable for the normal functioning of basic neurophysiological processes in the adult brain, the absence or dysfunction of microglia has a significant impact on activity-dependent physiological processes such as memory formation, similarly to that seen after brain injury or neurodegeneration (Perry et al., 2010; Parkhurst et al., 2013; Salter and Stevens, 2017). Selective, temporal elimination of microglia during adulthood by the CSF1 receptor (CSF1R) antagonists PLX3397 or PLX5622 is not associated with illness or robust behavioural alterations, it has no major impact on the number and functioning of neurons, endothelial cells, pericytes or glial cells and does not compromise the

integrity of the BBB (Elmore et al., 2014; Szalay et al., 2016; Huang et al., 2018). However, to understand the role of microglia in the adult brain under physiological conditions, further depletion- and selective microglia-manipulation studies are needed. For example, it is currently unclear, how long-term (several months long) elimination of microglia would influence physiological processes, aging or complex behavioural paradigms associated with learning, adaptation or formation of long-term memory. Furthermore, after experimental stroke and other forms of acute/subacute neuronal injury induced by diphtheria toxin in CaM/Tet-DT_A mice or by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) treatment in a mouse PD model, neuronal loss was markedly augmented (Rice et al., 2015; Szalay et al., 2016; Yang et al., 2018). On the other hand, improved cognitive performance, reduced neuronal loss and improved synaptic reorganisation have been reported following cranial irradiation, in mouse models of Alzheimer's disease or during recovery from diphtheria toxin-induced acute neuronal injury in the absence of functional microglia (Dagher et al., 2015; Rice et al., 2015; Acharya et al., 2016; Spangenberg et al., 2016). Markedly different outcomes after different forms of brain injury in microglia-depleted mice suggest that an in depth mechanistic insight into the main processes regulating microglia-neuron communication at different temporal and spatial scales will be essential to identify the most appropriate therapeutic targets in different forms of brain injury and neurodegeneration.

Microglia-neuron interactions at synaptic/dendritic/axonal level

Perhaps the best documented microglia-neuron interactions take place at the level of synapses. During development, vast amounts of synapses are generated during an intensive period of synaptogenesis. Following this, the refinement of neuronal networks is brought about by synaptic pruning, a process governing activity-dependent elimination of redundant synapses, in which complement-dependent microglial phagocytosis is considered to play a key role (Schafer et al., 2012; Jung and Chung, 2018). Unwanted developing synapses in the reticulogeniculate system are tagged

with the complement protein C1q (Stevens et al., 2007) and phagocytosed by microglia (Schafer et al., 2012). C1q-knockout mice have been shown to have an increased number of axonal boutons of pyramidal cells in cortical layer V and these animals were prone to epileptogenesis (Chu et al., 2010). The fractalkine (CX3CL1) - fractalkine receptor (CX3CR1) pathway has also been implicated in synaptic pruning (Kettenmann et al., 2013). CX3CR1-deficiency leads to a reduction of microglial surveillance and impaired development of hippocampal and thalamocortical synaptic circuitries (Hoshiko et al., 2012; Pagani et al., 2015). Microglial phagocytosis and interactions with neuronal elements also determine the fate of individual synapses in the adult brain (Tremblay et al., 2010). Furthermore, the dysregulation of these actions is thought to contribute to synapse loss in neurodegenerative processes, such as seen in Alzheimer's disease (Hong et al., 2016). Synapse elimination in the neuropil can be distinguished from a related process, synaptic stripping, which is characterized by the removal of somatic synapses from neuronal perikarya (Trapp et al., 2007; Yamada et al., 2008). Recently, it has been demonstrated that synapse elimination is the consequence of microglial phagocytosis or trogocytosis of presynaptic elements (Weinhard et al., 2018). Interestingly, microglia are not only responsible for the elimination of synapses, but microglial contacts have also been shown to induce synapse formation in the developing somatosensory cortex, while genetic ablation of microglia altered spine density, reduced functional excitatory synapses and the relative connectivity (Bessis et al., 2007; Miyamoto et al., 2016). Interleukin-10, which is produced by both microglia and astrocytes in the brain (Lobo-Silva et al., 2016) also appears to be involved in microglia-dependent synaptogenesis (Lim et al., 2013). Microglia also regulate synaptic plasticity (Rogers et al., 2011; Pascual et al., 2012; Pfeiffer et al., 2016; Sipe et al., 2016). The molecular mechanisms involved in this process include the modulation of the NMDA receptor glycine binding site (Hayashi et al., 2006), signalling by fractalkine through its receptor (Paolicelli et al., 2011; Rogers et al., 2011; Hoshiko et al., 2012), modulation of Cl- gradient in neurons through microglial BDNF release (Coull et al., 2005), purinergic signalling (Tsuda et al., 2010) and glial TNF-α release (Stellwagen and Malenka, 2006) among others. During hippocampal LTP, microglia alter their

morphological dynamics by increasing the number of their processes and by prolonging their physical contacts with dendritic spines, and these effects are absent in the presence of an NMDA receptor antagonist (Pfeiffer et al., 2016). Furthermore, after transient cerebral ischemia, the duration of microglia–synapse contacts are markedly prolonged (ca. 1 h) and are occasionally followed by the disappearance of the presynaptic bouton (Wake et al., 2009). Synaptic alterations have also been shown to occur in response to the loss-of-function mutation of DAP12, a transmembrane protein associated with microglial TREM2, leading to enhanced hippocampal LTP and changes in glutamatergic transmission (Roumier et al., 2004, 2008). Interestingly, mutations in the genes encoding microglial DAP12 or TREM2 are responsible for the development of Nasu-Hakola disease, which is characterized by progressive presenile dementia associated with bone cysts (Hakola and Iivanainen, 1973; Paloneva et al., 2000). Microglia also promote the formation of proper functioning networks by gathering around growing axons, and also contribute to fiber reorganization during development (Dalmau et al., 1998). It has been described that microglia accumulate at decision points along axonal tracts, and modulate axonal growth and pathfinding with the CX3CL1-CX3CR1-, complement- and DAP12 pathways involved (Squarzoni et al., 2014). Other studies have confirmed that the proper formation of corpus callosum and axonal fasciculation depend on the presence of microglia (Pont-Lezica et al., 2014). Overall, proper microglial function together with both direct and indirect interactions with different neuronal subcompartments are instrumental for appropriate development, and also contribute to homeostasis of neuronal networks in adults.

Microglia-neuron interactions at the cellular level

Microglia play a major role in patterning the CNS during development by regulating neuronal proliferation and survival (Morgan et al., 2004; Ueno et al., 2013). Evidence from many different brain regions show a global ability for microglia to instruct programmed cell death (reviewed in Bilimoria and Stevens, 2015). Various signalling systems have been functionally associated with

processes: for example, neuronal-derived lysophosphatidylcholine (Lauber et al., 2003), DNA (Cox et al., 2015) or nucleotides (Elliott et al., 2009) can serve as “find me” signals for microglia, while fractalkine (Truman et al., 2008) and sphingosine-1-phosphate (Gude et al., 2008) also attract microglia/macrophages to dying cells. Outcomes in this context after the manipulation of microglial activity and responses are also largely model-dependent and complex. For example, the absence of microglial CSF1R can lead to increased neuronal density (Erblich et al., 2011), while others have found increased number of neurons undergoing apoptosis in CX3CR1 KO mice (Ueno et al., 2013), suggesting the importance of several different microglial signalling pathways in neuronal proliferation and survival. Meanwhile, microglia are also key players in the migration and differentiation of neural precursor cells via releasing soluble factors (Aarum et al., 2003). Altogether these data suggest a balancing role of microglia in the brain, which appears to be increasingly important during the early stages of brain development.

The hippocampal formation is indispensable for learning and memory, whilst neurogenesis occurs during adulthood in the dentate gyrus. Microglia-mediated actions contribute to both learning and neurogenesis. The absence of CX3CR1 leads to reduced adult neurogenesis in the dentate gyrus (De Lucia et al., 2016; Sellner et al., 2016), while exogenous fractalkine reverts age-dependent decrease of hippocampal neurogenesis (Bachstetter et al., 2011). Disrupted CX3CR1 signalling results in deficits in adult neurogenesis-linked functions, like spatial and motor learning (Rogers et al., 2011), or exercise induced increase in neuronal density (Vukovic et al., 2012). The fact that the absence of fractalkine did not reproduce the phenotype of CX3CR1 KO animals (Sellner et al., 2016) proposes the role of other ligands of CX3CR1, possibly IL-34 (Wang et al., 2012). Microglial IGF-1 also seems to promote an increase in neuronal density of the dentate gyrus, suggesting the role of this growth factor in neurogenesis and neuronal survival (Kohman et al., 2012; Ueno et al., 2013). The integration of newborn cells into functioning circuits is also supported by microglia, as they clear the region from the residues of apoptotic cells (Sierra et al., 2010). The CD200-CD200R signalling axis with CD200 expressed on neurons and its receptor on microglia, also contributes to the regulation of

microglial activity and their interactions with neurons. Retinal microglia in CD200 KO mice have been shown to display decreased ramification and increased levels of CD11b and CD45, which is associated with augmented inflammatory responses in different experimental models of neuropathology (reviewed extensively in Walker and Lue, 2013).

Microglial processes have been reported to contact different areas of neurons during surveillance activity (Nimmerjahn et al., 2005), while in zebrafish larvae it was proposed that contacts with perikarya were established in more active neurons resulting in the decay of their activity (Li et al., 2012). The activity-dependence of somatic microglia contacts was further strengthened by observations under hypoxic circumstances, when microglial processes gathered around neurons with elevated intracellular calcium levels (Szalay et al., 2016). Furthermore, there is also evidence for a direct contact between microglia and axon initial segment of cortical neurons in different species through which microglia exert a neuroprotective effect following neuronal hyperactivity (Baalman et al., 2015; Kato et al., 2016). The molecular mechanisms involved in microglia-mediated control of neuronal activity are not well understood. Neuronal ATP and glutamate have been shown to attract microglial processes (Kato et al., 2016) that may allow microglia to sense changes in neuronal activity. ATP during microglia-neuron interaction has been shown to be released by pannexin hemichannels or by via volume-activated anion channels (Dahl, 2015; Kato et al., 2016). However, recent data show that no ambient purinergic signalling is required to maintain microglial ramification and surveillance whilst the role of P2Y12 receptors may also be dispensable for these actions (Madry et al., 2018a, 2018b). GABA is also known to influence microglial process motility, and selectively blocking GABA-ergic neurotransmission increases the volume of tissue sampled by individual microglial cells (Nimmerjahn et al., 2005). In turn, mediators released from microglia including proinflammatory cytokines such as IL-1β and TNFα, have been shown to shape neuronal activity and also markedly contribute to neuronal injury (Coull et al., 2005; Nygård et al., 2009; Hewett et al., 2012; Pascual et al., 2012; Béchade et al., 2013; Kato et al., 2016; Cantaut-Belarif et al., 2017). In spite of the extensive knowledge available about the molecular pathways regulating microglia-

neuron communication (see also Eyo and Wu, 2013; Tsuda and Inoue, 2016 for further details), how these different signals are integrated in individual microglial cells that contact 5-12 neurons in average and nearby capillaries at the same time (Szalay et al., 2016) and at the level of large neuronal networks, is currently unclear (See also Box1.).

Microglia-neuron interactions at the network level

The interactions between microglia and neurons are likely to be present at different organizational levels. Unlike astrocytes, microglial cells are not interconnected via gap junctions in mammals with individual microglia occupying a given volume of tissue in the brain (Nimmerjahn et al., 2005). It is currently not well understood whether and how coordinated actions of several microglial cells could shape the connectivity or activity of complex neuronal networks. The network-level manifestations of these interplays are emerging as the complex sum of direct cell-to-cell interactions supplemented by more diffuse paracrine or volume-transmission effects. Although the clear dissection between cellular- and network-level mechanisms of microglia-neuron interactions is currently not possible, increasing number of studies has provided evidence for the network-level effects of microglial functions within neuronal systems. Local hippocampal and whole-brain depletion of microglia led to decreased spatial learning performance (Torres et al., 2016), while the absence of microglia resulted in dysregulated neuronal network activity patterns in the injured brain (Szalay et al., 2016). fMRI measurements of CX3CR1-knockout mice revealed a decreased functional connectivity between prefrontal cortical and hippocampal areas (Zhan et al., 2014). Microglia-dependent BDNF-TrkB signalling controls overall neuronal excitability via the regulation of inhibitory transmission in neuronal networks(Tanaka et al., 1997; Frerking et al., 1998; Baldelli et al., 2005; reviewed extensively by Ferrini and De Koninck, 2013), while the absence of microglia or BDNF alone decreased learning coupled structural plasticity (Parkhurst et al., 2013). Neuroinflammation in general is thought to be involved in the generation of neuronal hyperexcitability (reviewed in

Vezzani and Viviani, 2015). Purinergic signalling, the glutamatergic pathway and Kv7/M-channels are also contributors to this process (Henshall and Engel, 2015; Tzour et al., 2017). Thus, these results highlight the potential importance of microglial functions in regulating higher-level properties of neuronal networks. Some important molecular pathways implicated in microglia-neuron interactions are summarized on Fig. 1.

Multi-cellular interactions

One major future challenge of microglia research is to resolve the mechanism of highly complex multi-level interactions, in which the communication among several different brain cell populations is integrated or summed to generate given physiological or pathophysiological readouts. Some of these complex multi-step interactions have already been identified. For example, the developing vasculature serves as a main support and provides critical guidance clues for the migration of GABAergic neurons (Won et al., 2013). Disturbances of microglial functions may lead to disrupted vasculature development, as microglial signals can trigger vessel sprouting (Rymo et al., 2011), and this way the proper migration and positioning of GABAergic neurons will be affected negatively. On the other hand, the direct trophic effects of microglia on GABAergic cells via the BDNF-TRKb axis provide another level of complexity during development (Baldelli et al., 2002; Mizoguchi et al., 2003). The exact dissection of these parallel actions will require carefully designed experiments with multiple points of selective interventions. Another example for multi-step cellular interactions is astrocyte-microglia communication, which is evidenced by some interesting, but controversial findings. Activated microglia have been shown to induce the neurotoxic transformation of astrocytes (Liddelow et al., 2017), while others found that microglia can induce the transformation of astrocytes into a neuroprotective phenotype after brain injury (Shinozaki et al., 2017). The involvement of a purinergic-glutamatergic cascade between microglia, astrocytes and neurons has also been implicated (Tzour et al., 2017). Recently, astrocyte-derived interleukin-33 (IL-33) has been

described to play an important role in microglial synaptic pruning (Vainchtein et al., 2018). Furthermore, the instrumental role of microglial functions via their interactions with different cell types during development is also known (Mosser et al., 2017; Mallard et al., 2018), as discussed above. Thus, beyond the need to understand different intercellular interactions and the mechanisms involved, clues for the integration of complex signalling- and intercellular cascades will also be essential, in which systems biology approaches appear to be indispensable. Please note, that this section only aims to demonstrate that the effect of microglia-dependent actions on neurons may also be mediated via other cell types and not intended to give a comprehensive review of inter-glial interactions.

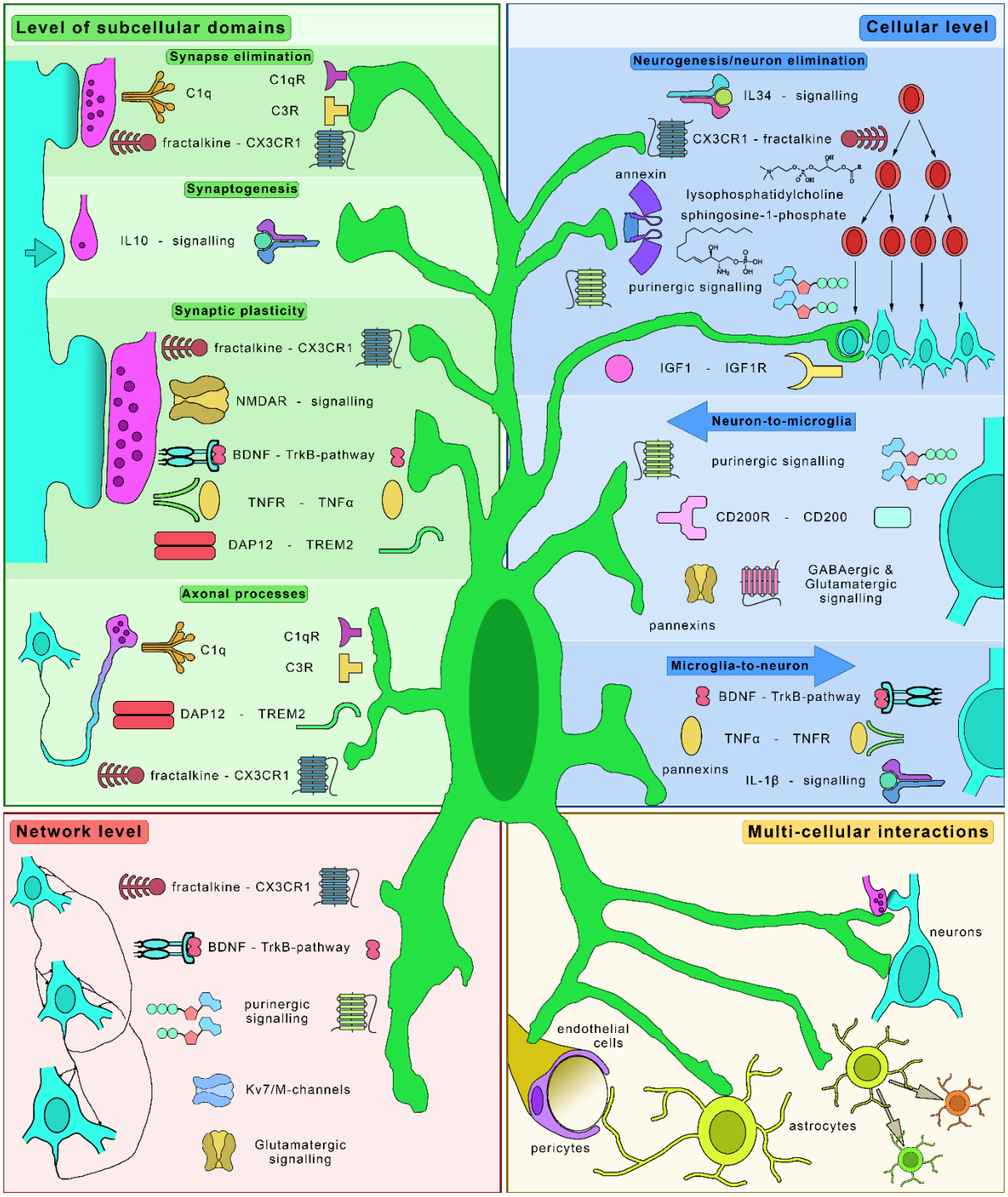

Fig1. Molecular pathways involved in microglia-neuron interactions. The schematic figure shows a simplified view of the main signalling pathways that are implicated in the bidirectional communication between microglia and neurons. The four main levels of interactions (Level of subcellullar domains, Cellular level, Network level and Multi-cellular interactions) follow a bottom-up approach. Please note, that the pathways depicted on the figure are restricted to those discussed in the review (please refer to details in the text). Abbreviations: BDNF – brain derived neurotrophic factor, C1q – complement component 1q, C1qR – C1q-receptor, C3R – complement receptor 3, CD200 – cluster of differentiation 200, CD200R – CD200 receptor, CX3CR1 – C-X3-C-motif chemokine receptor, DAP12 – DNAX activation protein of 12kDa, IGF1 – insulin-like growth factor 1, IGF1R – IGF1 receptor, IL-1β – interleukin 1 beta, IL10 – interleukin-10, IL34 – -interleukin 34, Kv7/M – M-

type potassium channel, NMDAR – N-methyl-D-aspartate receptor, TNFα – tumor necrosis factor alpha, TNFR – TNF receptor, TREM2 – Triggering receptor expressed on myeloid cells 2, TrkB – Tropomyosin receptor kinase B.

Challenges and future directions of microglia research

In this chapter, we aim to highlight a few research areas that may be important for a better understanding of the complex role of microglia in the CNS (summarized in Box 1.). Several additional important topics exist that have not been discussed herein due to space limitations. The investigation of steady-state microglia is hampered by the fact that microglia – by their very nature – rapidly respond to any disturbances of the brain microenvironment, which is a common confounder of any invasive method, surgical manipulation or treatment that disturbs brain homeostasis. Prompt microglial activation, changes in process motility and directed process recruitment are triggered by parenchymal injections, insertion of capillaries into the brain, or even by the preparation of cranial windows for *in vivo* imaging (Kozai et al., 2012; Eles et al., 2017). All these data highlight the importance of using non-invasive or minimally invasive methods when studying microglia *in vivo*. Good examples for these are the minimally invasive and/or chronic cranial window techniques with intact dura mater (Grutzendler et al., 2002; Davalos et al., 2005; Holtmaat et al., 2009, 2012; Szalay et al., 2016) as well as different skull clearing methods (Zhang et al., 2015; Steinzeig et al., 2017) widely used for *in vivo* two-photon imaging and other optical techniques. Models that allow prolonged imaging of microglial actions (beyond the assessment of process motility as a single readout) in response to non-invasive neuronal manipulation induced by chemogenetic approaches (e.g. Designer Receptors Exclusively Activated by Designer Drugs, DREADDs) or optogenetic stimulation would also greatly advance the knowledge of this field. However, optogenetic and chemogenetic constructs used to induce depolarization or hyperpolarization in neurons will need to be fine-tuned to better reflect intracellular calcium dynamics and evoke physiological actions of microglia.

Another area, where microglia research is standing before a big leap forward is the use of specific, microglia-selective manipulations. So far one of the most straightforward and robust methods to investigate the involvement of microglia in specific physiological or pathophysiological processes has been the selective depletion of these cells with CSF1R antagonists (Rice et al., 2015; Acharya et al., 2016). In the last couple of years, the field obtained critically important data with this method, some of these re-shaping our conceptual understanding of the role and nature of microglia (Szalay et al., 2016; Huang et al., 2018). However, microglia depletion studies may not provide detailed information regarding the specific mechanisms, the molecular pathways or the additional regulatory factors involved. Long term genetic alteration of the microglial system – especially during brain development – can trigger compensatory mechanisms, resulting in phenotypes that are not solely attributable to the direct effects of microglial manipulations. Even during adulthood, temporal elimination of microglia may also induce compensatory actions in neurons, ranging from the level of synapses to complex neuronal networks, or influence other cell types, which should be taken into consideration for the interpretation of the results from these studies. This necessitates the use of more specific microglial interventions in future research, in addition to cell-specific depletion studies. Development of models to selectively induce or inhibit microglial activation in real time by genetic manipulation of microglia-specific proteins, selective inhibitors of microglia-specific signalling pathways and the selective chemogenetic and optogenetic control of microglial actions will also be of great importance.

Several different molecular signalling pathways have been implicated in physiological neuron to microglia communication in recent years that were discussed in several excellent reviews in recent years (Kettenmann et al., 2013; Bilimoria and Stevens, 2015; Mosser et al., 2017; Salter and Stevens, 2017; York et al., 2017; Mecca et al., 2018). However, this area still has many critical unanswered questions. It is well known, that microglia express a large variety of classical neurotransmitter receptors, several of which were described to be functional. For example, glutamate can act on microglial ionotropic and metabotropic receptors to modulate TNFα-release or regulate processes

linked to neurotoxicity/neuroprotection, respectively (Hagino et al., 2004; Taylor et al., 2005). GABAergic (Charles et al., 2003) and cholinergic (Shytle et al., 2004) signalling have also been shown to modulate cytokine release by microglia. Nevertheless, the exact role of neurotransmitter receptors and their regulation of microglial function are incompletely understood and the possibility of a direct synaptic input by neuronal axon terminals on microglia remains to be investigated. High-resolution microscopy studies to reveal the precise localization of neurotransmitter receptors, transporters and other molecular elements on microglia in the context of their connection with neurons are currently lacking. These data together with functional studies would help to understand the direct and paracrine signalling mechanisms through which synaptic activity shapes microglial responses in health and disease.

It is also vital to gain further mechanistic insight into the main pathways regulating microglia-neuron communication. At present, several experimental models implicate the functional role of microglia in the formation, functioning or dysfunction of neuronal networks, but the identity of the actual microglia-mediated effects is far from being understood. A typical example is the complex role of the fractalkine signalling pathway in health and disease. CX3CR1 KO mice have been shown to experience defective synaptic development and maturation in the hippocampus and the barrel cortex (Paolicelli et al., 2011; Hoshiko et al., 2012; Zhan et al., 2014), while other studies describe unaltered synaptic development and activity-dependent plasticity in the visual cortex in the absence of CX3CR1 (Lowery et al., 2017; Schecter et al., 2017). In line with this, CX3CR1-deficient microglia show increased or decreased level of proinflammatory cytokine production and altered phagocytic activity or contact with neurons in a model-dependent manner, whereas CX3CR1 KO mice display reduced neuronal death in response to acute brain injury with impaired outcomes in several models of neurodegeneration (Cardona et al., 2006; Denes et al., 2008; Ransohoff, 2016a). These controversies are likely to be due to differences in the experimental models used, the presence or absence of BBB injury, the time-scales of observation and the differences in disease pathophysiology among others, which will need to be investigated in future studies.

ATP and its derivatives are well-known chemoattractants and regulators of microglial motility mainly via P2Y12 receptors, whilst P2X7, P2X4 and other purinergic receptors regulate microglial cytokine production, migration and phagocytosis (Davalos et al., 2005; Haynes et al., 2006; Shieh et al., 2014; Fabbrizio et al., 2017; He et al., 2017; Suurväli et al., 2017). To date, the P2Y12 signalling pathway has mainly been implicated in responses of microglia to pathological changes (Inoue, 2002; Haynes et al., 2006; Tozaki-Saitoh et al., 2008; Gu et al., 2016; Tsuda and Inoue, 2016), and recent studies suggest that while microglial potassium fluxes – via the newly described two-pore domain THIK-1 channel – play an important role in the regulation of physiological surveillance of microglia, purinergic signalling participates only in case of injury or distress (Madry et al., 2018a, 2018b). However, it is still unknown where the different motility-regulating signalling pathways intersect and how they influence each other in physiological and pathological states. Furthermore, since neurons can release ATP also during their basal activity (Ho et al., 2015; Menéndez-Méndez et al., 2017), purinergic signalling via P2Y12 receptors is essential for synaptic plasticity (Sipe et al., 2016) and microglial P2Y12 signal decreases during activation (Haynes et al., 2006), the role of purinergic signalling in physiological microglial functions cannot be ruled out and should be investigated in detail.

Further on, the signals regulating the precise localization of microglia in the brain and their migration in response to injury are improperly defined. For example, motile microglial processes rarely contact processes of nearby microglia in the healthy brain, allowing individual microglia to occupy a given volume of brain tissue, whilst microglial cell bodies are largely localised and evenly distributed under physiological conditions (Nimmerjahn et al., 2005). The precise mechanisms through which microglial process dynamics is influenced by nearby microglial cells are presently unclear and warrant further investigation. However, repopulating microglia (upon the cessation of CSF1R blockade) migrate and proliferate in the uninjured brain parenchyma until the distribution of the cells becomes similar to that of naïve animals within a short period of time (Elmore et al., 2014; Huang et al., 2018). In line with this, tissue injury, cerebral ischemia, BBB breakdown and several

other processes induce the directed migration of microglia to areas of injury even from large distances. Thus, it is likely that the signalling mechanisms and interactions with the extracellular matrix and other cells regulating actions of microglia show subcellular heterogeneity, which remains to be understood. Furthermore, microglia display region-specific heterogeneity at the transcriptomic level in the brain (Doorn et al., 2015; De Biase et al., 2017) suggesting that the neurochemical phenotype and function of surrounding neurons shape microglial responses.

Direct physical contact between microglia and neurons seems to play an important, but presently undefined role in several physiological and pathophysiological processes. Microglia contact several domains of the neuron, such as synapses, dendrites or axon initial segments (Nimmerjahn et al., 2005; Wake et al., 2009; Tremblay et al., 2010; Baalman et al., 2015). However, the comprehensive mapping of domain-specific microglia-neuron interactions has not yet been performed. Additionally, the function of satellite microglia and their possible regulatory actions are also unknown. Fast and precise communication between different cellular components, together with the operation of localised regulatory mechanisms is a fundamental feature of neuronal networks. Obviously, the presence of paracrine effects, the relevance of “volume transmission” is not arguable, however, more and more signalling pathways – previously thought to operate only in a diffuse manner – have been shown to operate mainly in a fast, specific and localised manner. The ascending median raphe pathway, the cholinergic innervation of cortical areas and nitric oxide signalling are all good examples at the level of neuron-to-neuron interactions (Sarter et al., 2009; Varga et al., 2009; Garthwaite, 2016). These data suggest that the significance of direct and local mechanisms is still generally underestimated and highlight the importance of mapping direct microglia-neuron contacts and their functional contribution to the interactions between these cells.

The bidirectional communication between microglia and neurons under physiological and pathophysiological conditions has been widely studied. Under physiological conditions, among many others, microglial BDNF, IL-1ß and TNFα have been shown to contribute to this dialogue. However, the exact signals triggering or blocking dendritic spine formation, synaptogenesis etc. at areas of

microglial contacts, or those regulating repolarization and soothing of hyperactive neurons following microglia process recruitment remain to be defined. In a similar line, it is currently unclear how changes in neuronal activity contribute to complement-mediated synapse elimination by microglia (Stevens et al., 2007; Schafer et al., 2012). Furthermore, our knowledge about the interactions between microglia and neuronal synapses is focused mainly on the glutamatergic contacts. Studies are already emerging showing microglial regulation of non-glutamatergic synapses (Chen et al., 2014; Cantaut-Belarif et al., 2017; Um, 2017), but this area needs further research efforts.

Aside from some specific areas including the subventricular zone, the dentate gyrus and the olfactory bulb, neurons are not able to renew themselves. Latest data further strengthen the view that neurogenesis plays a smaller role in memory formation and renewal of neuronal populations that assumed previously (Sorrells et al., 2018) and suggest that most of our neurons stay with us throughout our entire life (Farzanehfar, 2018). On the other hand, most cell types in the brain have been thought to have a much shorter lifespan and a relatively high turnover rate. Interestingly, recent studies described that individual microglia have an unexpectedly long life, and a lower turnover rate (Füger et al., 2017; Réu et al., 2017). These results propose an interesting direction for future research, and change our conceptual understanding of microglial functions both in physiological states, and – perhaps more importantly – in the context of aging and neurodegeneration (Olah et al., 2018; Santoro et al., 2018).

The long-held view of microglial states (i.e.: “resting” and “activated”) is getting more and more outdated and should rather be replaced by a more integrative paradigm based on results from numerous imaging, transcriptomic and functional studies. The original concept led to the false and oversimplified view suggesting that in physiological state the only function of microglia is to wait for an insult as opposed to recent studies demonstrating constant surveillance activity and the fundamental homeostatic and neuroprotective roles of microglia (Kierdorf and Prinz, 2017). This area needs a lot of future work, but a new paradigm is clearly on the horizon. Similarly, the concept of microglial polarization and the existence of M1 and M2 states appear to be replaced by novel

concepts. Whilst microglial transcriptomes differ unambiguously from that of peripheral macrophages, single-cell RNA-seq studies have demonstrated that canonical markers of divergent polarization states were highly coexpressed in single cells (Ransohoff, 2016b). This is also supported by the findings that regional and age-dependent microglial profiles do not support the existence of either M1 or M2 transcriptomes under physiological or stress conditions (Grabert et al., 2016; Ransohoff, 2016b). This certainly raises important issues in the context of the plasticity of individual microglial cells with important implications to microglia-neuron interactions.

In the last decades, the rising frequency of neurological disorders imposes an extreme socio-economic burden on society (Pritchard et al., 2013), which is accompanied by the very limited success of clinical trials concerning new treatments for brain disorders like stroke or Alzheimer's disease (Hoyte et al., 2004; Anderson et al., 2017). The reasons for failure are likely to include the long-standing "neuron-centric" view of brain research (Verkhratsky et al., 2016). Throughout recent years the basic contribution of neuroinflammation to brain disorders has been confirmed, and microglia also stepped up as "central players" in neurodegenerative processes (Ransohoff, 2016a; Ramirez et al., 2017; Salter and Stevens, 2017). Future research should acknowledge the complexity of brain, and conceptually integrate all cellular elements in an unbiased manner (Masgrau et al., 2017). Among these, microglia-specific processes are clearly emerging as so-far unexplored targets for therapeutic interventions, urging for multiplied efforts in microglia-related translational research.

Conclusions

In this review, we have attempted to give a short and unbiased summary of microglia-neuron interaction research, and perhaps more importantly – besides the snapshot depicting the current status of the field – tried to provide an actual "shopping list" of the major questions and possible future directions of research. As usual, it is possible that some of the research frontlines identified will eventually turn out to be dead ends, and presently unexpected novel concepts will revolutionize

microglia research. Nevertheless, this field will give an inexhaustible source for investigation in the following years, presumably with important translational outputs.

Box 1. – Considerations when studying microglia-neuron interactions

Technological and experimental objectives

- Development and use of non-invasive models and imaging tools to study microglial actions under physiological and pathophysiological conditions *in vivo*
- Identifying novel markers of microglia for cell-specific manipulation
- Non-invasive and selective microglia manipulation techniques with emphasis on real-time manipulation
- Novel imaging, molecular anatomy and systems biology approaches to understand microglial function in health and disease
- Validation of microglia-manipulation approaches and disease models in a translational context

Some important research directions

- Revealing microglial transcriptome, translatome, proteome and secretome, and their changes during development, adulthood, aging and disease
- Unveiling molecular determinants of direct neuron-microglia communication
- Exploring microglial influence on neuronal activity
- Revealing the role of microglial neurotransmitter receptors in microglial activity and responses
- Understanding the mechanisms of complement-mediated synapse elimination and identification of further molecular pathways involved
- Understanding purinergic signalling in health and disease
- Identification of the mechanisms mediating regional specificity of microglia in the brain
- Understanding the role of microglial function during development and the impact of early-life events on lasting pathophysiological alterations
- Studying innate immune memory and epigenetic changes in microglia to understand the impact of central and systemic inflammatory responses on the brain
- Better understanding of the cooperation between glial cells
- The significance of long living microglia and their role in neuronal senescence and aging
- Exploring changes in neuron-microglia interactions in pathologic states
- Working towards effective utilization of microglia research discoveries in medicine

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