DUAL ROLE OF MICROGLIA IN HEALTH AND DISEASE: PUSHING THE BALANCE TOWARDS REPAIR

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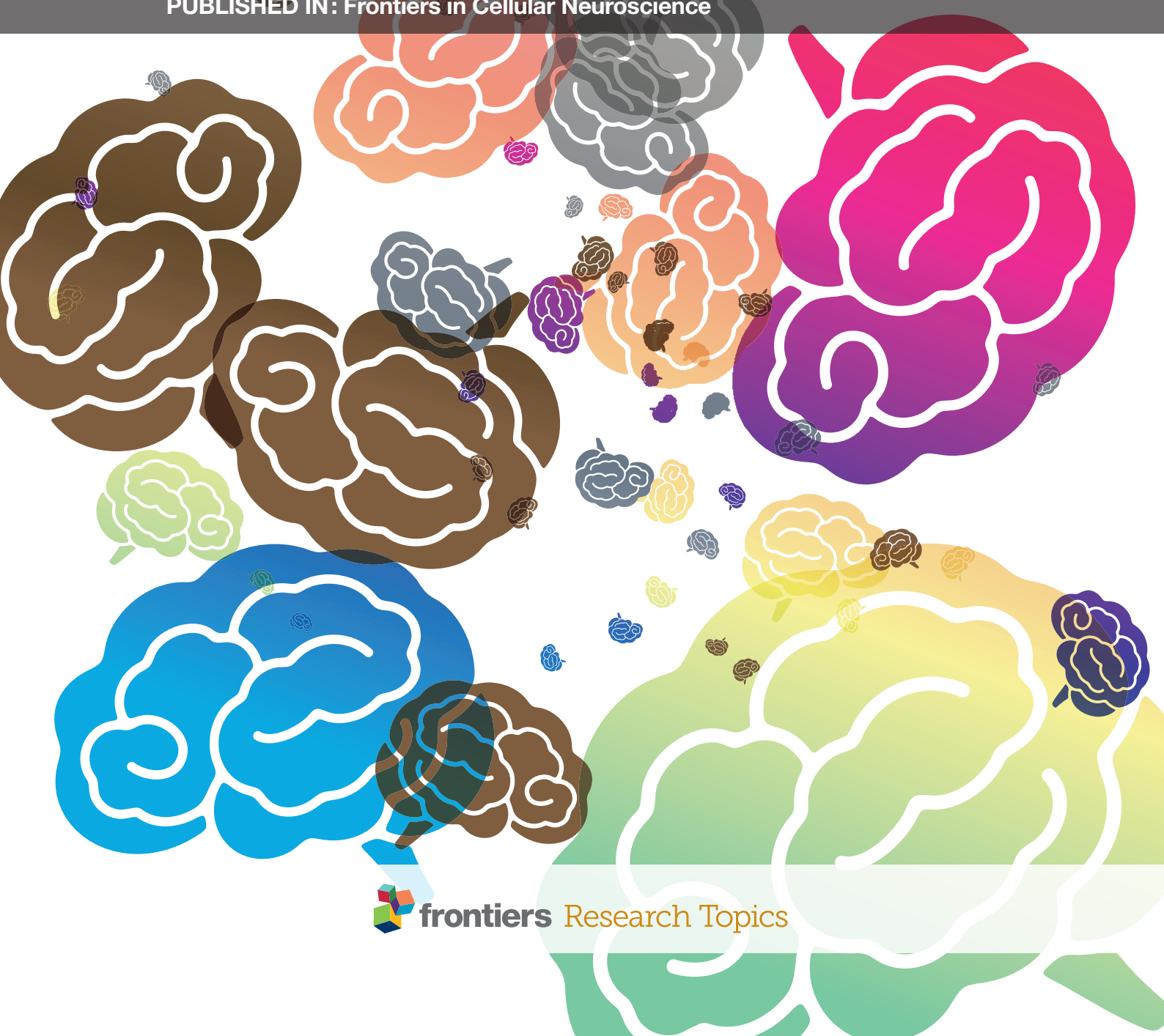

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DUAL ROLE OF MICROGLIA IN HEALTH AND DISEASE: PUSHING THE BALANCE TOWARDS REPAIR

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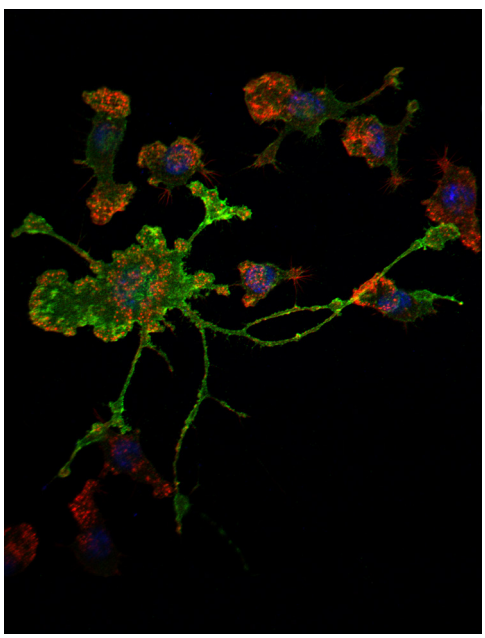

An activated microglia cell (enlarged cell in the center of the image) is interacting with restinglike microglia (surrounding cells). Microglial cells were revealed by the expression of CD11b (green) and actin cytoskeleton reorganization labelled by phalloidin (red). Hoechst 33342 unmasks nuclear staining in blue. Image by Raquel Ferreira

Microglial cells play a vital role in the innate immune response occurring in the Central Nervous System (CNS). Under physiologic conditions, microglia dynamically patrol the brain parenchyma and participate in the remodeling of active neuronal circuits. Accordingly, microglia can boost synaptic plasticity by removing apoptotic cells and by phagocytizing axon terminals and dendritic spines that form inappropriate neural connections. Upon brain and spinal cord injury or infection, microglia act as the first line of immune defense by promoting the clearance of damaged cells or infectious agents and by releasing neurotrophins and/ or proneurogenic factors that support neuronal survival and regeneration. Recently, two main pathways were suggested for microglia activation upon stimuli. Classical activation is induced by Toll-like receptor agonists and Th1 cytokines and polarizes cells to an M1 state, mainly leading to the release of TNF-alpha, IL-6 and nitric oxide and to grave neural damage. Alternative activation is mediated by Th2 cytokines and polarizes cells to an M2a state inducing the release of antiinflammatory factors. These findings have further fueled the discussion on whether microglia has a detrimental or beneficial action (M1 or M2-associated phenotypes, respectively) in the diseased or injured CNS and, more importantly, on whether we can shift the balance to a positive outcome. Although microglia and macrophages share several common features, upon M1 and M2 polarizing conditions, they are believed to develop distinct phenotypic

and functional properties which translate into different patterns of activity. Moreover, microglia/macrophages seem to have developed a tightly organized system of maintenance of CNS homeostasis, since cells found in different structures have different morphology and specific function (e.g. meningeal macrophages, perivascular macrophages, choroid plexus macrophages). Nevertheless, though substantial work has been devoted to microglia function, consensus around their exact origin, their role during development, as well as the exact nature of their interaction with other cells of the CNS has not been met.

This issue discusseshow microglial cells sustain neuronal activity and plasticity in the healthy CNS as well as the cellular and molecular mechanisms developed by microglia in response to injury and disease. Understanding the mechanisms involved in microglia actions will enforce the development of new strategies to promote an efficient CNS repair by committing microglia towards neuronal survival and regeneration.

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Dual role of microglia in health and disease: pushing the balance toward repair

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Keywords: microglia, inflammation, neuronal repair, neuron-glia crosstalk, neurodegenerative diseases

Microglial cells have been traditionally regarded as the rowdy foot soldiers of the Central Nervous System (CNS), always on the edge of causing massive destruction when provoked. Nevertheless, microglia are responsible and/or strongly contribute to the maintenance of CNS homeostasis, immune surveillance and synaptic plasticity. Until now, it was believed these complex processes depended on two polarizing stimuli received by microglia: the "bad" ones leading to a classical pro-inflammatory response (M1), and the "good" ones leading to a typical anti-inflammatory profile (M2). However, under M1 and M2 polarizing conditions, microglia can *differentiate* into different population subsets and develop specific patterns of activity. Gertig and Hanisch (2014) extensively review the heterogeneity of microglia responses and discuss how it impacts cytokine production, clearance of tissue debris, antigen presentation or the ability to sense neurotransmitters. The challenge will be the development of tools that allow us to select a subpopulation of interest and modulate its response specifically to an optimal therapeutic effect. Microglia are classically addressed as the resident macrophages of the CNS. In that sense, these cells are strongly committed to removal of foreign/infectious particles and clearance of cellular components to maintain a healthy brain parenchyma. Nau et al. (2014) distinguish two pathways by which microglia increase phagocytosis and intracellular elimination of pathogens albeit with different outcomes on neuronal survival. Stimulation of one or several toll-like receptors or nucleotide-binding oligomerization domain-containing protein 2 receptors leads to the release of pro-inflammatory products causing neuronal damage. Conversely, authors report that microglia activation by palmitoylethanolamide increases phagocytosis and intracellular elimination of pathogens without the release of pro-inflammatory mediators. The discovery of molecules that can eliminate pathogens without damaging surrounding neuronal tissue will likely strengthen the ability of the brain to resist infection. On the latter phagocytic function of microglia, Gitik et al. (2014) show that paxillin and cofilin activation promotes phagocytosis of degenerated myelin in SIRP-alpha knocked-down microglia, and show a positive correlation between paxillin and cofilin activation and phagocytosis. Myelin breakdown occurs following traumatic axonal injury and neurodegenerative disorders such as multiple sclerosis. Understanding the mechanisms underlying the removal of degenerated myelin will allow us to develop the necessary strategies to promote remyelination more efficiently while preserving nearby intact tissue. This study further emphasizes the importance of studying microglia responses and the nature of their interactions with other cell types at different stages of disease progression in order to maximize overall tissue recovery and/or survival. Chronic microglia activation also occurs in amyotrophic lateral sclerosis (ALS), the most aggressive form of adult motor neuron degeneration. Brites and Vaz (2014) extensively review the intricate crosstalk between motor neurons and glial cells, including microglia, and how the latter modulate ALS onset and progression. In an early stage of the disease microglia have a beneficial effect while overly activated and pro-inflammatory microglia in the later stages of the disease become neurotoxic. The study of neuron-microglia interactions and the selection of the adequate model to study microglia contribution to this dynamics warrants attention from Neiva et al. (2014). The group discusses important biochemical and physiological differences between immortalized microglia cell lines and primary microglia and alert to possible pitfalls when using artificial culture systems. On this note, authors suggest that supplementation of microglial culture media with fractalkine induces a more *in vivo*-like typical morphology and behavior compared to most *in vitro* models. Although the proposed model requires additional characterization it is important to be aware of the strategy we choose to validate microglia therapeutic or detrimental properties. An emerging target for the development of anti-neuroinflammatory strategies is histamine, mainly known for its role as a peripheral inflammatory mediator. Rocha et al. (2014) review current literature on histamine modulation of microglia activity but also present new data on how the secretome of histamine-stimulated microglia promotes dopaminergic neuronal death. By examining the effects of antihistamines on dopaminergic cell survival, authors unveil new perspectives on therapeutic platforms for Parkinson's disease. Microglia activity changes not only in a pathological context but also in the healthy aging brain. To better understand these mechanisms, Caldeira et al. (2014) propose an *in vitro* model of reactive and aged microglia. In their study, authors report that 16 days *in vitro* microglia evidence morphological and reduced inflammatory and functional activity corresponding to irresponsive/senescent cells. An increase in life expectancy and age-related neurodegenerative diseases highlights the importance of developing tools to control and recover microglia activity in the aging brain. Both aging and neurodegenerative disorders lead to neuronal loss albeit at different extents.

An approach to delay or halt neuronal death and/or to promote the replacement of dead/dying neurons is to stimulate neurogenesis. In this sense, the role of microglia on the modulation of the neurogenic niche has been increasingly addressed. Marshall et al. (2014) show how regional differences (neurogenic niches vs. cerebral cortex) can affect microglia proliferative ability in the brain. Authors conclude that microglia possess intrinsic and spatially-restricted characteristics that allow them to function as distinct populations independently of their *in vitro* environment. This distinction allows for microglia isolated from the neurogenic niches to be more efficient in promoting neurogenesis. Microglia not only modulate the neurogenic niche but interact and shape the activity of mature neurons. Cristovao et al. (2014) comment on the novel role of microglia on pre-synaptic differentiation as a means to better understand the formation of aberrant synapse formation occurring in neurodevelopment disorders. Authors show that activated microglia cause a significant increase in the axonal density of pre-synaptic marker synapsin I, which is not observed in the presence of non-primed microglia or in isolated axons. This study emphasizes the importance of dissecting microglia responses to maternal infection during pregnancy and how they may impact fetal synapse formation. In the present research topic, researchers presented their work and views on the cellular and molecular mechanisms developed by microglia in response to injury and disease, neuronal remodeling and aging. We expect the work presented herein can advance our understanding on microglia-mediated responses toward neuronal survival and regeneration and therefore promote the development of new therapeutic approaches to efficiently repair the diseased and/or injured CNS.

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Microglial diversity by responses and responders

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Microglia are the principal resident innate immune cells of the CNS. Their contributions to the normal development of the CNS, the maintenance and plasticity of neuronal networks and the safeguarding of proper functionality are becoming more and more evident. Microglia also survey the tissue homeostasis to respond rapidly to exogenous and endogenous threats, primarily with a protective outcome. However, excessive acute activation, chronic activity or an improper adaptation of their functional performance can foster neuropathologies. A key to the versatile response behavior of these cells is their ability to commit to reactive phenotypes, which reveal enormous complexity. Yet the respective profiles of induced genes and installed functions may build up on heterogeneous contributions of cellular subsets. Here, we discuss findings and concepts that consider the variety of microglial activities and response options as being based—at least in part—on a diversity of the engaged cells. Whether it is the production of proinflammatory cytokines, clearance of tissue debris, antigen presentation or the ability to sense neurotransmitters, microglial cells present with an unanticipated heterogeneity of their constitutive and inducible features. While the organizational principles of this heterogeneity are still largely unknown, functional implications are already perceptible.

Keywords: diversity, cytokines, immunity, innate, microglia, subtypes, TLR

The term "microglia" is commonly used as a plural word. The tenaciousness by which text processing software marks respective phrases for correction and insists on a singular form may inspire a reflection of the true meaning of "the" microglia as to their plurality. Several and especially most recent observations raise the question whether microglia do represent a uniform entity or a heterogeneous community—with subpopulations being distinguishable by their functional capacities and executed functions (Fitzner et al., 2011; Scheffel et al., 2012; Pannell et al., 2014).

Here we take examples from selected domains of microglia physiology, including monitoring of neuronal activity, synthesis of cytokines, expression of molecules for antigen presentation or the ability for clearing myelin material, to discuss the heterogeneity of microglia in serving housekeeping duties, sensing environmental signals and organizing their (mostly) adequate responses to a disturbed CNS homeostasis. Even though the evidence for such a heterogeneity by equipment (expressed molecules) and performance (function) is ubiquitous, the underlying determinants, the physiological significance and the pathophysiological impact remain largely enigmatic (Hanisch, 2013a,b,c).

IS THERE A NEED FOR ADDRESSING MICROGLIAL CONSTITUTION AND PERFORMANCE AT A SINGLE CELL LEVEL?

The characteristics as neuroimmune cells and the versatile response behavior of microglia upon infection or tissue injury, following to ischemic stroke or in neurodegenerative diseases, as part of an autoimmune disorder or by exposure to a tumor have been described already in detail–with increasing broadness regarding the accompanying molecular profiles and even the dynamics of activity changes (Pukrop et al., 2010; Kettenmann et al., 2011; Prinz et al., 2011; Hickman et al., 2013; Hanisch, 2013a; Butovsky et al., 2014). It is generally understood that a particular challenge within a given context can lead to a distinct reactive phenotype (Hanisch and Kettenmann, 2007; Eggen et al., 2013; Hanisch, 2013a).

Originally based on extraneural macrophages, reactive phenotypes were defined by triggering responses to cytokines and microbial agents, such as interferon-γ (IFNγ) from T helper cell type 1 (Th1) and natural killer cells, interleukin-4 (IL-4) from Th2 cells and Toll-like receptor (TLR) agonists, such as lipopolysaccharide (LPS), a cell wall component of gram-negative bacteria and a widely used standard tool for triggering proinflammatory reactions in myeloid cells. In a simplified concept, exposure to IFNγ or LPS would induce a "classical" activation, also commonly defined as M1 reaction, which comes with the production of proinflammatory mediators in support of defense-oriented Th1 type immune reactions. On the contrary, IL-4 would instruct an "alternative" activation, also termed M2, with a distinct profile of induced genes. This phenotype assists Th2 type immune responses, exerts anti-inflammatory effects, resolves inflammation and supports tissue repair (Hanisch, 2013b). While reciprocal expression of IL-12 and IL-10 served as a first indicator for M1 *versus* M2 commitment, and further molecules were identified with more or less biased association, the sets of regulated genes and functions were found way more complex and

not necessarily exclusive. With more activating scenarios being analyzed, it became clear that reactive phenotypes of either M1 or M2 tendencies can vary by the molecular signatures. Attempts of subclassification were very helpful. Major orientations could be defined. Yet a simple polarization by pure M1 *versus* M2 reactions may represent an extreme. Challenges by pathogen-associated molecular patterns (PAMPs) as derived from bacteria or viruses, confrontations with tumors or apoptotic cells, exposure to tissue debris or damage-associated molecular patterns (DAMPs) from impaired cells, a plethora of factors acting in concert simultaneously or in sequential order will tailor adapted macrophage responses. In their variety, they may almost appear as a continuum and cover combinations or simultaneous presence not easily fitting a dual classification system (Sierra et al., 2007; Mosser and Edwards, 2008; Nikodemova et al., 2013). Moreover, reactive phenotypes will also undergo adjustments (Kigerl et al., 2009). Early profiles for defensive actions may shift and deescalate upon successful elimination of the infectious agent or wound closure. At later stages, reactive phenotypes have to mature to promote tissue repair and to support restoration of the structural integrity and—as far as possible—functional recovery.

With the emerging concept of reactive phenotype diversity of macrophages it became also obvious for microglia that distinct stimuli trigger distinct responses (Hanisch and Kettenmann, 2007; Hanisch, 2013c). Defined experimental conditions *in vitro* helped to determine how microbial agents or cytokines lead to an expression of surface receptors and enzymes or to a release of soluble mediators, namely cytokines themselves. Increased motility and directed movements, phagocytic clearance, stimulation of T cell proliferation or impacts on neuronal survival were studied. *In vivo*, a characterization is made more difficult since infiltrating monocytes can intermingle with the resident microglia under various disease conditions and in their respective animal models. Morphological criteria or staining for marker proteins, such as CD11b or Iba1, are not sufficiently reliable, although some distinction can be done by the level of expression, as in the case of CD45. Options for segregating microglia from immune infiltrates have been improved by the introduction of genetically engineered mouse models expressing fluorescent proteins under the control of chemokine receptor promoters, such as for CCR2 and CX_3CR1 (Mizutani et al., 2012). Elegantly applied in diverse experiments, these studies revealed invaluable insights into the populational maintenance *versus* replenishment or the functional contributions of microglia in health and disease—and to address their versatile reactive behavior (Prinz et al., 2011).

However, in many (if not most) cases, a reactive phenotype is assigned to the affected or responding microglia as a bulk. It appears as an averaged profile. More or less equal contributions of individual cells are assumed to sum up. Measurements of cytokine contents in a culture supernatant or gene array analyses on tissue samples do not detect the parameters at cellular level. Studies with cellular resolution, such as conventional flow cytometry, ELISPOT assays, *in situ* hybridization, PCR analyses on laser capture samples or classical immunocytochemistry, are often restricted by the number of analytes. What if different reactive phenotypes and their prominent features are supported by distinct cells? What if microglial subpopulations take the lead in a response? What if functions are directed to specialized subtypes? These questions sound academic, on a first glance. They gain value when inspecting (and trying to interpret) the enormous complexity, overlaps and dynamics of phenotypic orientations and when considering targets and tools for potential (therapeutic) manipulations. Ideally, multiparametric information on both the molecular equipment and their functional translations should be available for circumscribed populations or individual cells in combination with their local position and changes over time.

For neurons, it is text book knowledge that subtypes exist by morphology, neurotransmitter use, coexpressed molecules and electrophysiological parameters. Distinctions are made also for astrocytes (Emsley and Macklis, 2006; Matyash and Kettenmann, 2010). For immune cells, like T cells, subsets are defined by expression and release properties with direct importance for supported immune functions. Still single-cell technologies are on the verge to be required—and to be at hand—for covering substantial contributions of rare subsets and to explore the individual phenotypic variability for identified cells at a given time point and with kinetic resolution (Shalek et al., 2013; Chattopadhyay et al., 2014).

HOW DOES INFORMATION ABOUT THE CATEGORY MICROGLIA EXPLAIN THE FUNCTION OF A CELL?

Microglia comprise a rather large population. By parenchymal localization in the brain, spinal cord as well as the retina, they differ from other myeloid cells within the cranial compartment, such as those in the meninges, the choroid plexus or the perivascular space (Ransohoff and Cardona, 2010; Kettenmann et al., 2011; Prinz et al., 2011). CNS regions vary by microglial density, morphology and expression of a substantial number of gene products, even though physiological implications are still barely addressed. Heterogeneity in cell shape and the constitutive or episodic expression of certain molecules has been noticed since a long time (Streit and Graeber, 1993), but not necessarily and always with a conclusive interpretation as to cellular subsets (Kettenmann et al., 2011; Hanisch, 2013b).

Recently, regional differences have been addressed more consciously and comprehensively by looking at expression profiles and associated functional properties, distinct responsiveness to activating stimuli and adjustments that occur with development, aging or diseases, whereas information on gender differences or the comparability between species is still scarce (McCluskey and Lampson, 2001; Sierra et al., 2007; de Haas et al., 2008; Lai et al., 2011, 2013; Hart et al., 2012; Nikodemova et al., 2013; Torres-Platas et al., 2014). Enormous efforts have now been taken to characterize at large scale the transcriptome and microRNA spectrum of microglia isolated from newborn, adult and aged mice, in comparison to other major CNS cell types, other immune cell populations or the human microglia counterpart—thereby not only delivering exhaustive data sets but also extracting microglial signatures (Hickman et al., 2013; Butovsky et al., 2014). The findings build up on and extend previous lines of research that demonstrate the uniqueness of microglia among the family of tissue macrophages as to their lineage origin within the

mononuclear phagocytic system, as to their self-maintenance as well as properties acquired and exhibited within the context of their tissue environment (Ginhoux et al., 2010; Gautier et al., 2012; Schulz et al., 2012; Davies et al., 2013; Greter and Merad, 2013; Kierdorf et al., 2013; Wynn et al., 2013).

Accordingly, microglia are distinguished as a class of cells. Tissue-architectural, cellular and biochemical specificities of the CNS regions may impose further adaptations. Yet particular actions as a helper in development, as a housekeeper and sentinel in homeostatic surveillance or as an innate immune cell in fighting infection and endogenous threats have to be organized by an individual cell in contact with its immediate neighborhood, which may instruct and require adapted microglial properties.

HOW MUCH INDIVIDUALITY CAN FIT INTO THE CURRENT CONCEPTS OF A MICROGLIAL CELL?

A distinction of microglia could be based on single proteins that are sufficiently different at constitutive expression levels or unequally induced on demand—or by combination of both if there is some coincidental or causal link. Microglia differing by CD40 expression reveal concomitantly either the ability or inability for the induction of inducible nitric oxide synthase (iNOS) and release of tumor necrosis factor (TNF) α upon stimulation with LPS (Kawahara et al., 2009). Classification by the presence *versus* absence of a "marker" does not yet reflect individuality. However, when features with dichotomic expression are combined the variety of patterns increases quickly.

Microglia express a range of neurotransmitter receptors (Pocock and Kettenmann, 2007; Kettenmann et al., 2011). Neuronal activity can thereby influence their migratory behavior, phagocytic activities or the release of cytokines, chemokines and other mediators. Impaired neuron-to-microglia signaling can cause severe dysregulations of microglial functions during development, as part of the aging process and, of course, in neurodegenerative diseases. In turn, abnormal microglia impacts on neuronal cells and their circuitry (Heneka et al., 2010; Zhan et al., 2014). Even though neurons can exert influences on microglia by a variety of ligand-receptor systems, including CD200/CD200R, SIRP1α/CD47, CX_3CL1/CX_3CR1 or CD22/CD45 (Kettenmann et al., 2013; Hanisch, 2013b), also classical neurotransmission could play critical roles in organizing daily surveillance and maintenance activities as well as controlling of innate immune actions upon a challenge. A just published study systematically investigated the functional expression of numerous neurohormone and neurotransmitter receptors in mouse microglia (Pannell et al., 2014). Not only does this work reveal richness in the sensory spectrum. It indicates an unanticipated heterogeneity as well (**Figure 1A**).

Following up former work (Seifert et al., 2011), and based on inducible calcium signals recorded with fluo-4, the study addressed the sensitivity of acutely isolated adult as well as cultured neonatal and adult mouse microglia to a panel of substances. Cells were exposed to angiotensin II, dopamine, endothelin, galanin, histamine, neurotensin, nicotine, serotonin, somatostatin, substance P or vasopressin at relevant concentrations. Only subpopulations responded to a given compound with a calcium transient, in most cases less than 20% of the cells. The subpopulational responses were similarly observed in the three preparations. Thus, and along with other technical aspects, isolation or culture artifacts could be ruled out. Pretreatment with agents inducing M1 (LPS and IFNγ) or M2 (IL-4) polarization led to individual increases and decreases in the size of the responder populations. While the patterns may vary with the age of the animals at which cells were harvested and with the influence of modulating factors, the essential message is diversity among cells. Combinatory delivery (by a consecutive treatment) of neurotransmitters/neurohormones led then to the conclusion that the respective receptor expressions are largely at random and that microglia present with an immense diversity.

Of course, isolation of microglia from larger brain parts for a study *in vitro* cannot correlate the response pattern to anatomical divisions. Features found for subsets may relate to the need for local communication with neurons and neurosecretory cells in a cortical layer or a nucleus. In this regard, and as an example of a compound included in the screening, substance P was found to exert distinct effects on the IFNγ-induced expression of MHCII molecules in the brainstem *versus* the hippocampus of rats, ranging from strong enhancement to very little (if any) impact (McCluskey and Lampson, 2001). Yet culture conditions as applied by Pannell and coworkers do not seem to override these adaptations and the functional receptor expression may not reside only in one subset (Pannell et al., 2014). Of course, this study cannot inform about (each or even a global) functional implication, but it estimates an organizational distinction among microglia at a level far above any differentiation based on single molecule expression (Kettenmann et al., 2011; Hanisch, 2013b). Correlations with functions are still more limited by number. Yet the examples at hand could further substantiate the understanding of "the" microglia as a generic term for a diversified cell community.

CAN DISCRETE SUBSETS OF MICROGLIA BE ASSIGNED TO PARTICULAR FUNCTIONS?

While the above example displays an enormous spectrum of microglia by sensory equipment, activation of a single receptor can also lead to distinct responses in subsets. TLR4 is known for recognizing LPS as a PAMP, but it also mediates responses to a range of disparate self-derived molecules acquiring the meaning of a DAMP in noninfectious settings (Hanisch, 2013a,c). Among its family members, TLR4 is the most complex by signaling options and interactions with other receptor and co-receptor proteins. We had reported that its function in mouse microglia undergoes a massive change during postnatal development, apparently correlating with some reorganization in signaling pathways, but also showing a learning process in the functional interpretation of structural LPS variants (Regen et al., 2011; Scheffel et al., 2012).

Stimulation of mouse microglial TLR4 triggered the panpopulational upregulation of MHCI, a surface structure required for antigen presentation to cytotoxic T cells. As expected, TLR4

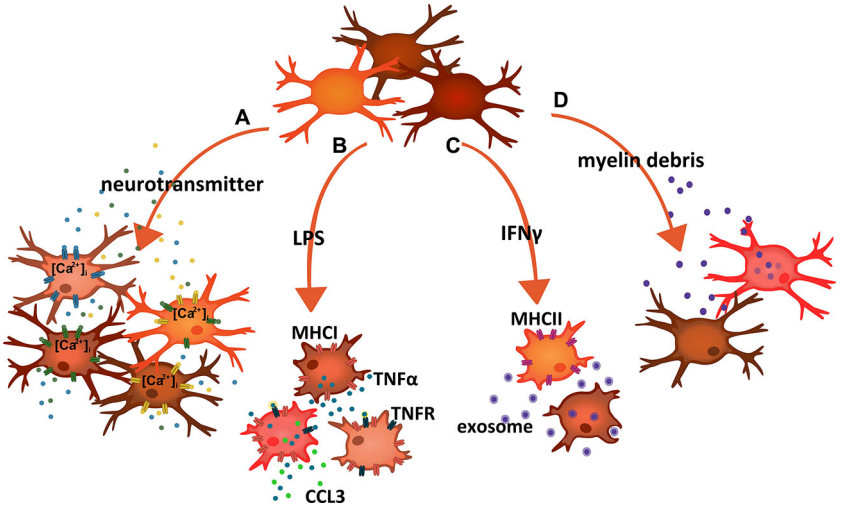

FIGURE 1 | Schematic summary of examples indicating microglial response heterogeneity. (A) Stimulation of microglia cells with neurotransmitters and neurohormones triggering calcium signals revealed that only small fractions responded to each compound. Combined stimulation and population analysis thereby suggested an enormous variety in terms of functional receptor expression. **(B)** Exposure to LPS induced a panpopulational expression of major histocompatibility complex I (MHCI) molecules, indicating that all microglia expressed TLR4. In contrast, only subsets also produced TNFα and/or CCL3. **(C)** Treatment of microglia with IFNγ caused the expression of major histocompatibility complex II (MHCII) in some but not all cells. On the other hand, MHCII-expressing microglia did not clear myelin-laden exosomes, a function that rather associated with MHCII-negative cells. **(D)** Exposure of cells to myelin debris resulted in the phagocytic uptake of the material in a subset of microglia. Adapted and expanded from Scheffel et al. (2012).

activation also led to the release of TNFα. However, when resolved at single cell level, only a subpopulation carried this activity (**Figure 1B**). The demarcation of the subpopulation got sharper with increasing postnatal age of the mice. Including also CCL3 (MIP-1α) as a T cell-attracting chemokine in the analysis, the TNFα$^+$ microglia subpopulation could be further separated into cells with a combined production and those only producing TNFα (**Figure 1B**). Focusing on CNS regions, the subsets were found in the cerebral cortex, the cerebellum and the spinal cord. With a delivery of the pleiotropic and proinflammatory cytokine relying on a subset and responses being imposed on many more cells expressing TNF receptors (including other microglia and CNS-resident as well as invading immune cells), a principle of "master instruction" could be established, in which a small set of TNFα-producing microglia takes an essential role in coordinating tissue responses (Clausen et al., 2008; Lambertsen et al., 2009). It is important to note that the detection of a subpopulation responding to TLR4 stimulation with TNFα cannot be explained by unequal TLR4 expression (Scheffel et al., 2012). Almost all cells showed TLR4-mediated induction of MHCI. Organization of a discrete production of the cytokine is thus an inherent property of the TLR4 signaling cascade in some but not all cells. Notably, selective responses can also be triggered through other receptor systems.

IFNγ can trigger the expression of MHCII structures for professional antigen presentation. Microglia respond in this way both *in vitro* as well as *in vivo* (Fitzner et al., 2011; **Figure 1C**). Different regions of the CNS thereby reveal a different response sensitivity and/or induction capacity (McCluskey and Lampson, 2001), and it is again only a subset of microglial cells that present with a detectable expression (Fitzner et al., 2011). A subset-dependent expression is also seen for the costimulatory molecules CD80 and CD86, which act in support of MHCII function. A demonstration of heterogeneous MHCII induction in microglia upon IFNγ injections into the CNS also considered potential gradient effects, which may feignedly reveal MHCII$^-$ microglia in vicinity to MHCII$^+$ cells, but just because of insufficient stimulation. Nevertheless, the MHCII$^+$ cells were rather scattered and blended with those lacking any staining signal. Moreover, pathohistology data from human postmortem material corroborated the findings in rodents as to a restricted microglial expression of respective human leucocyte antigen molecules. Apparently, not all microglia would be able to act as an antigen-presenting cell (APC). Heterogeneity of microglia as to APC functions has been noticed also on the basis of CD11c expression and actual performance (Remington et al., 2007). Interestingly, intracellular MHCII molecules may also serve some additional and previously unknown function in TLR4 signaling (Liu et al., 2011). This link could integrate in the subset organization under TLR4 activation (Hanisch, 2013b).

The very same study addressed the activity of microglia to clear myelin-laden exosomes from oligodendrocytes, under normal conditions and as a housekeeping function in support of myelin turnover (Fitzner et al., 2011; **Figure 1C**). This would well fit to the notion of microglia as never-resting sentinels and servants, which continuously scan their environment, but also "nurse" and shape synaptic connections (Davalos et al., 2005; Nimmerjahn et al., 2005; Haynes et al., 2006; Wake et al., 2009; Graeber, 2010; Paolicelli et al., 2011; Tremblay et al., 2011; Schafer et al.,

2012; Kettenmann et al., 2013; Zhan et al., 2014). The exosome removal, as performed by macropinocytosis, associated with some but not all cells. Surprisingly, when combining a detection of the MHCII surface structures upon IFNγ treatment with an analysis of exosome uptake, populations were largely exclusive, meaning that $MHCII^+$ microglia would not be concerned with myelin clearance and *vice versa*. This principle was, therefore, termed immunologically silent myelin removal (Fitzner et al., 2011). Thought to the end, it suggests a compartmentalization where self-derived material destined for degradation is sequestered from a pool of cells which can present material to the adaptive immunity for appropriate attack. Here, two subpopulations exercise a division of labor, probably to avoid a dangerous collision of functions. Conceivably, an accidental spill of myelin material into the cellular domain potentially serving APC functions could come with a risk of autoimmune responses, as they occur in multiple sclerosis.

Microglia can remove myelin debris resulting from damage and autoimmune destruction, an essential contribution to allow for repair and to reduce further damage (van Rossum et al., 2008; Gitik et al., 2011; Hadas et al., 2012). Uptake of myelin material is preferentially seen in a subpopulation of cells (Regen et al., 2011; Scheffel et al., 2012; **Figure 1D**). Similar to the disposal of the exosome-wrapped myelin, phagocytosis of myelin debris is suppressed by TLR4 activation, although the mechanisms of incorporation *per se* significantly differ from each other. On the other hand, activity of TLR signaling can rather enhance clearance of pathogens, such as Gram-negative and –positive bacteria (Ribes et al., 2009, 2010), a function that also appears to be associated with microglial subsets. It would be very interesting to determine such a subpopulational split for other clearance cargo as well.

Taking together, various lines of evidence combine in a notion of microglial diversity by both spontaneous and inducible functions. Such a concept could have multiple implications. Thus far, heterogeneity with regard to microglia has been mainly seen in the light of different reactive phenotypes, i.e., diversity by expressed genes and activities. Underlying organization principles and the contributions by individual cells were mostly neglected or not explicitly scrutinized. A split into subsets for a single protein expression or a given task can repeatedly be observed in many cases. Their comprehensive compilation, however, would probably give a complex matrix of microglial activity and response options. Anatomical resolution would be required to sort such subsets to CNS regions.

Some features and feature combinations may concentrate in some brain structures and may not be distributed throughout others, such as the proliferative potential (Walton et al., 2006; Marshall et al., 2008; Thored et al., 2009). Cells in the subventricular zone were found to be distinguished by their proliferative capacity and the support of neurogenesis. Proliferative potential would be important for the self-renewal of microglia, but might be a function especially carried out by some cells (Gomez Perdiguero et al., 2013). Similarly, support of neurogenesis and oligodendrogenesis, as a demonstrated activity of microglia (Butovsky et al., 2006), could be organized in some (functional) niches. How more locally situated cells could deploy and offer their specialized functions at other sites is not quite obvious. Even though microglia use their motile processes for scanning, they are rather stationary, at least under normal conditions. On the other hand, they can migrate on demand.

While microglia may have distinct capacities among themselves they differ from monocytes. Infiltration of peripheral immune cells in CNS pathologies could be essential whenever some requested know-how is simply not offered by microglia, not even by their specialized subsets. In such situations, the CNS may depend on extraneural monocytes/macrophages. What kind of expertise would then not be properly covered by the microglial spectrum? Certain aspects of phagocytosis, assistance in certain immune functions? However, these question cannot be answered satisfyingly right now—and it is also unclear whether all the observed heterogeneity by subsets has a fundament in truly distinct subtypes of microglia. In other words, how would individual microglial cells acquire distinct functional and reactive behavior?

HOW COULD FUNCTIONAL DIVERSITY OF MICROGLIA BE INSTALLED?

Various mechanisms could be envisaged. As major and fundamental—but still hypothetical—alternatives for an explanation one may consider a rather stable installation of differences in "subtypes" or one that is more instructed by environmental cues. Still another theory could be based on an entirely stochastic process.

In series of seminal studies, the ontogenetic origin and the maintenance of a stable microglia population have been dissected, identifying sources, critical transcription factors and steps of microgliogenesis (Naito et al., 1996; Ginhoux et al., 2010; Gautier et al., 2012; Gomez Perdiguero et al., 2013; Schulz et al., 2012; Yona et al., 2012; Kierdorf et al., 2013). It is speculative to assume that dissection of lineage origin and stages could still leave room for a late split into cells that later give rise to functionally distinct microglia subtypes. If so, how many distinct versions could be generated to accommodate the variety of microglial responses by distinguished responders? How and when would they be distributed throughout the CNS tissues? Such "hardware-based" concepts may quickly run into a dead end since it would imply too many bifurcations and too much of a logistic effort.

Instructions of microglial cells by the (micro) environment appear to be more feasible. The cellular neighborhood, the extracellular matrix (ECM) or features of the blood-brain barrier as varying by CNS region could impose a myriad of soluble and immobilized cues and signals to organize the properties of microglia (Hanisch, 2013b). The ECM is seen as a provider of compartments and as an organizer of functional microdomains (Dityatev et al., 2010). Factors circulating in the blood may have restricted entry to the CNS parenchyma at particular sites of the CNS, where the local microglia expresses respective sensor molecules (Perry et al., 1992). Heterogeneity of microglia may go hand in hand with the heterogeneity of other glial cells (Emsley and Macklis, 2006; Kitada and Rowitch, 2006; Matyash and Kettenmann, 2010). The prevailing neurotransmitter in a cortical layer or a nucleus may govern microglial assets as to

receptors as much as microglial cells participate in the maturation of synaptic connectivity (Parkhurst et al., 2013; Zhan et al., 2014). Instruction by multiple inputs at a specific location could offer advantages for explaining microglial heterogeneity. First, it would define cellular properties locally, and not beforehand for a whole set of microglia which still would need to be properly placed. Second, combination of numerous instructing signals—rather than a single "master" factor—could then also better organize a range of distinct features. Microglia themselves may take influences. At a lesion site, attracting microglia and causing higher than normal cell density, profiles of cytokines and chemokines could result from unequal release activities as organized by cooperative action. As stressed above, some cells may take the lead. Early production of key factors by a cellular subset could subsequently govern the responses of others. Distinct specializations and respective subset sizes might be precisely arranged under such conditions to adapt to the actual needs.

We have shown that microglia undergo a postnatal maturation process, regarding signaling properties of TLRs and including the ability of TLR4 to distinguish between PAMP ligand variants as to inducible cytokine profiles (Scheffel et al., 2012). In mice, a window between postnatal days P21 and P49 was thereby found to be critical. The period coincides with other CNS maturation aspects, including the formation of myelin sheets. The fact that and also how microglial maturation continues after birth has been addressed in a recent study, showing that the period between P21 and about P60 comes with a strong expression of genes that define the signature of (adult) microglia (Butovsky et al., 2014). This work also identified transforming growth factor (TGF) β as a key molecule in this process.

Features acquired within a defined environment during a particular developmental window may either remain stable or stay subject to adaptations and re-instruction. This could apply to microglia in general as well as to their potential subsets. It would be interesting to test for the stability of regional properties by exchanging local populations and determining characteristic features in a new environment. The observation that microglia can be harvested at given developmental stages and kept *ex vivo* with distinct features may suggest that they preserved at least some of the properties acquired within the tissue (Scheffel et al., 2012; Pannell et al., 2014).

How long would a regional imprint last without renewal by the critical cues? Could cells also change properties upon exposure to key signals or following a drastic activation? We had recently discussed issues of the postactivation fate of microglia in which experience of a previous challenge may alter responses to a subsequent encounter of the same or a different stimulus (Hanisch, 2013a,b). Epigenetic mechanisms could provide the molecular script for such "learning" processes. The long-lived microglia may also accumulate over the years (and decades) traces of repeated minor challenges and thus slowly alter surveillance or response properties in the aging CNS. Some diversity may thereby build up also with time, rendering some cells less efficient in providing support for maintenance, or yielding cells which do not properly respond to calming and activating signals anymore. Instructed during normal development or by episodic influences, diversity among microglia would in all these cases rely on a preserved assignment of particular functions to certain cells. However, a solution for the question of how microglia reveal distinct performance could also be found in a principle not depending on any determination at all.

Subpopulations as to the induction of a gene could be "created" by chance, resulting from a stochastic process of transcription (Hume, 2000). Variable stability of mRNA in cells and the consequence for the respective translation is a key in this concept, which explicitly applies to leukocyte differentiation and activities. It could also explain heterogeneity in TNFα synthesis upon TLR4 stimulation (Ravasi et al., 2002). The tempting and data-supported principle could thus help saving efforts in identifying complex instructions. Environmental factors as well as intrinsic mechanisms as based on probability may cooperate to create a situational response diversity, as transcriptional regulation in macrophages still seems to be complex (Lawrence and Natoli, 2011). Microglia may leave the synthesis of an important cytokine to a subset by chance, as long as it is secured that some cells will "feel responsible". Yet it would be more difficult to imagine a complex interaction of numerous gene products, as for example needed for antigen processing and presentation. Genes with grouped functions might be regulated *en bloc*, but would a stochastic process suffice a timed and synergistic performance? What about the heterogeneity observed for microglial responses to neurotransmitters and neurohormones (Pannell et al., 2014)? Is the respective receptor equipment expressed ubiquitously and at random or in correlation to the neuronal community? Would a sufficiently strong stimulation of a receptor with panexpression in microglia (like TLR4) but still triggering a response in only some of them (like the production of TNFα) at one point drive the induction in the entire population? Or would the competence for it remain a subset feature?

Regardless of which explanation will turn out to be suitable, the diversity in microglial actions remains as a phenomenon. It may stay a subordinated aspect in the organization of reactive phenotypes or an annotation when deciphering housekeeping functions. On the other hand, it could provide a clue to the understanding of the multi-faceted activities of microglia taking place on a daily basis throughout the CNS and in situations of emergencies.

CONCLUSION

There is virtually no neuropathological event which remains unnoticed by microglia. Their involvement is indicated by signs of "activation". The term, however, does not tell much about the actual activities (Hanisch and Kettenmann, 2007). Microglia may themselves be part of the pathogenetic process or rather attempt to contain its aggravation. Failure in protection would be detrimental. Excessive acute, sustained (chronic) activity or improperly adjusted reactive phenotypes may also render microglia a disease-driving element. On the other hand, and in the past, there has been no CNS disease declared to be directly and solely linked to a microglial defect, in terms of a cell-specific gene mutation or a selective functional deficit. More recent studies, however, provide strong arguments for a fundamental importance of microglia in normal development. They reveal how disturbances in cellular communication as well as executive functions of microglia could

underly impairment of even higher CNS activities and even identify essential gene products therein (Chen et al., 2010; Derecki et al., 2012; Parkhurst et al., 2013; Zhan et al., 2014). Maybe, a massive failure of microglia has an early fatal outcome. Other essential contributions, later in life and serving the homeostasis on a daily basis, are probably missed because they are mostly effective. Many of the minor, locally restricted and transient insults to neural cells may never surface with symptoms due to a rapid protective engagement of microglia. It is probably a subtle deficiency in a particular microglial activity which can then build up to a harmful consequence. It is thus of foremost relevance to dissect the pervasive performance and the emergency measures of microglia—in their duality as a major CNS as well as immune cell and with subpopulational resolution by and within regions.

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Strategies to increase the activity of microglia as efficient protectors of the brain against infections

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In healthy individuals, infections of the central nervous system (CNS) are comparatively rare. Based on the ability of microglial cells to phagocytose and kill pathogens and on clinical findings in immunocompromised patients with CNS infections, we hypothesize that an intact microglial function is crucial to protect the brain from infections. Phagocytosis of pathogens by microglial cells can be stimulated by agonists of receptors of the innate immune system. Enhancing this pathway to increase the resistance of the brain to infections entails the risk of inducing collateral damage to the nervous tissue. The diversity of microglial cells opens avenue to selectively stimulate sub-populations responsible for the defence against pathogens without stimulating sub-populations which are responsible for collateral damage to the nervous tissue. Palmitoylethanolamide (PEA), an endogenous lipid, increased phagocytosis of bacteria by microglial cells *in vitro* without a measurable proinflammatory effect. It was tested clinically apparently without severe side effects. Glatiramer acetate increased phagocytosis of latex beads by microglia and monocytes, and dimethyl fumarate enhanced elimination of human immunodeficiency virus from infected macrophages without inducing a release of proinflammatory compounds. Therefore, the discovery of compounds which stimulate the elimination of pathogens without collateral damage of neuronal structures appears an achievable goal. PEA and, with limitations, glatiramer acetate and dimethyl fumarate appear promising candidates.

Keywords: microglia, blood–brain barrier, blood–CSF barrier, innate immune system, bacteria, phagocytosis, palmitoylethanolamide

INTRODUCTION

In healthy young and middle-aged individuals beyond the neonatal period, infections of the central nervous system (CNS) are rare events. Although the blood–brain and blood–cerebrospinal fluid (CSF) barriers, i.e., the endothelium of the vessels situated in the brain parenchyma and the epithelium of the choroid plexuses, strongly impede the entry of pathogens into the CNS, it probably is not uncommon that pathogens reach the brain tissue or the CSF. In the CSF space, meningeal and perivascular and few circulating macrophages can eliminate pathogens (Engelhardt and Coisne, 2011). Moreover, pathogens can be transported by the CSF bulk flow (in humans approx. 20 ml/h) either through the arachnoid granulations into the blood or along cranial and spinal nerve roots into regional lymph nodes (Davson et al., 1987). Meningeal macrophages and the glia limitans, composed of astrocytic foot processes and a parenchymal basement membrane, impede the spread of pathogens from the CSF space into the brain tissue.

Bacteria can cross the blood–brain and blood–CSF barrier by (a) destruction of the endothelial cell layers (e.g., by pneumococcal pneumolysin), (b) traversal in between the cells of the barriers by disruption of the tight junctions, and (c) transcytosis, an intracellular transport route designed to transport molecules and vesicles through cells from the apical to basolateral side (Tenenbaum et al., 2009; Iovino et al., 2013). The adhesion of *Escherichia coli*, which reaches the brain by transcytosis, to membrane receptors of the cerebrovascular endothelium triggers a cascade of host cell signal transduction pathways resulting in host cell actin cytoskeleton rearrangements. This process involves host actin binding proteins and signaling molecules (e.g., Rac-1) and microbial determinants (e.g., IbeA and OmpA; Maruvada and Kim, 2012). *Streptococcus pneumoniae* preferentially adheres to the subarachnoid vessels, and only at the later stages of infection interacts with the endothelium of the choroid plexus (Iovino et al., 2013).

We hypothesize that the entry of a pathogen into the CNS probably is not uncommon. Yet, in an immunocompetent host the vast majority of pathogens, which eventually reach the brain tissue, are either eliminated or controlled in a latent form by the immune cells of the brain parenchyma, in particular, the microglial cells.

The immune defense of the CNS has been compared with a medieval castle. The blood–brain and blood–CSF barriers serve as the outer walls of the castle. The castle moat is represented by the CSF space. The second wall is represented by the glia limitans and resident macrophages. Inside the castle, i.e., the CNS parenchyma, "the royal family of sensitive neurons resides" protected by (micro)glial cells (Engelhardt and Coisne, 2011). Evidence for protective and reparative functions

of microglial cells in the CNS has been found in diverse neurologic diseases, particularly in Alzheimer's disease, stroke and excitotoxic brain injury (Anrather et al., 2011; Naert and Rivest, 2011; Woo et al., 2012). The beneficial aspects of the immune response in the nervous system are beginning to be appreciated and their potential as pharmacologic targets in neurologic disease is being explored (Graber and Dhib-Jalbut, 2009). In a mouse model of Alzheimer's disease, repeated systemic injections of monophosphoryl lipid A, a LPS-derived Toll-like receptor 4 (TLR4) agonist that exhibits immunomodulatory properties at non-pyrogenic doses induced a potent phagocytic response by microglia, reduced the amyloidβ load in the brain and enhanced cognitive function (Michaud et al., 2013).

The density of microglial cells in brain tissue depends on the brain region. The healthy mouse brain contains an average of approximately 7000 cells/μl, i.e., the same order of magnitude as the density of leukocytes in the blood (calculated from Lawson et al., 1990 assuming a volume of the adult mouse brain of 0.5 ml). Microglia are the most abundant immune cells of the CNS. In their "resting" state, they continuously survey their environment with highly mobile processes (Nimmerjahn et al., 2005; Raivich, 2005). "Microglia are not notorious miscreants lurking in the CNS to harm neurons on any occasion. They are not placed there simply as a risk factor" (Hanisch, 2013). We are convinced that they are the key players to eliminate or at least control the replication of pathogens, which have entered the brain and spinal cord despite the fortifications surrounding the nervous tissue.

Statement: in healthy individuals, the protection of the CNS against infections relies both on the integrity of the blood–CSF and blood–brain barrier and on resident phagocytes, in particular microglial cells and perivascular and meningeal macrophages.

PATHOPHYSIOLOGICAL ASPECTS OF ACUTE OR CHRONIC CNS INFECTIONS

Bacterial meningitis, meningoencephalitis, and brain abscess are life-threatening diseases with a high incidence in neonates, infants and in the immunocompromised and elderly. Besides classical pathogens (*S. pneumoniae, Neisseria meningitides, Haemophilus influenzae*) the causative agents of CNS infections in persons with an impaired immune system are other bacteria including Gram-negative aerobic rods, group B streptococci, *Nocardia* spp. and *Listeria monocytogenes* (Bouadma et al., 2006; Cabellos et al., 2008, 2009; Gaschignard et al., 2011; Lubart et al., 2011). Immunocompromised patients also are susceptible to meningitis and encephalitis caused by a variety of fungi, most frequently *Cryptococcus neoformans* in AIDS patients and *Aspergillus* spp. in patients on glucocorticoids and/or immunosuppressants. In bacterial and fungal meningitis, pathogens probably frequently cross the choroid plexus (i.e., the blood–CSF barrier; Tenenbaum et al., 2009), whereas in encephalitis and brain abscess, they often cross the cerebrovascular endothelium (i.e., the blood–brain barrier). In meningitis, encephalitis, and brain abscess, the causative pathogens also can enter the brain through a skull defect or along (thrombosed) vessels crossing the skull.

Many viruses have a propensity to cause latent infections and persist in cells of the central or peripheral nervous system. The majority of these viruses belong to the family of *Herpesviridae* (Traylen et al., 2011): herpes simplex virus (HSV)-1, HSV-2, varicella zoster virus (VZV), Epstein–Barr virus (EBV), cytomegalovirus (CMV), human herpesvirus (HHV)-6 and HHV-7. VZV, the most frequent viral cause of a reactivated infection of the nervous system (Herpes zoster, encephalitis, meningitis, myelitis, vasculitis), principally persists in neurons, and only occasionally in other cells of the CNS (Cohen, 2007). Whereas the incidence of HSV-1 encephalitis apparently is not increased in the immunocompromised host, the strongest risk factor for the development of VZV reactivation is age because of the age-related natural decline in cellular immunity to VZV. Furthermore, VZV, CMV, HSV-2, and HHV-6 and -7 typically cause reactivated infections in immunosuppressed individuals.

Despite antiretroviral therapy (ART), HIV infection is responsible for cognitive dysfunction and neurodegeneration through persistent viral replication in the CNS, inflammation and release of neurotoxic compounds from infected and/or activated macrophages/microglia (Cross et al., 2011). Because of the poor penetration of many antiretroviral drugs across the blood–brain and blood–CSF barrier, the CNS frequently is a site of continued HIV replication even when the viral load in blood is below the detection limit (Letendre et al., 2008; Nau et al., 2010; van Lelyveld et al., 2010).

Of the family of *Polyomaviridae*, JC virus causes progressive multifocal leukencephalitis (PML). The incidence of PML is <0.3 per 100,000 persons/year in the general population. In persons with an immune defect, either by an underlying disease or by the administration of monoclonal antibodies such as natalizumab, rituximab, efalizumab, and infliximab, or other immunosuppressants in the treatment of autoimmune or malignant disease the incidence is increased (Bellizzi et al., 2013) to 2.4 cases per 1000 persons/year in HIV-infected individuals without combination antiretroviral therapy (cART) and to 2.1 cases per 1000 patients/year in multiple sclerosis (MS) patients treated with natalizumab (Hirsch et al., 2013). Because natalizumab blocks α4-integrin-dependent lymphocyte entry into the brain, not the overall cellular immunodeficiency but the failure of the brain's immune surveillance is considered responsible for the development of PML (Hirsch et al., 2013). JCV DNA was detected in oligodendrocytes, astrocytes and cerebellar granular cell neurons of the brains of humans without PML. The most common site for viral latency was cortical oligodendrocytes (65% of the samples analyzed). Immunocompromised patients more frequently harbored JCV DNA in cerebellar granular cell neurons than immunocompetent patients (Bayliss et al., 2012). This indicates that JCV DNA is present in cells of the human brain without clinical symptoms of PML and supports the hypothesis that reactivation of latent brain JCV may be central to disease pathogenesis (Bayliss et al., 2011, 2012).

The obligate intracellular parasite *Toxoplasma gondii* can infect and replicate within mammalian or avian cells including those residing in the brain. Circulating white blood cells, particularly dendritic cells and macrophages, are intracellularly infected

and allow the parasite to spread hematogeneously to the brain and muscle (Kamerkar and Davis, 2012). *In vitro*, approximately 30% of the microglial cells compared to 10% of neurons and astrocytes were intracellularly infected with *T. gondii* (Lüder et al., 1999). *In vitro*, microglial cells and astrocytes were able to inhibit parasite replication upon activation (Chao et al., 1993). Traditionally, latent infections in humans were assumed to be largely asymptomatic, but recently behavioral abnormalities including schizophrenia have been linked with latent *T. gondii* infections (Webster, 2007). In HIV-infected individuals receiving no effective antiretroviral therapy, cerebral toxoplasmosis became a major complication and an AIDS-defining disease. When the host's cerebral immune response weakens, parasite tissue cysts rupture and release bradyzoites which convert to rapidly dividing tachyzoites and cause *T. encephalitis* (Sullivan et al., 2009).

Statement: few typical pathogens only are able to overcome the immune defense of the healthy brain (e.g., *S. pneumoniae*, *N. meningitidis*, and *H. influenzae* causing acute meningitis and HSV-1 causing acute encephalitis). Conversely, in the immunocompromised host a wide spectrum of pathogens can cause acute, subacute or chronic CNS infections. The example of *T. gondii* illustrates that latent, principally well-controlled CNS infections even in immunocompetent hosts may lead to behavioral abnormalities.

RESISTANCE OF THE HEALTHY BRAIN AGAINST INFECTIONS

The resistance of the brain to infections can be studied by direct injection of rapidly multiplying pathogens in a small volume into the brain parenchyma. With this experimental approach, the immune response in the first hours determines whether the host organism survives or eventually succumbs to an infection. Since circulating leukocytes generally need several hours to migrate into the central nervous compartments (Ernst et al., 1983), the ability of the host to eliminate invaded pathogens in the first hours depends on the local immune defense, i.e., the activity of microglial cells.

For a variety of highly virulent pathogens, the healthy brain possesses a remarkable resistance to infection, e.g., the brain of an immunocompetent young mouse can clear up to 3×10^7 colony-forming units (CFU) of *Staphylococcus aureus* injected into the forebrain without developing brain abscess or meningitis (**Figure 1**). To overcome the local defense of the mouse against this pathogen and reliably produce brain abscesses in experimental conditions, *S. aureus* had to be embedded into agarose beads (Kielian and Hickey, 2000).

Even when *S. pneumoniae*, the most frequent agent causing community-acquired bacterial meningitis, is injected into the murine neocortex in a low number, depending on the injected inoculum and the mouse strain used some animals are capable to overcome the infection without development of meningitis. The inoculum size necessary to induce lethal meningitis with *E. coli*, a pathogen predominantly causing meningitis in newborns, infants, old, or immunosuppressed people, lies between the inocula necessary to induce *S. pneumoniae* and *S. aureus* meningoencephalitis (**Figures 2** and **3**).

Although white blood cells are not at the site of intracerebral infection in the first hours, granulocytopenia leads to a strong increase of the susceptibility of mice to an intracerebral injection of

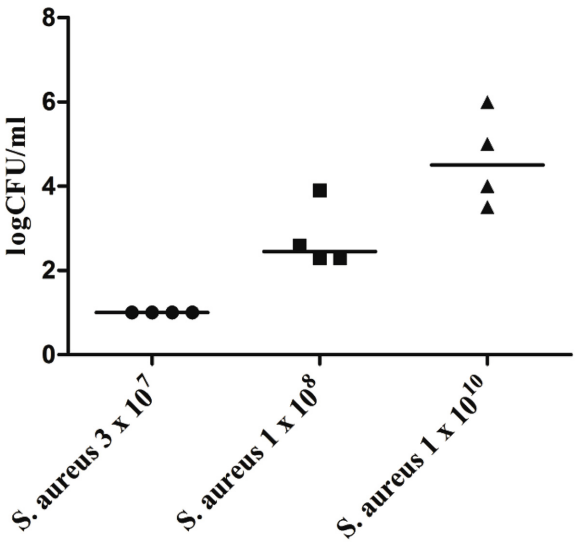

FIGURE 1 | Bacterial concentrations of *Staphylococcus aureus* American Type Culture Collection (ATCC) 29213 in cerebellar homogenates ($n = 4$ animals per inoculum) 168 h after intracerebral infection with different inocula of *S. aureus* under anesthesia. Horizontal bars indicate median values, the detection limit was 10 colony-forming units (CFU)/ml (Marija Djukic, unpublished data). Please note that all mice were able to clear an inoculum of 3×10^7 bacteria within 1 week.

S. pneumoniae or *E. coli* (**Figures 2** and **3**). This observation underlines the importance of the cross-talk between resident immune cells, the vascular endothelium and circulating leukocytes (Perry et al., 2003) for the proper functioning of microglial cells. In experimental septicaemia-induced *S. pneumoniae* meningitis, microglia appeared to sense bacterial adhesion to the endothelial cells and were activated immediately, before meningitis developed (Iovino et al., 2013).

Microglia as part of the mammalian innate immune system express germ line-encoded pattern recognition receptors (PRRs) that are crucial in the recognition of pathogens. Toll-like receptors (TLRs), RIG-like receptors (RLRs), and nucleotide-binding oligomerization domain-leucine rich repeat containing (NLR) proteins serve as PRRs that recognize different microbial structures, so-called pathogen-associated molecular patterns (PAMPs). TLR signaling and the resulting transcriptional activation of immune response genes requires the adaptor molecule myeloid differentiation factor 88 (MyD88), except for TLR3 signaling. Intact TLR → MyD88 signaling was essential for the survival in the acute phase of infection and for control of bacterial replication in both intracerebrally inoculated *E. coli* K1 (Ribes et al., 2013) and intracisternally injected *S. pneumoniae* (Koedel et al., 2004). In our experience, Myd88$^{-/-}$ mice rapidly succumbed to *E. coli* meningoencephalitis even at a very low inoculum size (**Figure 3**). After oral infection with *T. gondii*, MyD88 was essential in establishing the protective host response in the CNS (Torres et al., 2013). *T. gondii*, an intracellular protozoan parasite, establishes a latent chronic infection primarily in the brain after replication of the parasite (tachyzoite form)

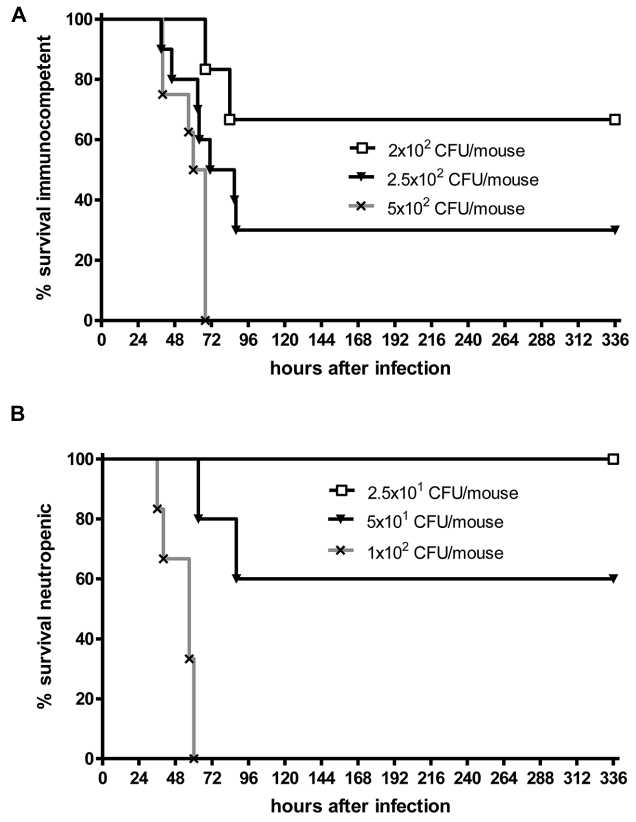

FIGURE 2 | Kaplan–Meier survival curves after intracerebral injection of different numbers of *Streptococcus pneumoniae* D39 (kindly provided by Prof. Dr. Sven Hammerschmidt, University of Greifswald, Germany). (A) C57Bl/6 immunocompetent mice, **(B)** C57Bl/6 mice rendered neutropenic with 50 μg of an anti-Ly-6G monoclonal antibody (1A8 clone). Meningitis was induced by bacterial inoculation into the right frontal neocortex under anesthesia. Please note that 70% of immunocompetent mice survived an intracerebral injection of 200 colony-forming units (CFU). Conversely, all neutropenic mice were killed by an intracerebral inoculum of 100 CFU in less than 3 days suggesting that microglia need the cross-talk with granulocytes across the blood–brain barrier to function properly (Sandra Ribes, unpublished data).

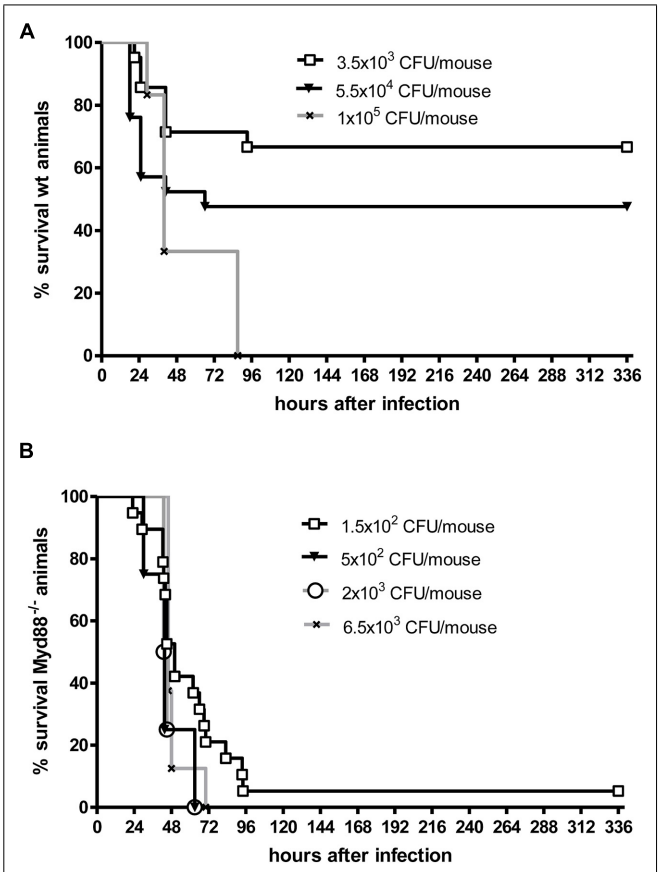

FIGURE 3 | Kaplan–Meier survival curves from (A) C57Bl/6J wild-type (wt) mice and (B) C57Bl/6J MyD88$^{-/-}$ mice following intracerebral injection of different numbers of *Escherichia coli* K1 (kindly provided by Dr. Gregor Zysk, Düsseldorf). Meningitis was induced by bacterial inoculation into the right frontal neocortex under anesthesia. Please note that 70% of immunocompetent mice survived an intracerebral injection of 3500 colony-forming units (CFU). Conversely, over 90% of MyD88$^{-/-}$ mice were killed by an intracerebral inoculum of 150 CFU in less than 4 days suggesting that MyD88 as a central signaling molecule of the innate immune system is essential for the inactivation of bacteria by parenchymal microglia and macrophages (Sandra Ribes, unpublished data).

in various organs during the acute stage of infection. While other organs (e.g., liver, lungs, heart, and small intestine) were not affected, in Myd88$^{-/-}$ mice mortality was caused by severe toxoplasmic encephalitis which correlated with low numbers of CD8^{+} T cells and a significantly higher infiltration of the brain with CD11b^{+} and F4/80^{+} cells compared to Myd88$^{+/+}$ control mice (Torres et al., 2013). F4/80^{+} cells were together with CD11c^{-} and CD11b^{+} cells responsible for parasite dissemination to the brain (Courret et al., 2006; Torres et al., 2013). Macrophage/microglial activation was observed all over different brain regions of *T. gondii*-infected Myd88$^{-/-}$, and only deficient mice showed numerous clusters of ameoboid microglial cells (Torres et al., 2013).

TLR3 is a sensor for viral double-stranded RNA (dsRNA) which signals through the Toll–interleukin-1 (IL-1) receptor (TIR)-domain-containing adaptor inducing IFN-β (TRIF). A protective role of the TLR3-TRIF-mediated pathway was reported in response to Poliovirus (PV), the causative agent of poliomyelitis (Abe et al., 2012). TLR3 and TRIF signaling pathways inhibited PV replication in non-neural tissues thereby influencing the viral invasion of the CNS. TRIF-deficient mice (trif^{lps2}) were not more susceptible to intracerebral *E. coli* K1 infection than wild-type mice of the same age and background (Ribes et al., 2013).

Statement: the resistance of the CNS of healthy hosts to infection as assessed by direct injection of pathogens into the brain tissue is higher than often perceived. The infection resistance relies on the ability of microglial cells and resident macrophages to phagocytose and kill pathogens, which is strengthened by the cross-talk with circulating immune cells.

MICROGLIA CAN BE STIMULATED TO PHAGOCYTOSE AND KILL BACTERIA AND FUNGI

In vitro, unstimulated microglia phagocytosed *E. coli* and *S. pneumoniae* at a low rate. Phagocytosis was stimulated by TLR2, 3,

4, and 9 agonists and by the nucleotide-binding oligomerization domain-containing protein 2 (NOD2) agonist muramyl dipeptide (Ribes et al., 2009, 2010a,b, 2012). These agents also increased intracellular bacterial killing. The presence of a bacterial capsule as one important virulence factor decreased the rate of phagocytosis of *E. coli* and *S. pneumoniae* by one order of magnitude compared to unencapsulated strains (Ribes et al., 2009, 2010a,b, 2012). Agonists of the innate immune system also enhanced the phagocytosis of *Cryptococcus neoformans* (Redlich et al., 2013). The increase of phagocytosis by stimulation of microglial cells via agonists of receptors of the innate immune was accompanied by a release of NO and proinflammatory cytokines.

Phagocytosis of *Mycobacterium tuberculosis* by microglial cells can be inhibited by antibodies against CD14, which together with TLR4 forms the LPS receptor (Peterson et al., 1995). Microglial cells also can phagocytose the predominantly intracellular pathogen *L. monocytogenes*. After bacterial ingestion, microglia appeared to act as Trojan horses, transporting and releasing the phagocytosed bacteria inside the brain tissue after systemic infection (Serbina et al., 2008; Remuzgo-Martínez et al., 2013). To our knowledge, intracellular killing of mycobacteria and *L. monocytogenes* by microglial cells has not been studied. Among human monocytes, two subsets indistinguishable by the expression of cell surface markers involved in the phagocytosis of microbes were observed: approximately 75% of all monocytes were not very active in phagocytosing *L. monocytogenes*, but restricted intracellular growth. Approximately 25% could ingest a large number of bacteria and permitted intracellular growth of *L. monocytogenes* (Zerlauth et al., 1996). Monocytes which were able to kill *L. monocytogenes*, secreted more TNFα than those who allowed intracellular replication of bacteria (Zerlauth et al., 1996). *In vitro*, microglias were able to phagocytose and inhibit the growth of *T. gondii* in a NO-dependent manner, which was stimulated by IFNγ and LPS. In contrast to microglial cells, uptake of *T. gondii* into astrocytes was parasite-driven, and astrocytes were unable to inhibit multiplication of tachyzoites suggesting that astrocytes may provide a safe harbor for *T. gondii* (Peterson et al., 1993).

For the phagocytosis and intracellular killing of many pathogens, data on microglial cells are incomplete. The contribution of monocytes to the control of other pathogens able to cause CNS infections including fungi is compiled in a recent review (Serbina et al., 2008).

In *E. coli* experimental meningitis, pre-treatment with the TLR9 agonist cytosine-guanine oligodeoxynucleotide 1668 (CpG) strongly increased survival of neutropenic C57Bl6 mice. The protective effect was associated with long-term increased levels of IL-12/IL-23p40 in serum and spleen. CpG-treated neutropenic mice had reduced bacterial concentrations in brain and spleen 42 h after infection (Ribes et al., 2014). Although the administration of CpG caused sickness behavior and the release of proinflammatory cytokines, no long-term neurological abnormalities were noted in surviving CpG-treated mice. This work indicates that systemic immunostimulation with a TLR9 agonist can not only protect against systemic bacterial infections but also against an intracerebral bacterial challenge (Ribes et al., 2014). In microglial-neuronal co-cultures, stimulation of microglia by Pam_3CSK_4 (TLR2), LPS (TLR4), and CpG (TLR9) caused injury to the neurons starting at the axons (Iliev et al., 2004; Schütze et al., 2012). Therefore, the approach may entail the risk of inducing collateral damage to the nervous tissue.

Statement: microglia can be stimulated by a variety of compounds to phagocytose and kill pathogens *in vitro*. Preliminary data suggest that systemic administration of these compounds can also increase the resistance of the brain to infections. This approach, however, may entail the risk of inducing collateral damage to the nervous tissue

MICROGLIAL CYTO- AND CHEMOKINE RELEASE DEPENDS ON THE PROINFLAMMATORY STIMULUS AND THE SUBSET OF MICROGLIAL CELLS ACTIVATED

The microglial response upon contact with products of infectious agents or synthetic analogs of them is not uniform: in primary microglial cultures from newborn mice, incubation of microglia with agonists of the TLR2 (0.1 μg/ml Pam_3CSK_4), TLR4 (0.01 μg/ml LPS), and TLR9 (1 μg/ml CpG) at the lowest concentrations inducing maximum NO release caused a comparable release of TNFα (Ribes et al., 2009). *In vitro* and *in vivo*, however, synthesis of TNFα is not a common feature of all microglia, but restricted to a specific subset. The proportion of TNFα-producing microglia increased from approx. 30% in neonatal to 75% in young adult microglial cells (Scheffel et al., 2012; Hanisch, 2013). The synthesis of CCL3 (macrophage inflammatory protein 1α, MIP-1α) was restricted to a rather small subpopulation, and most of the CCL3-synthesizing cells belonged to the (larger) population of TNFα producers (Scheffel et al., 2012; Hanisch, 2013).

Unlike TNFα, stimulation of microglia with the same TLR agonists at equal potencies resulted in a divergent release of CXCL1. Pam_3CSK_4 at 0.1 μg/ml was the stimulus that induced higher CXCL1 concentrations in the cell culture supernatants compared to 0.01 μg/ml LPS and 1 μg/ml CpG (Ribes et al., 2009). While stimulation with the TLR2 agonist Pam_3CSK_4 led to a predominant release of IL6, incubation with the TLR4 ligand LPS produced the highest levels of CCL5 (RANTES) (Ribes et al., 2010b). The subset distribution of the cells releasing these cyto- and chemokines remains to be determined. Our data, however, demonstrate that microglial cells do not respond uniformly to infectious stimuli, but are able to mount a nuanced reaction.

Human innate immune cells stimulated by TLR agonists selective for TLR7 or TLR8 also did not react in a uniform way: TLR7 agonists directly activated plasmacytoid dendritic cells and, to a lesser extent, monocytes. On the contrary, TLR8 agonists directly activated myeloid dendritic cells, monocytes, and monocyte-derived dendritic cells. TLR7-selective agonists were more potent than TLR8-selective agonists at inducing IFN-inducible protein and IFN-inducible T cell alpha chemoattractant from human mononuclear cells. Conversely, TLR8 ligands were more effective than TLR7 agonists at inducing TNFα, IL-12 and MIP-1α (Gorden et al., 2005). In microglia, the TLR4 co-receptor CD14 played an important role in controlling the profiles of cyto/chemokine production. Dependent on the TLR4 agonist (different classes of LPS, fibronectin) and the activation of co-receptors, distinct signaling

routes are activated leading to a modulation of response profiles of key cytokines (Regen et al., 2011). Hence, for the immune response of microglial cells it also appears of importance which TLR and which co-receptor(s) are stimulated.

Statement: unlike previously thought, an accumulating body of evidence demonstrates the diversity of microglial cells. This opens avenue to selectively stimulate sub-populations responsible for the defence against pathogens without stimulating sub-populations which are responsible for collateral damage to the nervous tissue.

THE EFFICIENCY OF MICROGLIA TO PREVENT CNS INFECTIONS DEPENDS ON CO-OPERATION WITH CIRCULATING IMMUNE CELLS

Microglia and CNS macrophages cross-talk with circulating blood cells by several mechanisms (Perry et al., 2003): (a) the bi-directional passage of circulating cytokines or pro-inflammatory pathogen compounds through leaks of the blood–brain barrier (physiologically in the circumventricular organs, under pathological conditions at lesion sites with an impaired blood–brain barrier), (b) the activation of endothelial cells and perivascular macrophages either by circulating or brain-derived compounds, (c) via the vagus nerve. $CD11b^{+}Ly\text{-}6G^{+}Ly\text{-}6C^{int}$ granulocytes and $CD11b^{+}Ly\text{-}6G^{-}Ly\text{-}6C^{high}$ monocytes in the bloodstream were required for the control of intracerebrally injected *E. coli* K1 (Ribes et al., 2013). Animals that received intraperitoneally an anti-Gr-1 antibody (clone RB6-8C5) showed an earlier and higher mortality than anti-IgG_{2b}-treated mice (92.3 vs. 11.1%, $P = 0.0002$). Depletion of $CD11b^{+}Ly\text{-}6G^{+}Ly\text{-}6C^{int}$ granulocytes from the systemic circulation by intraperitoneal injection of the anti-Ly-6G antibody (clone 1A8) also had an influence on the survival of *E. coli* K1 infected mice (mortality 59.2% compared to 34.6% in anti-IgG_{2a}-treated animals, $P = 0.049$). We hypothesize that after intracerebral bacterial infection the primary immune response determines whether the infected organism will survive without antibiotic treatment or will eventually succumb to the infection. This is supported by the course of the clinical score in mice. All animals which develop serious symptoms of infection eventually die, unless they are treated with antibiotics. Animals which eventually survive either show no signs of infection or are mildly lethargic in the first hours after infection and then recover. Immediately after inoculation of bacteria, before bacteria start to multiply, resident phagocytes as the primary line of defence have the opportunity to phagocytose and kill the inoculum. Data from experimental *S. pneumoniae* meningitis models suggest that after bacterial inoculation granulocytes need approx. 12 h (e.g., Ernst et al., 1983) to migrate into the CSF, i.e., in the first hours after infection the resident phagocytes are on their own. When the inoculum is high enough to overcome the primary immune defence of resident phagocytes, invading granulocytes are not able to control multiplication of bacteria in the intracranial compartments. Granulocyte invasion therefore represents a futile secondary immune response in this condition. In the CSF of rabbits rendered leukopenic by nitrogen mustard, the growth rate of *S. pneumoniae* was slightly increased (mean generation time 60 vs. 67 min), and ultimate bacterial density in the CSF was slightly higher than in immunocompetent rabbits, i.e., leukocytes did not effectively slow or limit the growth of pneumococci in the CSF *in vivo* (Ernst et al., 1983). For these reasons, the strong detrimental effect of neutropenia on the resistance of the brain to intracerebral *E. coli* infection was unexpected and can be only explained by the cross-talk of resident phagocytes and circulating leukocytes across the blood–brain and blood–CSF barrier (Ribes et al., 2013).

Statement: the protective action of microglia against infections critically depends on the cross-talk with circulating granulocytes and monocytes in the first hours, before circulating leukocytes enter the brain and CSF. For this reason, immunosuppressive measures affecting all or specific subsets of circulating leukocytes probably bear the risk of also affecting microglial function by impairing this cross-talk.

PHAGOCYTOSIS AND KILLING OF PATHOGENS BY MICROGLIA DOES NOT NECESSARILY REQUIRE NITRIC OXIDE OR CYTOKINE RELEASE

Bacterial clearance by phagocytes of the central nervous compartments apparently does not always require the presence of interferonγ (IFNγ) and nitric oxide (NO): in experimental *S. pneumoniae* meningitis, $IFN\gamma^{-/-}$ mice showed reduced gene expression of NO synthase, but bacterial clearance was enhanced in $IFN\gamma^{-/-}$ compared to wild-type infected animals (Mitchell et al., 2012). *In vitro*, the addition of 100 U/ml IFNγ did not increase the uptake of an unencapsulated strain of *E. coli* by TLR4-stimulated microglia while it moderately increased the bacterial ingestion by TLR2- and TLR9-stimulated cells (**Figure 4**). In microglial cultures pre-stimulated with different agonists of the innate immune system, NO release and phagocytosis of bacteria do not strongly correlate (Ribes et al., 2012; **Figure 5**).

At present, it is unclear whether all microglial cells or only a fraction of them is involved in the phagocytosis and inactivation of pathogens. Myelin uptake under pathophysiological conditions apparently is executed by a fraction of microglia (Scheffel et al., 2012; Hanisch, 2013). For this reason, it is probable that a subgroup of microglial cells bears phagocytic activity against pathogens (Hanisch, 2013). Selective stimulation of the activity of the subgroup(s) of microglial cells, which phagocytose pathogens, appears a feasible goal of future research.

Statement: since NO release probably is a main cause of collateral damage to nervous tissue after microglial activation, the finding that elimination of pathogens does not necessarily depend on NO release encourages the search for stimulants which increase pathogen clearance without inducing damage to the nervous tissue.

ANTIBIOTIC TREATMENT: REDUCING THE RELEASE OF PROINFLAMMATORY COMPOUNDS OF PATHOGENS IN ORDER TO PREVENT EXCESSIVE MICROGLIAL ACTIVATION

Injury and mortality of bacterial meningitis is caused by the joint action of multiplying bacteria and released bacterial products, the local immune response of the brain and by granulocytes and monocytes invading the subarachnoid space and nervous tissue from the blood (Nau and Brück, 2002; Mook-Kanamori et al., 2011; Nau et al., 2013).The contribution of these individual mechanisms depends on the pathogen and the host's immune response. Microglial cells stimulated by bacterial products can kill neurons

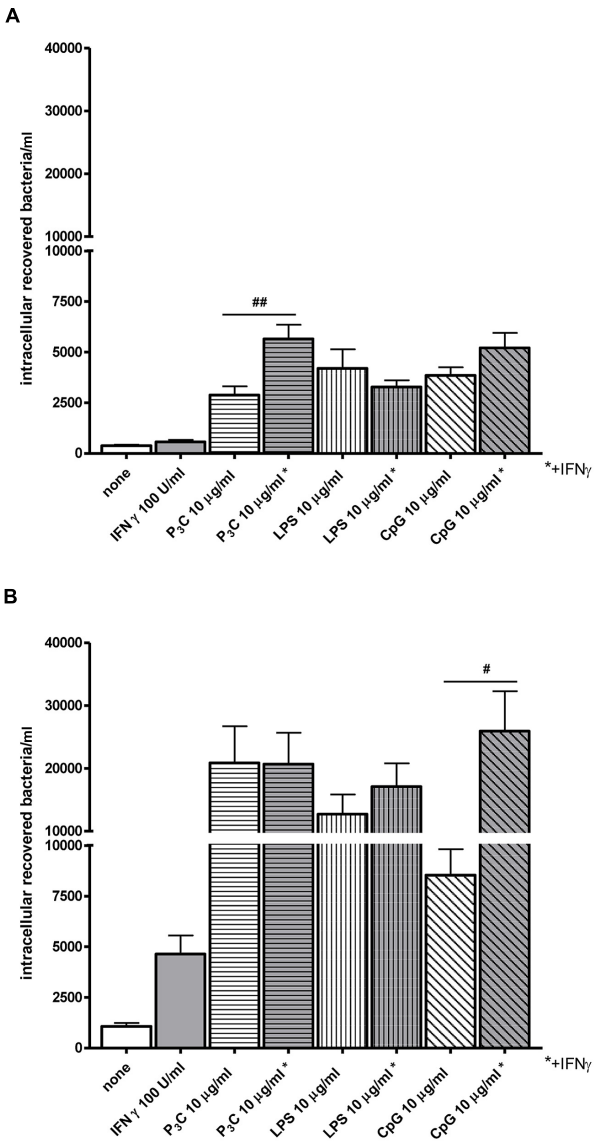

FIGURE 4 | Effect of interferonγ (IFNγ) on bacterial phagocytosis by Toll-like receptor-stimulated primary microglia. (A) 30 min and **(B)** 90 min of phagocytosis of *E. coli* DH5α by microglial cells after 24 h of stimulation with the TLR agonists: 0.1 μg/ml Pam_3CSK_4 (P3C), 0.01 μg/ml LPS and 1 μg/ml CpG alone or in combination with 100 U/ml IFNγ. After stimulation, cells were washed and bacteria were added for different time periods (30 and 90 min). Then, gentamicin (200 μg/ml) was added for 1 h to kill extracellular bacteria. Thereafter, bacteria were lysed in 0.1 ml of distilled water. The number of ingested bacteria was determined by quantitative plating of the cell lysates after the different incubation intervals. Data are shown as recovered bacteria (CFU) per well [mean ± SEM (error bars)]. Data were analyzed using one-way ANOVA followed by Bonferroni's multiple comparison test ($^{\#}P < 0.05$; $^{\#\#}P < 0.01$; Sandra Ribes, unpublished data).

in vitro (Iliev et al., 2004). Reduction of the amount of proinflammatory/toxic pathogen-derived products avoids overstimulation of resident and migrating immune cells including microglia and thereby protects the nervous tissue. For this reason, it appears desirable to keep the concentrations of proinflammatory products of pathogens in the central nervous tissue low during the whole course of an infection including its treatment. This aspect of microglial stimulation has been most extensively studied in bacterial meningitis, but probably is of importance also for other CNS infections, particularly for those with a high pathogen load.

In clinical practice, a favorable outcome depends on rapid antibiotic treatment after hospital admission (Auburtin et al., 2006). Although many antibiotics commonly used in the management of bacterial meningitis cause bacterial lysis and subsequent release of proinflammatory or directly cytotoxic compounds, rapid antibiotic treatment stops bacterial replication and production of bacterial products and therefore on the long run reduces the concentrations of proinflammatory/toxic bacterial products in the CNS (Stuertz et al., 1998; Spreer et al., 2003). *In vitro*, temporarily the pneumolysin release was higher in ceftriaxone-treated *S. pneumoniae* compared to untreated *S. pneumoniae* cultures. After 3 h, however, the pneumolysin release during spontaneous growth exceeded the release after initiation of ceftriaxone treatment. In particular, a strong increase of extracellular pneumolysin was observed when untreated pneumococci reached the end of the logarithmic growth phase (Spreer et al., 2003).

In contrast to the classical view considering bactericidal and bacteriolytic synonyms, the cidal and the lytic effects of an antibiotic do not necessarily coincide. With cell-wall active antibacterials, in particular beta-lactam antibiotics, lytic and cidal action are tightly linked. Bactericidal antibiotics acting by the inhibition of RNA or protein synthesis or DNA replication (rifamycins, macrolides, clindamycin, ketolides, and with some limitations also quinolones) circumvent or at least delay bacterial lysis (for review, see Nau and Eiffert, 2002, Nau et al., 2013). In animal models of bacterial meningitis, rifampicin, clindamycin and daptomycin reduced inflammation, mortality, neuronal injury or/and neurological long-term sequelae compared to the standard therapy with beta-lactam antibiotics (Nau et al., 1999; Gerber et al., 2003; Böttcher et al., 2004; Grandgirard et al., 2007, 2012; Spreer et al., 2009; Barichello et al., 2013; Nau et al., 2013).

Statement: the reduction of potentially deleterious pathogen-derived compounds by rapid initiation of an effective antibiotic therapy or by choosing compounds which do not release large amounts of pathogen products is a promising strategy to avoid overstimulation of microglial cells and decrease neuronal injury.

PLEIOTROPIC COMPOUNDS INHIBITING MICROGLIAL FUNCTION

The role of corticosteroids in infections of the CNS has been debated for decades.

In vitro, dexamethasone inhibited the release of TNFα and IL-1β by microglia after exposure to LPS (Forshammar et al., 2013). After dexamethasone exposure, pronounced transcriptional effects were observed in microglia, where 257 genes were differentially expressed. The majority of these genes were related to the immune function (Jenkins et al., 2013). Conversely, corticosteroids can delay the production of myelin. In high-purity cell cultures, however, oligodendrocyte lineage cells were not influenced by exposure to dexamethasone.

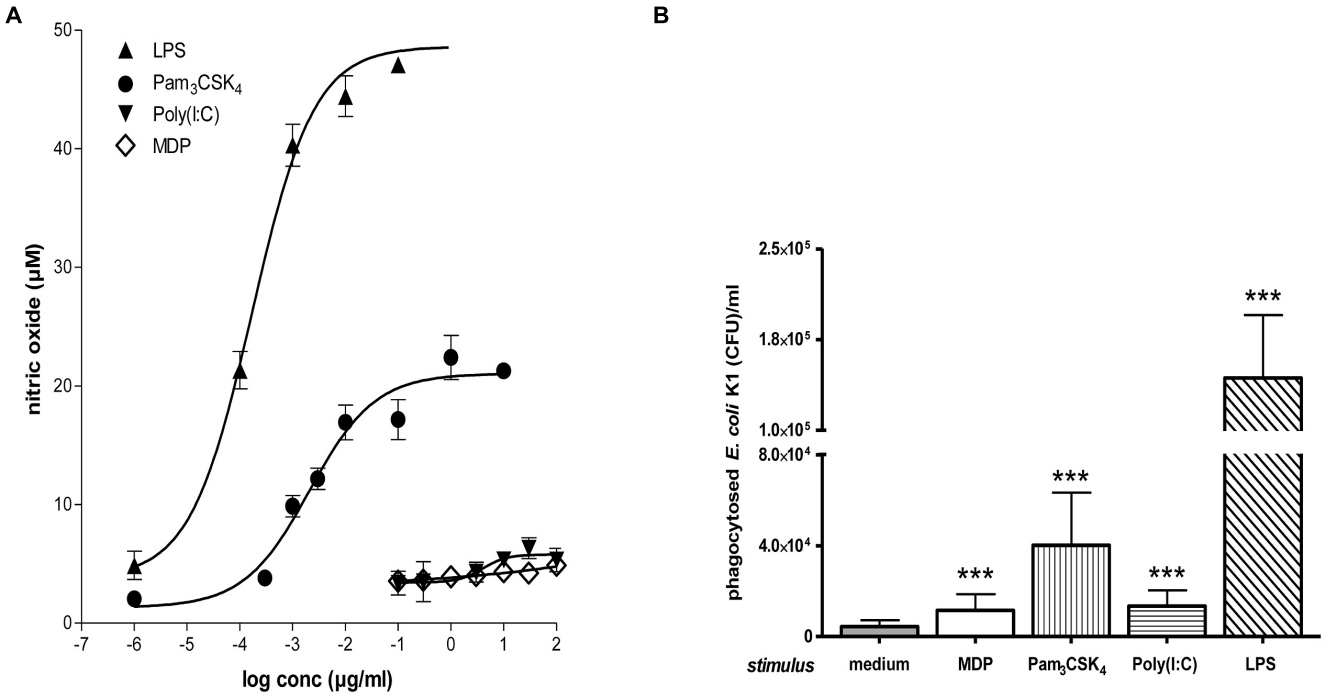

FIGURE 5 | Microglial NO release and phagocytosis of bacteria. **(A)** Stimulation with TLR2 or 4 agonists induced stronger microglial nitric oxide (NO) release than stimulation with a TLR3 agonist or muramyl dipeptide (MDP). Dose–response relations after 24 h treatment with various concentrations of agonists of TLR1/2 (Pam_3CSK_4), TLR3 [poly(I:C)], TLR4 (LPS), and NOD2 (MDP; Ebert et al., 2005; Ribes et al., 2009; Ribes et al., 2012). **(B)** Phagocytosis of *E. coli* K1 strain by microglial cells after 24 h of stimulation with the agonists at the lowest concentration inducing maximum NO release: 0.1 µg/ml Pam_3CSK_4, 10 µg/ml poly(I:C), 0.01 µg/ml LPS, and 10 µg/ml MDP (means ± SD, ***$p < 0.001$ vs. medium-treated cells); Student's *t* test and correction for repeated testing by the Bonferroni–Holm method. Please note that although stimulation with poly(I:C) and MDP released low amounts of NO, both compounds led to a substantial increase of bacterial phagocytosis (Figure reproduced from Ribes et al., 2012, with permission of the publisher).

Untreated microglial cell cultures showed a branching ramified morphology indicative of the "resting" state. Dexamethasone exposure dramatically reduced cell densities, and thereafter many cells showed a rounded appearance. Corticosteroid-treated microglia may demonstrate an impaired ability to migrate toward and phagocytose cells/synapses and myelin debris and probably thereby indirectly affects myelination (Jenkins et al., 2013). Whether corticosteroids also inhibit phagocytosis and intracellular inactivation of pathogens by microglia, remains to be studied.

Dexamethasone as an adjunct to antibiotic treatment in the adult rabbit model of *S. pneumoniae* and in the infant rat models of *Streptococcus* group B and *S. pneumoniae* meningitis aggravated apoptotic neuronal injury in the hippocampal dentate gyrus and impaired long-term learning capacity of surviving rats in the Morris water maze compared to rats treated with an antibiotic only (Leib et al., 1996, 2003; Zysk et al., 1996; Spreer et al., 2006; Nau et al., 2013). There is, however, no evidence of an aggravation of human hippocampal injury in bacterial meningitis by adjunctive dexamethasone (Brouwer et al., 2013; Nau et al., 2013). Data from clinical trials at present point to a beneficial effect of corticosteroids as an adjunct to antibiotic therapy in community-acquired acute bacterial and Tuberculous meningitis (Thwaites et al., 2009; Brouwer et al., 2013). In other CNS infections (e.g., cerebral toxoplasmosis, brain abscess, HSV encephalitis), glucocorticoids should be used only in the presence of a life-threatening brain edema.

Cytostatics such as mitoxantrone and cyclophosphamide and the antimetabolites methotrexate and azathioprine act on a variety of proliferating cells and as immunosuppressants probably also inhibit proliferation and function of microglial cells (e.g., Vairano et al., 2004; Li et al., 2012). New agents used for the treatment of MS also have pleiotropic effects including a direct influence on microglial activity. Fingolimod inhibits autoreactive lymphocytes from infiltrating the CNS, but also downregulates the production of the pro-inflammatory cytokines TNFα, IL-1β, and IL-6 by activated microglia (Noda et al., 2013).

Statement: the long-term use of pleiotropic compounds affecting microglial function increases the risk of CNS infections. In community-acquired bacterial meningitis, dexamethasone as an adjunct to antibiotic treatment at present represents the standard therapy to inhibit the systemic inflammatory and the local microglial response of the host. Corticosteroids or other pleiotropic immunosuppressive compounds probably are not the ideal agents for this purpose, because they affect the ability of microglia to clear pathogens. This is of particular importance, when antiinfectives are not able to kill all causative pathogens.

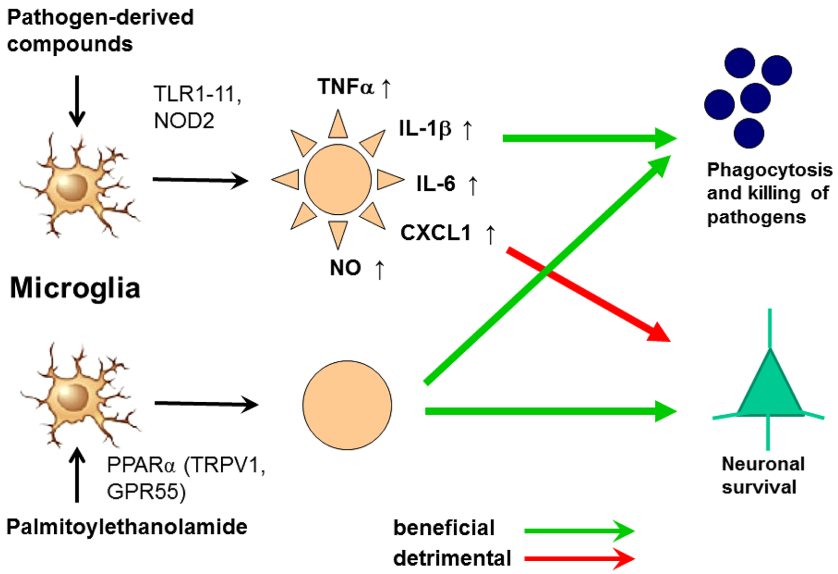

FIGURE 6 | Activation of microglia by Toll-like receptor (TLR) agonists and by palmitoylethanolamide (PEA). The activation by both ways leads to an increase of phagocytosis and intracellular killing of pathogens. Stimulation of one or several TLR or nucleotide-binding oligomerization domain-containing protein 2 (NOD2) receptors causes the release of proinflammatory products from microglial cells causing neuronal injury in microglial-neuronal co-cultures and probably also in vivo. PEA also increases phagocytosis and intracellular killing of pathogens. To our knowledge, it does not release proinflammatory mediators. For this reason we hypothesize that it will not cause collateral neuronal injury. PEA probably acts via the peroxisome proliferator-activated receptor (PPAR)α, but also is a ligand of the transient receptor potential vanilloid-1 (TRPV1) and the orphan G-protein coupled receptor GPR55 (De Petrocellis et al., 2001; LoVerme et al., 2005, 2006; Ryberg et al., 2007; Esposito and Cuzzocrea, 2013).

COMPOUNDS STIMULATING PHAGOCYTOSIS BY MICROGLIAL CELLS WITHOUT INDUCING AN INFLAMMATORY REACTION

Palmitoylethanolamide (PEA) is a small endogenous lipid (molecular mass 299.5 g/mol) which is widely present in cells including microglia (Muccioli and Stella, 2008), tissues and body fluids. It has analgesic, anticonvulsant, neuroprotective, antipyretic and anti-inflammatory properties. Its actions depend mainly on the peroxisome proliferator-activated receptor (PPAR)α, but it also is a ligand of the transient receptor potential vanilloid-1 (TRPV1) and the orphan G-protein coupled receptor GPR55 (De Petrocellis et al., 2001; LoVerme et al., 2005, 2006; Ryberg et al., 2007; Esposito and Cuzzocrea, 2013). In a murine Theiler's virus model of chronic MS, treatment with PEA (5 mg/kg) between days 60 and 70 post-infection resulted in a strong improvement of motor deficits caused by a reduction of microglial activation observed in untreated mice (Loría et al., 2008).

In spite of its anti-inflammatory properties, 30 min pre-treatment with PEA stimulated phagocytosis of *S. pneumoniae* (EC_{50} 5.9 nM) and *E. coli* (EC_{50} 23 nM) by microglial cells *in vitro*. It was not toxic to microglial cells up to a concentration of 1000 nM. Unlike pre-stimulation with TLR and NOD2 agonists, the PEA-mediated increase of microglial bacterial uptake was not accompanied by a release of pro-inflammatory cyto-/chemokines [TNFα, IL-6, and CXCL1 (KC)], avoiding the risk of concomitant neuronal injury (Redlich et al., 2012). Preliminary data suggest that PEA also decreases the susceptibility of the brain to intracerebral injection of bacteria (Sandra Redlich, unpublished data). From 1969 to 1979, PEA was tested under the brand name Impulsin^R (SPOFA United Pharmaceutical Works, Prague, Czechoslovakia) in prophylactic and therapeutic clinical trials (five in adults, one in children): it reduced the incidence and severity of acute respiratory infections and influenza (Kahlich et al., 1979; Keppel Hesselink et al., 2013). More than 3600 patients received PEA at daily doses from 600 to 1800 mg, and no severe adverse effects were reported (Kahlich et al., 1979; Keppel Hesselink et al., 2013).These properties illustrate that PEA is a true immunomodulator and not an immunosuppressant and make PEA a promising agent to enhance the resistance of the brain against infection without carrying the risk of inducing neuronal injury (**Figure 6**). This effect may be of clinical value both in preventing bacterial CNS infections in high-risk groups and in reducing the invasion of pathogens into brain tissue in manifest meningeal infections.

Glatiramer acetate also increased phagocytosis *in vitro* (Pul et al., 2011, 2012): the ingestion of fluorescent beads was greater in monocytes from MS patients treated with glatiramer acetate than in those from healthy controls or non-treated MS patients. Only monocytes co-expressing CD16 were observed to phagocytose, and addition of IL-10 did not decrease phagocytosis (Pul et al., 2012). Moreover, in primary rat microglia glatiramer acetate promoted the phagocytosis of fluorescent latex beads and increased IL-10 secretion, whereas it decreased the release of TNFα and did not affect NO release (Pul et al., 2011). Phagocytosis of bacteria, however, was not studied. Glatiramer acetate, a random polymer of four amino acids found in myelin basic protein (glutamic acid, lysine, alanine, tyrosine) as a relatively large hydrophilic compound (molecular mass approx. 600 g/mol) in contrast to PEA

probably crosses the blood–brain and blood–CSF barrier to a small extent only. It remains to be studied, whether the glatiramer acetate CNS concentrations are high enough to influence the phagocytic activity of microglia.

Dimethyl fumarate is a small lipophilic compound (molecular mass 144.1 g/mol). Treatment of HIV-infected human monocyte-derived macrophages with dimethyl fumarate attenuated HIV replication in a dose-dependent manner, as determined by reverse transcriptase concentrations in the culture supernatants. Dimethyl fumarate also inhibited NF-κB translocation and the release of TNFα from phytohemagglutinin-activated macrophages and reduced HIV-induced TNFα release from macrophages (Cross et al., 2011; Gill and Kolson, 2013). Dimethyl fumarate together with other esters of fumaric acid has been marketed for several years as an antipsoriasis drug. Lymphocytopenia and eosinophilia are frequent side effects, and the compound appears to increase the risk of PML, Kaposi sarcoma and nocardiosis (manufacturer's information, http://www.akdae.de/Arzneimittelsicherheit/RHB/20130625.pdf).

Statement: the data obtained with PEA and, with limitations, glatiramer acetate and dimethyl fumarate underline that the search for modulators instead of inhibitors of microglial activity appears promising.

CONCLUSION

Phagocytosis of pathogens by microglial cells can be stimulated by agonists of receptors of the innate immune system. The use of this signaling pathway to increase the resistance of the brain against infections entails the risk of inducing collateral damage to the nervous tissue. As a consequence of microglial diversity, it appears possible to identify compounds that increase pathogen uptake and elimination and thereby may contribute to the protection of the brain without a concomitant measurable proinflammatory effect. In this context, PEA, an endogenous lipid which had been tested clinically in the 1970s and apparently decreased the incidence of respiratory infections without severe side effects, appears to be most promising. Based on *in-vitro* data, glatiramer acetate and dimethyl fumarate both used for the treatment of MS appear as other potential candidates.

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Phagocytic receptors activate and immune inhibitory receptor SIRPα inhibits phagocytosis through paxillin and cofilin

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The innate immune function of phagocytosis of apoptotic cells, tissue debris, pathogens, and cancer cells is essential for homeostasis, tissue repair, fighting infection, and combating malignancy. Phagocytosis is carried out in the central nervous system (CNS) by resident microglia and in both CNS and peripheral nervous system by recruited macrophages. While phagocytosis proceeds, bystander healthy cells protect themselves by sending a "do not eat me" message to phagocytes as CD47 on their surface ligates immune inhibitory receptor SIRPα on the surface of phagocytes and SIRPα then produces the signaling which inhibits phagocytosis. This helpful mechanism becomes harmful when tissue debris and unhealthy cells inhibit their own phagocytosis by employing the same mechanism. However, the inhibitory signaling that SIRPα produces has not been fully revealed. We focus here on how SIRPα inhibits the phagocytosis of the tissue debris "degenerated myelin" which hinders repair in axonal injury and neurodegenerative diseases. We tested whether SIRPα inhibits phagocytosis by regulating cytoskeleton function through paxillin and cofilin since (a) the cytoskeleton generates the mechanical forces that drive phagocytosis and (b) both paxillin and cofilin control cytoskeleton function. Paxillin and cofilin were transiently activated in microglia as phagocytosis was activated. In contrast, paxillin and cofilin were continuously activated and phagocytosis augmented in microglia in which SIRPα expression was knocked-down by SIRPα-shRNA. Further, levels of phagocytosis, paxillin activation, and cofilin activation positively correlated with one another. Taken together, these observations suggest a novel mechanism whereby paxillin and cofilin are targeted to control phagocytosis by both the activating signaling that phagocytic receptors produce by promoting the activation of paxillin and cofilin and the inhibiting signaling that immune inhibitory SIRPα produces by promoting the inactivation of paxillin and cofilin.

Keywords: microglia, macrophage, phagocytosis, CD47, SIRPα, paxillin, cofilin, cytoskeleton

INTRODUCTION

Phagocytosis, the engulfment and internalization of large particles by phagocytes, is an innate immune function which is carried out in the central nervous system (CNS) parenchyma by resident microglia and in both CNS parenchyma and peripheral nervous system (PNS) nerves by recruited bone marrow-derived macrophages. Phagocytosis is essential for homeostasis as it clears apoptotic and aging cells, tissue repair when it removes tissue debris, fighting infection since it scavenges pathogens and combating malignancy while it eliminates cancer cells. During such phagocytosis, bystander healthy cells are protected from being phagocytosed. CD47-SIRPα interactions provide this protection when CD47 on the surface of healthy cells binds and activates the immune inhibitory receptor signal regulatory protein-α (SIRPα; also known as CD172α and SHPS-1) on the surface of phagocytes and SIRPα then produces the signaling that inhibits phagocytosis (Barclay and Brown, 2006; Matozaki et al., 2009; Oldenborg, 2013). Such observations led to define CD47 as a marker of "self" which sends a "do not eat me" message to phagocytes (Oldenborg et al., 2000).

This protective mechanism becomes detrimental under pathological conditions when tissue debris (Gitik et al., 2011) and malignant cells (Chao et al., 2012; Kim et al., 2012) employ CD47-SIRPα interactions to inhibit their own clearance. We (Gitik et al., 2011) and others thereafter (Han et al., 2012) documented in this regard that the tissue debris "degenerated myelin" inhibits its own phagocytosis as CD47 on degenerated myelin ligates SIRPα on macrophages and microglia. Intact myelin is a specialized extension of Schwann cells in the PNS and oligodendrocytes in the CNS, and further, it surrounds the larger diameter of PNS and CNS axons, enabling them fast conduction of electrical activity. Myelin breaks down in Wallerian degeneration following traumatic axonal injury (Waller, 1850; Vargas and Barres, 2007; Rotshenker, 2011) and in neurodegenerative diseases such as multiple sclerosis (Stadelmann and Bruck, 2008). The rapid phagocytosis of the tissue debris degenerated myelin so

produced is essential since it impedes repair and exacerbates disease by arresting the regeneration of severed adult axons (David and Aguayo, 1981), preventing remyelination (Kotter et al., 2006) and advancing the production of membrane attack complexes that damage nearby intact tissue (Mead et al., 2002). Thus, understanding how SIRPα inhibits the phagocytosis of degenerated myelin is of utmost importance.

Previous studies documented that SIRPα signaling involves the recruitment of tyrosine phosphatases SHP-1 and SHP-2 to the cytoplasmic domain of SIRPα, and subsequently, SHP-1 and SHP-2 may dephosphorylate phosphotyrosine sites in their immediate downstream target molecules (Barclay and Brown, 2006; Matozaki et al., 2009; Oldenborg, 2013). However, the molecular events further downstream to the SIRPα/SHP-1/2 complex have not been fully elucidated.

The phagocytosis of degenerated myelin is mediated by phagocytic receptors complement receptor-3 (CR3), scavenger receptor SRA-I/II (SRA) and Fcγ receptor (FcγR) (Reichert et al., 2001; Reichert and Rotshenker, 2003; Rotshenker, 2003). CR3 ligates both unopsonized and C3bi-opsonized degenerated myelin (i.e., opsonized by complement protein C3bi), SRA ligates unopsonized degenerated myelin, and FcγR ligates IgG-opsonized degenerated myelin (i.e., opsonized by anti-myelin antibodies). Further, degenerated myelin can simultaneously ligate several receptors. We focus in this study on the cytoskeleton as a potential target through which SIRPα may inhibit the phagocytosis of degenerated myelin which is mediated mostly by CR3, to a lesser level by SRA, and not at all by FcγR since phagocytosis was assayed in the absence of anti-myelin antibodies.

We previously documented that CR3 and SRA mediated phagocytosis of degenerated myelin involves structural changes in phagocytes. First, filopodia and lamellipodia extend and engulf the myelin-debris, and then, filopodia/lamellipodia retract, and pull-in the myelin-debris into the phagocyte where it is degraded (Hadas et al., 2012). Extension and retraction of membrane protrusions are driven by mechanical forces that are generated by the cytoskeleton. The production of membrane protrusions depends on the remodeling of filamentous actin (F-actin); i.e., the breakdown of old filaments and the production of new ones (Wang et al., 2007; Bernstein and Bamburg, 2010). Cofilin/ADF (actin depolymerizing factor) is a family of proteins that controls F-actin remodeling. Active unphosphorylated cofilin advances the remodeling F-actin and thereby the production of filopodia/lamellipodia whereas inactive phosphorylated cofilin (p-cofilin) stabilizes F-actin and thereby reduces the production of filopodia/lamellipodia. We previously documented in this regard that (a) filopodia/lamellipodia are involved in the phagocytosis of degenerated myelin (Hadas et al., 2012); (b) active unphosphorylated cofilin advances whereas inactive p-cofilin reduces phagocytosis (Hadas et al., 2012); (c) the small GTPase RhoA, which signals through ROCK, stabilizes F-actin, and further down-regulates phagocytosis (Gitik et al., 2010). The retraction of filopodia/lamellipodia can be driven by mechanical forces that are generated by contraction which is based on the interaction between F-actin and non-muscle-myosin in which motor activity was triggered (Clark et al., 2007; Vicente-Manzanares et al., 2009). We previously documented in this regard that the phagocytosis of degenerated myelin is advanced by myosin light chain kinase (MLCK) after it triggers motor activity in non-muscle-myosin which then leads to F-actin/non-muscle-myosin-based contraction (Gitik et al., 2010).

Taken that (a) active cofilin advances the phagocytosis of degenerated myelin by CR3 (an αM/β2 integrin; Hadas et al., 2012), (b) integrins promote the activation the scaffold/adaptor protein paxillin through focal-adhesion-kinase (FAK) and Src and, (c) paxillin in its active phosphorylated state (p-paxillin) can indirectly activate cofilin (Deakin and Turner, 2008), we hypothesize that SIRPα could inhibit phagocytosis by promoting the inactivation of both paxillin and cofilin (**Figure 1**). Observations made in this study support this working hypothesis, further suggesting that phagocytosis is determined by the balance between CR3 and SRA advancing phagocytosis by promoting paxillin and cofilin activation and SIRPα inhibiting phagocytosis by promoting paxillin and cofilin inactivation.

MATERIALS AND METHODS

ANIMALS

Animals Balb/C mice that were obtained from Harlan (Israel) were handled in accordance with the national research council guide for the care and use of laboratory animals and the approval of the institutional committee.

PRIMARY MICROGLIA

Primary microglia were isolated from brains of neonate mice as previously described (Reichert and Rotshenker, 2003). In brief, brains were stripped of their meninges, enzymatically dissociated, cells plated on poly-L-lysine coated flasks for 1 week, replated for 1–2 h on bacteriological plates and non-adherent cells washed away. The vast majority (over 95%) of adherent cells are microglia judged by morphology and positive immunoreactivity to Galectin-3/MAC-2, CR3, and F4/80 (Reichert and Rotshenker, 1996, 1999)

GENERATION OF MICROGLIA WITH STABLE REDUCED SIRPα EXPRESSION

Generation of microglia with stable reduced SIRPα expression was previously described and documented by us (Gitik et al., 2011). Reduction of SIRPα expression was achieved through lentiviral infection of wild-type Balb/C microglia with short hairpin RNAs directed against mouse SIRPα mRNA (SIRPα-shRNA) using pLKO.1 puro plasmids (Sigma, Israel). Three different shRNA sequences were used. All were effective in reducing SIRPα expression (60, 70, and 90% of levels in wild-type microglia). The one used in this study, as in our previous study (Gitik et al., 2011), was the SIRPα cDNA coding sequence 5′CCGGTGGTTCAAAGAACTCGAGTTCTTGCCCATCTTTGAACCATTTTTG-3′. The plasmid was transfected into a 293T-based packaging cell line, and the resulting culture supernatant was used for lentiviral infection. Infected microglia were selected on the basis of their resistance to puromycin brought by the pLKO.1 plasmid. Levels of SIRPα protein expression were monitored by immuno blot analysis. As a control, microglia were infected in a similar way with the shRNA sequence 5′CTTACGCTGAGTACTTCGA-3′ against the non-target firefly Luciferase gene. We refer to these microglia

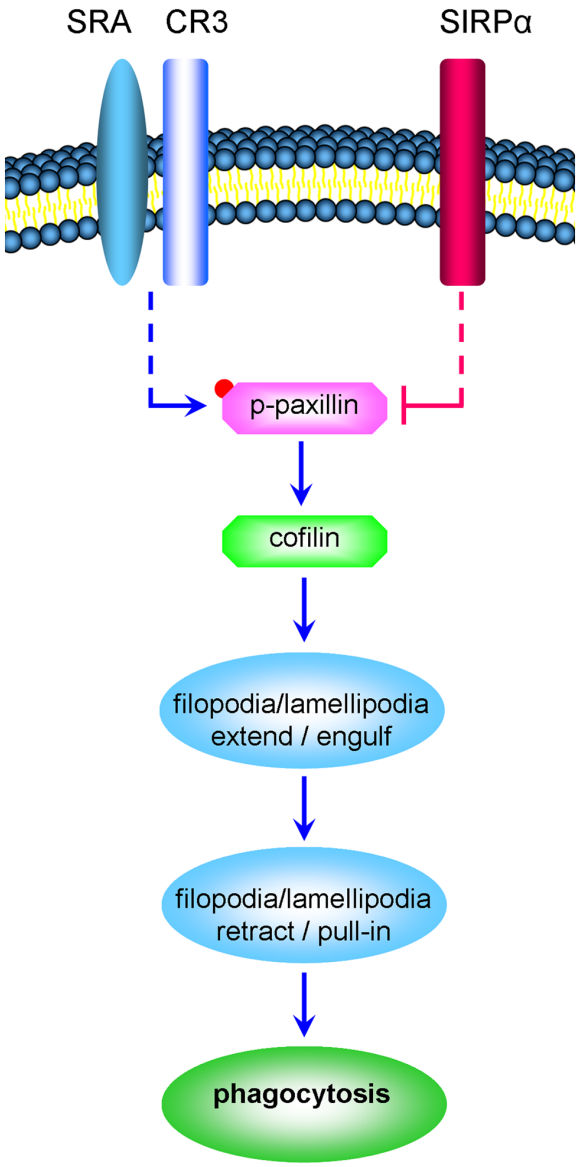

FIGURE 1 | CR3 and SRA advance the phagocytosis of degenerated myelin by promoting the activation of paxillin and cofilin whereas SIRPα inhibits phagocytosis by promoting the inactivation of paxillin and cofilin (a schematic representation of the working hypothesis). Degenerated myelin ligates simultaneously phagocytic receptors CR3 and SRA and immune inhibitory receptor SIRPα. CR3 and SRA produce the signaling that culminates in structural changes (marked by ellipses); filopodia and lamellipodia first engulf the myelin-debris as they extend and then pull-in the myelin-debris as they retract. We hypothesize the following. Phagocytic receptors promote paxillin activation through phosphorylation (paxillin → p-paxillin) leading to cofilin activation through dephosphorylation (p-cofilin → cofilin). In turn, cofilin promotes the production of filopodia and lamellipodia and so phagocytosis is advanced. In contrast, SIRPα promotes the inactivation of paxillin through p-paxillin dephosphorylation (p-paxillin → paxillin) leading to cofilin inactivation through phosphorylation (cofilin → p-cofilin). In turn, the production of filopodia and lamellipodia is reduced and so phagocytosis is inhibited. Retraction of filopodia and lamellipodia is promoted by MLCK triggering motor activity in non-muscle-myosin which then initiates F-actin/non-muscle-myosin-based contraction (not illustrated). Dashed lines mark indirect interactions; blue lines mark activation and red lines inhibition of phagocytosis.

as control microglia in text and Control Luciferase (Con-Luc) microglia in figures.

MYELIN ISOLATION

Myelin isolation from mouse brains has been previously described (Slobodov et al., 2001) and also visualized (Gitik et al., 2011). The isolated myelin is "degenerated myelin" since isolation involves breakdown of intact myelin.

PHAGOCYTOSIS OF DEGENERATED MYELIN

Phagocytosis of degenerated myelin was assayed in microglia that were plated in 96-well tissue culture plates at a density that minimizes cell-cell contact ($0.25-1.5 \times 10^4$/well) in the presence of Dulbecco's Modified Eagle Medium (DMEM)/F12 supplemented by 10% FCS. Non-adherent microglia were washed out after 2 h and adherent microglia left to rest overnight. Next, phagocytes were washed and degenerated myelin added in the presence of serum for the indicated time periods. Then, unphagocytosed degenerated myelin was washed out and levels of phagocytosis determined by enzyme linked immunosobent assay (ELISA).

ELISA ASSAY TO QUANTIFY THE PHAGOCYTOSIS OF DEGENERATED MYELIN

ELISA assay to quantify the phagocytosis of degenerated myelin is based on the detection of myelin basic protein (MBP) in microglia lysates. Since MBP is unique to myelin and is not produced by phagocytes, MBP levels detected in phagocyte cytoplasm are proportional to levels of degenerated myelin that is phagocytosed. In brief, after non-phagocytosed degenerated myelin is washed away and remaining degenerated myelin has been phagocytosed/internalized, phagocytes are lysed (0.05 M carbonate buffer, pH 10), lysates transferred to high protein absorbance plates (Nalge Nunc International, NY, USA) and levels of MBP determined by ELISA using anti-MBP monoclonal antibody. A detailed protocol is given in (Slobodov et al., 2001) where we also determined that more than 95% of the detected MBP arises from phagocytosed/internalized degenerated myelin. We further verified the validity of this phagocytosis assay by documenting over 95% inhibition of the phagocytosis of degenerated myelin in the presence of cytochalasin-D (not shown).

QUANTIFICATION OF PHAGOCYTOSIS

Quantification of phagocytosis was carried out in the following way. When phagocytosis by SIRPα-KD microglia (i.e., microglia in which SIRPα expression was knocked-down with SIRPα-shRNA) was compared to phagocytosis by control microglia (i.e., microglia infected with non-target Luciferase-shRNA), phagocytosis by each population was first normalized to the number of respective microglia counted in 1 mm^2 areas at the center of wells. Normalizing phagocytosis to cell number is required since SIRPα-KD and control microglia may differ in their adherence properties, thus resulting in different number of adherent microglia even when the same number of cells was initially seeded. To this end, microglia in replicate plates were fixed (i.e., instead of being lysed for the phagocytosis assay), stained, and counted. Phagocytosis normalized to cell number by control microglia was defined 100% and phagocytosis normalized to cell number by SIRPα-KD

microglia was calculated as percentage of phagocytosis by control microglia. Statistical analysis was carried out as detailed in figure legends.

IMMUNOBLOT ANALYSIS

Immunoblot analysis Microglia were plated in 10 cm tissue culture plates at a density that minimizes cell–cell contact (3×10^6 cells per plate) in the presence of DMEM supplemented by 10% FCS, and left to rest overnight. Phagocytes were washed in fresh DMEM supplemented by 10% FCS, degenerated myelin added in the presence of serum for the indicated time periods and unphagocytosed degenerated myelin washed out. For lysis, microglia were washed in PBS and lysed in ice cold lysis buffer (Tris HCL 1 M pH 7.5, $MgCl_2$ 1 M, NaCl 4 M, 0.5% NP-40, 0.1% DTT, and 0.1% NaVa) supplemented with protease and phosphatase inhibitors cocktail (Sigma–Aldrich, Israel), cellular debris was removed by centrifugation, and total protein content determined using Bradford reagent (Sigma–Aldrich, Israel). Equal protein content from whole cell lysates was separated on SDS-PAGE to detect SIRPα, paxillin, cofilin, and GAPDH. Proteins were blotted to nitrocellulose membranes, blocked with 10% non-fat milk or 5% BSA in TBS (Tris-buffered saline) for 1 h at RT, incubated over night at 4°C in the presence of primary antibodies mouse anti-rat SIRPα/CD172α (Serotec, Oxford, England), mouse anti-human GAPDH, rabbit anti-cofilin, rabbit anti-pS^3-cofilin-1, and rabbit anti-pY^{118}-paxillin (Santa Cruz Biotechnology, USA), rabbit anti-paxillin (Cell Signaling, USA), and mouse anti-actin monoclonal antibody (MP biomedicals, CA, USA). Blots were washed with TBST and incubated with respective secondary antibodies goat anti-rat, goat anti-rabbit, and goat anti-mouse conjugated to HRP (Jackson ImmunoReserach, USA) for 40-min at RT. Proteins were visualized with EZ-ECL kit for HRP detection (Beit Haemek, Israel). The intensities of immunoblot bands were determined by ImageJ software and quantification and statistical analysis was carried out as detailed in figure legends.

RESULTS

SIRPα PROMOTES THE INACTIVATION OF COFILIN

We previously documented the following with regard to the phagocytosis of degenerated myelin by microglia in the absence of anti-myelin antibodies: (a) phagocytosis is mostly mediated by CR3 and to a lesser degree by SRA (Rotshenker, 2003), (b) phagocytosis is advanced by cofilin (Hadas et al., 2012), (c) phagocytosis is inhibited by SIRPα (Gitik et al., 2011). Thus, SIRPα could inhibit phagocytosis by promoting the inactivation of cofilin. In this case, reducing SIRPα in phagocytes is expected to promote the activation of cofilin by shifting the balance from inactive p-cofilin (cofilin phosphorylated at serine site S^3) to active unphosphorylated cofilin (cofilin) concurrent with augmenting phagocytosis. We addressed this issue by examining p-cofilin levels before and during phagocytosis in microglia in which SIRPα expression was knocked-down (SIRPα-KD) by lentiviral infection with SIRPα-shRNA, and further, in control microglia that were similarly infected with the non-target firefly Luciferase-shRNA.

Indeed, SIRPα levels were reduced (**Figure 2A**) and phagocytosis was augmented (**Figure 2B**) in SIRPα-KD microglia

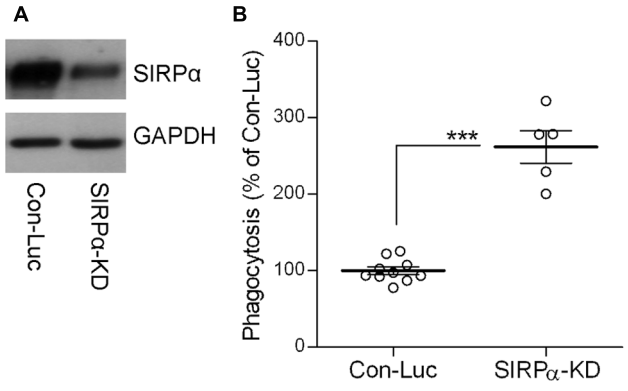

FIGURE 2 | The phagocytosis of degenerated myelin is augmented in SIRPα-KD microglia. (A) A representative (one of three) immunoblot of SIRPα and GAPDH. SIRPα levels are reduced in Balb/C microglia infected with SIRPα-shRNA (SIRPα-KD) compared with control microglia infected with non-target Luciferase-shRNA (Con-Luc). **(B)** Phagocytosis of degenerated myelin is augmented in SIRPα-KD microglia compared with phagocytosis by control microglia (Con-Luc). Phagocytosis by SIRPα-KD microglia was calculated as a percentage of phagocytosis by control microglia that was defined as 100%. Values of individual experiments each performed in triplicates and averages ± SE are given. Significance of difference by Mann–Whitney is $***p < 0.001$.

compared with control microglia, thus confirming our previously reported observations (Gitik et al., 2011). Levels of p-cofilin were then determined by immunoblot analysis using an antibody raised against cofilin that is phosphorylated at serine site S^3 (**Figures 3A,C**). Levels of p-cofilin were reduced in control microglia after 10 min of phagocytosis down to about 75% of those in non-phagocytosing control microglia. Then, after 30 min of phagocytosis, levels of p-cofilin returned to and rose above their initial levels in non-phagocytosing control microglia. These findings confirm our previously reported observations (Hadas et al., 2012).

SIRPα-KD microglia differed from control microglia with respect to p-cofilin levels before and during phagocytosis (**Figures 3B,C**). Levels of p-cofilin were reduced in non-phagocytosing SIRPα-KD microglia down to about 75% of those in non-phagocytosing control microglia. After 10 and 30 min of phagocytosis, p-cofilin levels were reduced further down to about 60% of those in non-phagocytosing control microglia. Taken together, cofilin was transiently activated during prolonged phagocytosis in control microglia but continuously activated in SIRPα-KD microglia, suggesting that normally SIRPα promotes the inactivation of cofilin through serine (S^3) phosphorylation.

SIRPα PROMOTES THE INACTIVATION OF PAXILLIN

Taken that the SIRPα/SHP-1/2 complex dephosphorylates phosphotyrosine sites in its immediate target molecules (Barclay and Brown, 2006; Matozaki et al., 2009; Oldenborg, 2013) and our present findings that SIRPα promotes cofilin inactivation by serine phosphorylation, SIRPα could not have inactivated cofilin directly. However, SIRPα could inactivate cofilin indirectly through paxillin. This proposition is based on previous observations that paxillin is activated by tyrosine phosphorylation

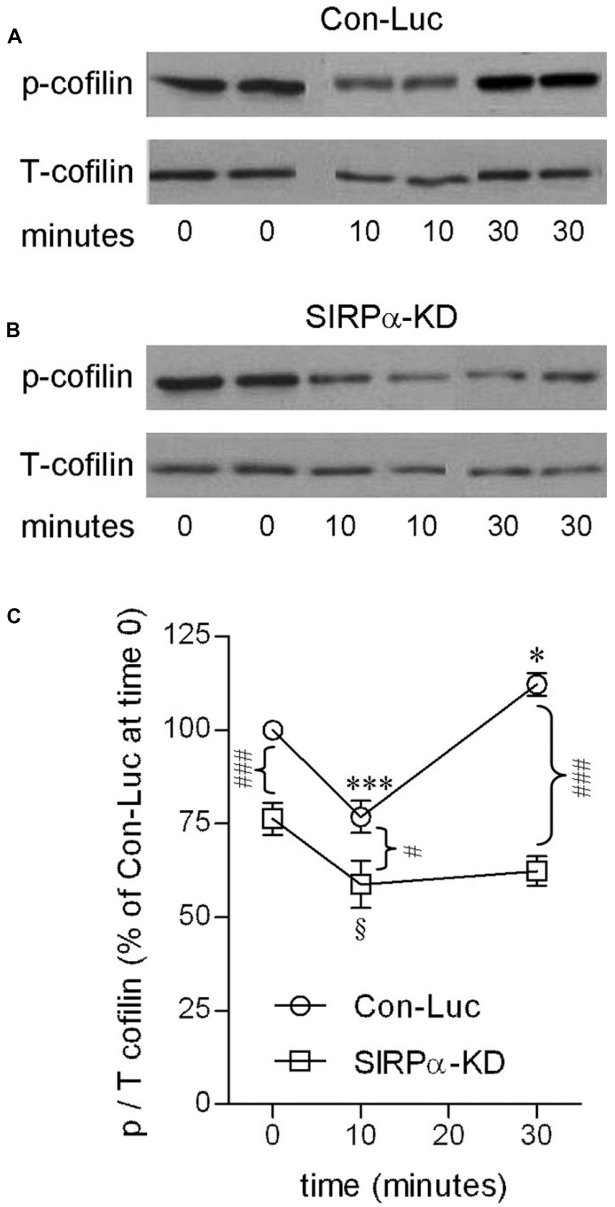

FIGURE 3 | Cofilin activation is transient in phagocytosing control microglia but continuous in phagocytosing SIRPα-KD microglia. Immunoblot analysis of phosphorylated and total cofilin-1 (p- and T-cofilin) in **(A)** control (Con-Luc) and **(B)** SIRPα-KD microglia before the onset of phagocytosis (time 0), and after 10 and 30 min of phagocytosis. The antibodies used for immunoblot analysis identify cofilin and cofilin-1 that is phosphorylated at S^3. **(C)** Quantitation of the ratio p/T based on immunoblot analysis. The ratio p/T in non-phagocytosing Con-Luc microglia (i.e., at time 0) was defined 100%. Then, p/T in all other non-phagocytosing and phagocytosing microglia was calculated as percentage of p/T in non-phagocytosing Con-Luc microglia. Average values ±SEM of four to six experiments, each performed in duplicates, are given. Significance of differences between initial values at 0 min and those at 10 and 30 min by one way ANOVA and the Dunnet post test are $^{*}p < 0.05$ and $^{***}p < 0.001$ for Con-Luc microglia and $^{\S}p < 0.05$ for SIRPα-KD microglia. Significance of difference between Con-Luc and SIRPα-KD microglia by two way ANOVA and the Bonferroni post test are $^{\#}p < 0.05$ and $^{\#\#\#}p < 0.001$. Significance of difference between 10 and 30 min in Con-Luc microglia by one way ANOVA and the Tukey's post test is $p < 0.001$ (not marked).

(paxillin phosphorylated at tyrosine site Y^{118}), and further, that p-paxillin can indirectly activate cofilin (Deakin and Turner, 2008). Thus if SIRPα promotes the inactivation of paxillin, then levels of active p-paxillin are expected to be higher in SIRPα-KD microglia than in control microglia.

Levels of p-paxillin were determined by immunoblot analysis using an antibody raised against paxillin which is phosphorylated at tyrosine site Y^{118} (**Figures 4A,C**). Levels of p-paxillin increased in control microglia to about 160% of those in non-phagocytosing control microglia after 10 min of phagocytosis. Then, after 30 min of phagocytosis, p-paxillin levels decreased significantly to about 120% of those in non-phagocytosing control microglia.

SIRPα-KD microglia differed from control microglia with respect to p-paxillin levels before and during phagocytosis (**Figures 4B,C**). Levels of p-paxillin were about 145% higher in non-phagocytosing SIRPα-KD microglia than in non-phagocytosing control microglia. After 10 and 30 min of phagocytosis, levels of p-paxillin increased further to about 160 and 170% of those in non-phagocytosing control microglia. Taken together, paxillin was transiently activated during prolonged phagocytosis in control microglia and continuously activated in SIRPα-KD microglia, suggesting that normally SIRPα promotes the inactivation of paxillin through phosphotyrosine (pY^{118}) dephosphorylation.

PAXILLIN ACTIVATION, COFILIN ACTIVATION AND PHAGOCYTOSIS POSITIVELY CORRELATE WITH ONE ANOTHER

It has been suggested that active p-paxillin can indirectly activate cofilin by promoting the transition from inactive p-cofilin to active unphosphorylated cofilin (Deakin and Turner, 2008). If this is the case during the phagocytosis of degenerated myelin, then levels of active p-paxillin and levels of inactive p-cofilin should negatively correlate with one another during phagocytosis, and further, phagocytosis augmentation should positively correlate with the activation of both paxillin and cofilin. Indeed, control microglia displayed transient increases in p-paxillin and transient decreases in p-cofilin during phagocytosis, and further, SIRPα-KD microglia exhibited higher levels of p-paxillin and lower levels of p-cofilin compared with control microglia both before and throughout phagocytosis which was augmented in SIRPα-KD microglia compared with control microglia. To obtain a quantitative measure to these apparent correlations, all values of p-paxillin and p-cofilin presented in **Figures 3** and **4** were subjected to linear regression and correlation analysis (**Figure 5**). Levels of p-paxillin and p-cofilin displayed significant ($p < 0.05$) negative correlation with an r^2 value of 0.79. Thus paxillin activation (reflected by higher levels of p-paxillin) and cofilin activation (reflected by lower levels of p-cofilin) positively correlated with one another. Since levels of phagocytosis increased in SIRPα-KD microglia compared with control microglia at the same time as levels of both paxillin and cofilin activation increased, all three (i.e., levels of phagocytosis, paxillin activation, and cofilin activation) positively correlated with one another.

DISCUSSION

Observations made in this study suggest a novel mechanism whereby paxillin and cofilin are targeted to control the

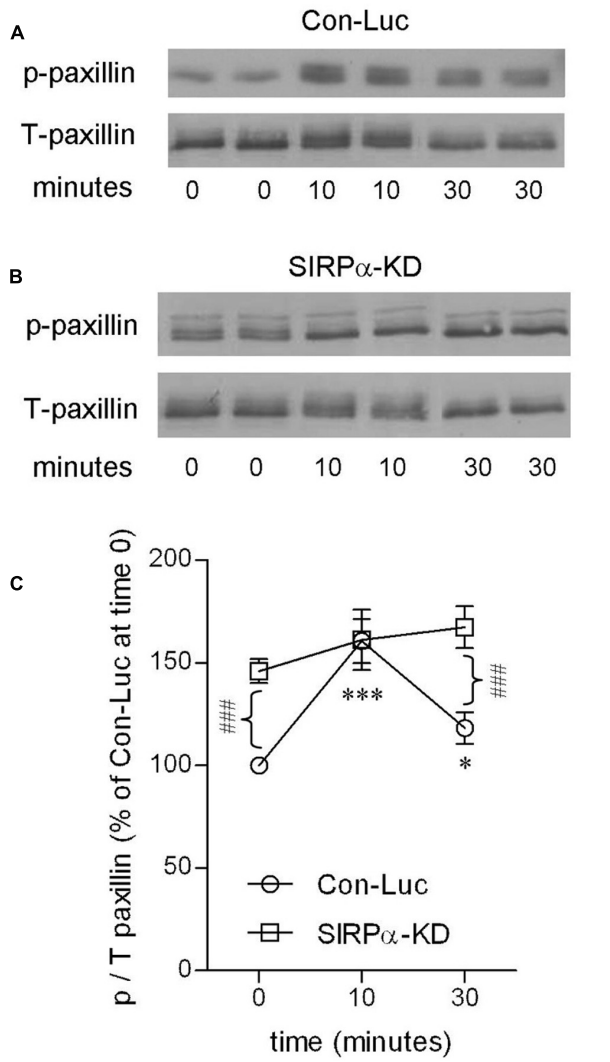

FIGURE 4 | Paxillin activation is transient in phagocytosing control microglia but continuous in phagocytosing SIRPα-KD microglia. Immunoblot analysis of p- and T-paxillin in **(A)** control (Con-Luc), and **(B)** SIRPα-KD microglia before the onset of phagocytosis (time 0), and after 10 and 30 min of phagocytosis. The antibodies used for immunoblot analysis identify paxillin and paxillin that is phosphorylated at Y^{118}. **(C)** Quantitation of the ratio p/T based on immunoblot analysis. The ratio p/T in non-phagocytosing Con-Luc microglia (i.e., at time 0) was defined 100%. Then p/T in all other non-phagocytosing and phagocytosing microglia was calculated as percentage of p/T in non-phagocytosing Con-Luc microglia. Average values ±SEM of four to eight experiments, each performed in duplicates, are given. Significance of differences between initial values at 0 min and those at 10 and 30 min by one way ANOVA and the Dunnet post test are $^{*}p < 0.05$ and $^{***}p < 0.001$ for Con-Luc microglia. Significance of difference between Con-Luc and SIRPα-KD microglia by two way ANOVA and the Bonferroni post test is $^{\#\#\#}p < 0.001$. Significance of difference between 10 and 30 min in Con-Luc microglia by one way ANOVA and the Tukey's post test is $p < 0.01$ (not marked).

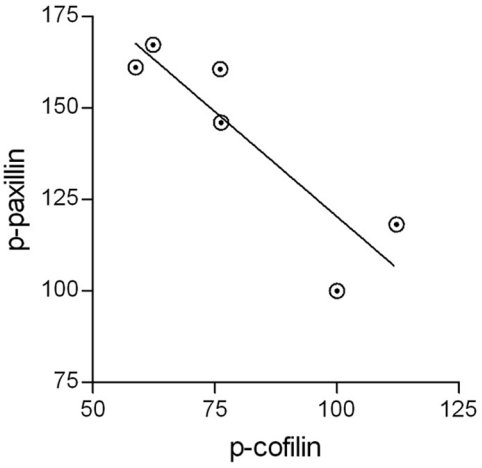

FIGURE 5 | The activation of paxillin and cofilin are linked. All values of active p-paxillin and inactive p-cofilin that are presented in **Figures 3** and **4** were subjected to linear regression and correlation analysis. The two display a negative correlation with an r^2 value of 0.79. Significance of correlation by the Pearson double tailed test is $p < 0.05$.

phagocytosis of degenerated myelin by both the activating signaling which phagocytic receptors CR3 and SRA produce and the inhibiting signaling that immune inhibitory receptor SIRPα produces. In this regard, phagocytic receptors advance phagocytosis by promoting the activation of paxillin and cofilin whereas immune inhibitory receptor SIRPα inhibits phagocytosis by promoting the inactivation of the two.

The activation of phagocytosis by paxillin and cofilin is suggested by the findings that phagocytosis positively correlates with the activation of both paxillin and cofilin in both control and SIRPα-KD microglia. In control microglia, paxillin and cofilin were activated concurrent with the activation of phagocytosis, and in SIRPα-KD microglia, levels of paxillin and cofilin activation increased simultaneously with the increase in phagocytosis.

The role cofilin plays in the phagocytosis of degenerated myelin was recently reported and discussed by us (Hadas et al., 2012). Cofilin advances phagocytosis by promoting the remodeling of F-actin and thereby the production of filopodia/lamellipodia which engulf the myelin-debris. We presently suggest that cofilin activation is promoted by paxillin. This proposition is based on the observations that paxillin can indirectly activate cofilin (Deakin and Turner, 2008) and our present findings that levels of paxillin activation and levels of cofilin activation positively correlated with one another. The involvement of p-paxillin in signaling phagocytosis by CR3 and SRA which is documented here could also take place in signaling phagocytosis by FcγR since paxillin is tyrosine phosphorylated during FcγR mediated phagocytosis (Greenberg et al., 1994).

That SIRPα inhibits the phagocytosis of degenerated myelin by promoting the inactivation of both paxillin and cofilin is suggested by the synchronized occurrence of three events in SIRPα-KD microglia. First, phagocytosis is augmented in SIRPα-KD microglia compared with control microglia; second, the activation of both paxillin and cofilin increases in non-phagocytosing as well as in phagocytosing SIRPα-KD microglia compared with non-phagocytosing and phagocytosing control microglia; third, the kinetics of the activation of both paxillin and cofilin switched from transient in phagocytosing control microglia to continuous

in phagocytosing SIRPα-KD microglia. SIRPα dependent inactivation of paxillin and cofilin was not reported before for any of the functions that SIRPα is involved in. We further raise the possibility that SIRPα could directly inactivate paxillin through dephosphorylation since paxillin is activated through tyrosine phosphorylation (Deakin and Turner, 2008) and the SIRPα/SHP-1/2 complex dephosphorylates phosphotyrosine sites (Barclay and Brown, 2006; Matozaki et al., 2009; Oldenborg, 2013).

In conclusion, the phagocytosis of degenerated myelin is determined by the balance between the signaling produced by CR3 and SRA which activates paxillin and cofilin and the signaling produced by SIRPα which inactivates paxillin and cofilin. SIRPα could also target paxillin and cofilin while inhibiting phagocytosis which is mediated by additional phagocytic receptors such as FcγR (Oldenborg et al., 2001) and during the phagocytosis of particles other than myelin-debris such as aging red blood cells (Oldenborg et al., 2000) and tumor cells (Chao et al., 2012; Kim et al., 2012).

ACKNOWLEDGMENT

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Microglia centered pathogenesis in ALS: insights in cell interconnectivity

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Amyotrophic lateral sclerosis (ALS) is the most common and most aggressive form of adult motor neuron (MN) degeneration. The cause of the disease is still unknown, but some protein mutations have been linked to the pathological process. Loss of upper and lower MNs results in progressive muscle paralysis and ultimately death due to respiratory failure. Although initially thought to derive from the selective loss of MNs, the pathogenic concept of non-cell-autonomous disease has come to the forefront for the contribution of glial cells in ALS, in particular microglia. Recent studies suggest that microglia may have a protective effect on MN in an early stage. Conversely, activated microglia contribute and enhance MN death by secreting neurotoxic factors, and impaired microglial function at the end-stage may instead accelerate disease progression. However, the nature of microglial–neuronal interactions that lead to MN degeneration remains elusive. We review the contribution of the neurodegenerative network in ALS pathology, with a special focus on each glial cell type from data obtained in the transgenic SOD1G93A rodents, the most widely used model. We further discuss the diverse roles of neuroinflammation and microglia phenotypes in the modulation of ALS pathology. We provide information on the processes associated with dysfunctional cell–cell communication and summarize findings on pathological cross-talk between neurons and astroglia, and neurons and microglia, as well as on the spread of pathogenic factors. We also highlight the relevance of neurovascular disruption and exosome trafficking to ALS pathology. The harmful and beneficial influences of NG2 cells, oligodendrocytes and Schwann cells will be discussed as well. Insights into the complex intercellular perturbations underlying ALS, including target identification, will enhance our efforts to develop effective therapeutic approaches for preventing or reversing symptomatic progression of this devastating disease.

Keywords: amyotrophic lateral sclerosis, microglia activation phenotypes, motor neuron, neuroinflammation, neurodegeneration, pathological cell–cell communication, SOD1G93A transgenic mouse/rat

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a non-cell-autonomous disease targeting motor neurons (MNs) and neighboring glia, with microgliosis directly contributing to neurodegeneration (Beers et al., 2006; Lobsiger and Cleveland, 2007; Yamanaka et al., 2008a). Indeed, the neurodegenerative process in ALS was shown to be accompanied by a sustained inflammation in the brain and spinal cord (SC) (Bowerman et al., 2013). Recently, microglia were suggested to be implicated in ALS initiation (Gerber et al., 2012), as well as to lose their surveillance capacity by switching from an activated to a neurodegenerative phenotype as the disease progresses (Weydt et al., 2004; Dibaj et al., 2011). Therefore, a better therapeutic strategy should envisage the recovery of healthy microglia from those transformed cells near the most affected MNs in the SC. In such way we may preserve the passage of toxic mediators to environmental cells, and maintain the homeostatic conditions.

For the vast majority of patients with ALS, the etiology of the disorder is unknown. Actually, only some ALS cases (less than 10%) have been linked to mutations in a number of genes, including in the enzyme Cu, Zn superoxide dismutase 1 (SOD1), TAR DNA binding protein (TDP-43), fused in sarcoma (FUS), optineurin (OPTN), valosin-containing protein (VCP), ubiquilin 2 (UBQLN2), profilin 1 (PFN1), and chromosome 9 open reading frame 72 (C9ORF72) repeat expansions (Tovar et al., 2009a; DeJesus-Hernandez et al., 2011; Ince et al., 2011; Renton et al., 2011, 2014; Bertolin et al., 2013). Interestingly, both the non-genetic and the genetic forms of ALS are suggested to have common pathogenic mechanisms (Lilo et al., 2013), as well as similar clinical courses and dysfunctional features, such as the abnormal accumulation of neurofilaments in degenerating MNs (Julien, 2001). Actually, cytoplasmic aggregation of nuclear TDP-43 and FUS in the degenerating neurons and glia of ALS patients, and release of the accumulated cytoplasmic mutant SOD1 (mSOD1) to the extracellular space that can be taken up by other cells, are common features (Li et al., 2013; Ogawa and Furukawa, 2014). The identification of C9ORF72 repeat expansions in patients with ALS but without a family history of ALS challenged the division between genetic (familial) and non-genetic (sporadic) cases (Turner et al., 2013). As indicated by Kiernan (2014), the true substrate of

ALS may reside in a pathogenic signature of nuclear protein mishandling.

There are several *in vitro* and *in vivo* models of MN degeneration. *In vitro* experimental models include SC cultures, NSC-34 cell line expressing the mSOD1 and organotypic cultures, while the axotomy-induced MN death, the naturally occurring ALS models, and the transgenic models are the most commonly used *in vivo* models (Elliott, 1999; Tovar et al., 2009a). Among the various transgenic models used in the study of ALS pathogenesis (Weydt et al., 2004; Kato, 2008), the transgenic rodent overexpressing mSOD1, in particular the SOD1G93A strain, is the most utilized and characterized. Transgenic mice containing other mSOD1 genes (G85R, G37R, D90A, or G93A missense mutations or truncated SOD1) and the related mutant (G86R) mouse have also shown progressive neurodegeneration of the motor system and resemblance to ALS (for review, see Van Den Bosch, 2011). Distinctive injurious effects between SOD1G93A and SOD1H46R on two different genetic backgrounds were recently recognized (Pan et al., 2012). Additionally developed models are based on TDP-43 (Wegorzewska et al., 2009; Liu et al., 2013; Yang et al., 2014) and FUS mutations (Verbeeck et al., 2012), but none of these models is currently used to study the pathogenesis of ALS and to test new drugs. Thus, the human mSOD1 murine model is the most widely used in the evaluation of the involved molecular targets, biomarkers and novel drugs/treatments for ALS. Apart from developing loss of MNs and symptoms that resemble human ALS by mSOD1, the model evidences molecular links between genetic and non-genetic cases of ALS (Andjus et al., 2009; Synofzik et al., 2010). To note, that non-genetic perturbations of the wild-type (wt) SOD1 protein may lead to SOD1 misfolding with a conformation much similar to genetic SOD1 variants (Cereda et al., 2006). Therefore, in this review we will summarize the most recent developments obtained in the SOD1G93A transgenic model to give consistency and cohesion between the data disclosed, and because we admit that common factors and pathways are shared in both genetic and non-genetic derived ALS cases, in particular changes in microglia performance and in neuron–glia communication.

It was initially considered that the selective death of MNs expressing the mutant protein was the player in the disease onset. However, non-cell-autonomous processes associated with mSOD1 in glial cells are believed to be implicated not only in disease progression and extent, but also to be related with the onset and early stage of the disease, thus underlying MN dysfunction and loss. Indeed, healthy glia evidenced to delay the progression of the disease (Boillée et al., 2006b; Yamanaka et al., 2008b) and the replacement of mSOD1 microglia by wt microglia slowed disease progression and prolonged mice survival (Lee et al., 2012). This finding is in line with previous studies showing that mSOD1 in microglia leads to the disease (Clement et al., 2003) and that the reduction of the mutant levels in the cells slows ALS progression (Boillée et al., 2006b). Indeed, damage to MNs by neighboring cells expressing mSOD1 seems to be required for MN degeneration (Pramatarova et al., 2001; Lino et al., 2002). Accumulating knowledge on the active participation of different microglia phenotypes in ALS was recently obtained when microglia were isolated from SOD1G93A rats at presymptomatic, symptom onset and end-stage periods (Nikodemova et al., 2013). Microglia were shown to be regionally different and to evidence a heterogeneity of phenotypes with the disease progression. Thus, it will be interesting to investigate the influence of changes caused by aging in the performance of the mutated microglia, namely on the interconnectivity with neurons and other glial cells. Moreover, our preliminary data indicate a decreased phagocytic and migration ability of healthy microglia by the soluble factors (SFs) released by NSC-34 cells expressing mSOD1, thus causing the loss of important microglia properties (Cunha, 2012). In addition, when this microglia was co-cultured with the mMNs we observed a reduction of the activation of matrix metalloproteinases (MMP)-2 and -9 in the extracellular media after cultivation for 4 days in vitro (DIV) (Barbosa, 2013), evidencing the beneficial effect of the healthy microglia in decreasing mMN stress.

Finally, we provide an outlook on the extent to which a diverse cellular environment may determine different pathological windows along disease progression opening new opportunities to explore distinct therapeutic approaches to either trigger a less reactive microglia phenotype at the early-onset, or a recovery of microglia dynamics at late-stage ALS. It will be also summarized recent advances in regenerative medicine technologies with potential to reverse or halt ALS progression by slowing MN death.

NEURODEGENERATIVE NETWORKING IN ALS

Neuronal homeostasis and survival were shown to be compromised in ALS due to multiple aberrant biological processes and to deregulated communication between neurons and glial cells in the brain and in SC by the disease. ALS, once considered a MN disease, is now known to have multiple influences and regarded as a multi-cellular/multi-systemic disease (Nardo et al., 2011). In fact, MN death seems to be driven by a convergence of damaging mechanisms, including glial cell pathology and inflammatory conditions, such as microglial activation or the invasion of lymphocytes, and calcium dysregulation (Grosskreutz et al., 2010; Beers et al., 2011b). It can be also determined by excitotoxicity due to the selective loss of the astrocytic glutamate transporter GLT-1, and consequent accumulation of extracellular glutamate (Rothstein, 2009). Our late results with astrocytes isolated from SOD1G93A mice and cultivated for 13 DIV suggest that both GLT-1 and glutamate aspartate transporter (GLAST) are compromised (personal communication) and corroborate other findings indicating that expression of such transporters are less potently activated by lipopolysaccharide (LPS) in astrocytes from the mSOD1 model than in those from the wt mice (Benkler et al., 2013). Other contributing processes include from neurovascular changes and compromised barriers of the central nervous system (CNS), to dysfunctional communication between neurons, abnormal neuron–glia interactions, and microglia and astroglia loss-of-function.

In fact, for the optimal functioning of the CNS, i.e., brain and SC, accounts the constant immune surveillance promoted by cells such as microglia, and the blood–brain barrier (BBB), the blood–SC barrier (BSCB), and the blood–cerebrospinal fluid (BCSF) barrier that uniquely shield CNS from potential mediators of infection and damage. Recently, brain pericytes, as important components of the neurovascular unit in the CNS, have shown to

have pleiotropic and regulatory activities in brain vessel function and homeostasis, blood flow and barrier function (Lange et al., 2012). Astrocytes also participate in maintaining homeostasis and supporting neuronal function. In addition to microglia, neurons have lately been indicated to intervene in immune responses through control of T cells infiltration into the CNS and glial cell immunoreactivity (Czirr and Wyss-Coray, 2012; Tian et al., 2012; Liblau et al., 2013). This integrative network sustains ionic, energetic, and redox homeostasis for proper function.

Moreover, ALS progression was suggested to involve cell-to-cell transmission of mSOD1 aggregates involving MNs, microglia and astrocytes, similarly to prion disease (Chia et al., 2010; Munch et al., 2011). Interestingly, the transcellular spread of SOD1 aggregates evidenced to not require cell-to-cell contacts but to depend from their fragmentation and extracellular release. However, additional studies should demonstrate that similar findings also occur *in vivo*. Intriguingly, while extracellular mSOD1G93A has shown to not have direct toxic effects on MNs, it morphologically and functionally activates microglia, supporting the non-cell-autonomous nature of MN toxicity in ALS (Zhao et al., 2010). Indeed, it was shown that this extracellular mSOD1 can be endocytosed into microglia, determining the activation of caspase-1 and the up-regulation of interleukin (IL)-1β (Zhao et al., 2013).

Another interesting concept is that astrocyte and microglia activation, which is regulated by a variety of signaling pathways, should not be considered merely as pernicious for CNS homeostasis, once it promotes metabolic support, wound healing and repair. In fact, the production of cytokines and chemokines initiate and coordinate diverse cellular and intercellular actions. Although neurons and glial cells of the CNS express receptors for cytokines and chemokines, the biological consequence of receptor activation is not fully understood. In contrast, it must be considered as part of the pathological processes the excessive or deregulated signaling pathways leading to microglia and astrocyte abnormalities that culminate in abnormal CNS function.

Thus, progressive neurodegeneration of MNs in ALS may result from a combination of intrinsic MN vulnerability to mSOD1 aggregates and of non-cell-autonomous toxicity derived from neighboring cells (**Tables 1** and **2**). Therefore, pathological changes in ALS indicate a broken homeostasis in the CNS. We will focus on how CNS homeostasis is lost in ALS and in what way BBB and neural networks dysregulation contribute to neurodegeneration in ALS.

MOTOR NEURON DYSFUNCTION

A certain number of cases are linked to mutations in SOD1 and candidate mechanisms are the formation of protein aggregates and pro-oxidant effects. Therefore, most of the experiments are generally conducted in the MN-like hybridoma cell line NSC-34 expressing human mSOD1 (hSOD1G93A) (Tovar et al., 2009a) or in the transgenic mice generated by Gurney et al. (1994) that also over-express hSOD1G93A (Riboldi et al., 2011).

SOD1 aggregates are observed in both genetic and non-genetic ALS cases, but their contribution to MN toxicity remains to be established, although deregulation of Golgi, endoplasmic reticulum (ER), and mitochondria, together with axonal transport defects have been indicated (for review, see Boillée et al., 2006a; Rothstein, 2009). Interestingly, when working with the NSC-34/hSOD1G93A cells we found that one of the most sensitive indicators of SOD1 accumulation and neuronal dysfunction was the elevation of MMP-9, but not of MMP-2 that remained unchanged (Cunha, 2012; Vaz et al., 2014). Elevation of the MMP-9 levels was previously observed in the SC of ALS mice from pre-symptomatic phase, predominantly in MNs but also in glia (Soon et al., 2010). These authors suggest that circulating MMP-9 is associated with the disease onset and is mainly derived from degenerating SC MNs and circulating cells. Its inhibition was shown to enhance animal survival in more than 30% (for review, see Riboldi et al., 2011). It was also found in the CNS, muscles, plasma, and skin of patients. Major inducers are reactive oxygen species (ROS) and cytokines released by microglia. Once MMP-9 is indicated to promote the regeneration of the injured neuron, it may be hypothesized that its increase results from a MN response to the pathology. However, it was recently claimed that MMP-9 is indeed a determinant of the selective neurodegeneration (Kaplan et al., 2014). The Authors demonstrated that MMP-9 is only expressed by the selectively vulnerable fast MNs and can originate ER stress and axonal dye-back. This finding provides a basis for considering MMP-9 as a candidate target for novel therapeutic approaches to ALS.

Oxidative damage at the level of proteins, lipids, and DNA was also observed in the transgenic hSOD1 mice models (Liu et al., 1999; Casoni et al., 2005; Poon et al., 2005; Barbosa, 2013) where mSOD1 revealed to be the most severely oxidized protein mouse (Andrus et al., 1998). Also accounting to MN disturbance is the failure of the Keap1/Nrf2/ARE system in regulating stress proteins, as evidenced along disease progression in MNs from the SC of hSOD1G93A mice (Mimoto et al., 2012). In addition, failure in MN autophagy was revealed as critical in the pathogenesis and progression of ALS (for review, see Pasquali et al., 2009; Chen et al., 2012; Otomo et al., 2012).

Currently, there is no cure for the ALS disease, although Riluzole, the only drug approved by the U.S. Food and Drug Administration (FDA), was shown to prolong median patient survival by 2–3 months and to be more effective when administered at an early stage of the disease (Zoccolella et al., 2007; Lee et al., 2013a). More potent therapeutic strategies may derive from better target identification and clarification of signaling mechanisms able to be modulated and from the use of cell-based therapies, such as the administration of mononuclear cells from human umbilical cord blood (Garbuzova-Davis et al., 2012) and of mesenchymal stromal (stem) cells (Uccelli et al., 2012), as referred to in Section "Challenges to Nerve Regeneration in ALS." Bellow we introduce what is known about the most prominent MN membrane proteins and SFs suggested to be implicated in ALS pathology, but still deserving to be more explored.

Perturbations in glutamate handling

Glutamate excitotoxicity is one primarily cause of neuronal death by necrosis and apoptosis and a critical player in ALS onset and progression. Indeed the blockade of the glutamate transporter in a SC organotypic slice model from SOD1G93A rats has shown to result in an increased survival of MNs (Yin and Weiss,

Table 1 | Functional alterations of motor neurons (MNs) in amyotrophic lateral sclerosis (ALS): candidate molecular targets.

Changes in MN signaling by ALS	ALS stages	Reference
Elevation of matrix metalloproteinase (MMP)-9	Pre-symptomatic phase	Soon et al. (2010), Kaplan et al. (2014)
Impaired antioxidative Keap1/Nrf2/ARE system	Along disease progression	Mimoto et al. (2012)
Oxidative and nitrosative stress	Early phase	Drechsel et al. (2012)
Glutamate excitotoxicity	Onset and progression	Spalloni et al. (2013)
Release of ATP	Along disease progression	Glass et al. (2010)
Protein misfolding, aggregation, and accumulation	Onset and progression	Julien (2001), Li et al. (2013)
Changes in fractalkine (CX3CL1), CD200, and CCL21	Not clarified	–
Decreased high-mobility group box protein 1 (HMGB1) cellular expression	Advanced phase	Lo Coco et al. (2007), Fang et al. (2012)
Release of neuregulin-1 (NRG1)	Along disease progression	Song et al. (2012)
Up-regulation of major histocompatibility complex (MHC) class I and β2-microglobulin mRNAs	Along disease progression	Staats et al. (2013)

2012). Glutamate, by activating the glutamatergic ionotropic receptor *N*-methyl-D-aspartate (NMDAR), triggers the influx of Ca^{2+} into neurons, increasing its intracellular levels (for review, see Spalloni et al., 2013). In these conditions, changes in mitochondria dynamic properties are produced leading to excessive oxidative phosphorylation and increased generation of ROS and reactive nitrogen species (RNS) that culminate in apoptosis. In parallel, the NMDAR excitotoxicity also results in the release of Ca^{2+} from the ER. In addition, it was shown that ER stress resulting from the accumulation of aggregated mSOD1 and dysfunction of the unfolded protein response (UPR) activation contributes to the apoptotic signaling cascade in ALS (for review, see Sofroniew, 2009).

Besides the synaptic glutamate pool directly implicated in the excitatory neurotransmission it has been lately additionally considered the extra-synaptic glutamate pool that influences cell communication. This pool, mainly derived from astrocyte and microglia release, is greatly increased by pathological stimuli (for review, see Rodriguez et al., 2013). Besides being implicated in many physiological conditions glutamate contribution to neuron–glia alterations of homeostasis and to the pathophysiology of neurodegenerative diseases, such as ALS, needs to be explored in the near future, due to controversial results. In fact, although linkage between glutamate and neuroinflammation was suggested to have a role in potentiating MN death in a model mimicking ALS disease (Tolosa et al., 2011), other Authors have demonstrated that glutamate by itself was not able to induce MN damage in ALS (Tovar et al., 2009b). Indeed, it has been questioned the involvement of glutamate in ALS-induced MN death (Le Verche et al., 2011) and the cytotoxicity of the cerebrospinal fluid (CSF) from patients with ALS evidenced to not be related to glutamate (Gomez-Pinedo et al., 2013).

Membrane-bound and soluble fractalkine (CX3CL1)

Fractalkine (FKN) mRNA is dominantly expressed in neuronal cells, particularly in those at the cortex, hippocampus, caudate putamen, thalamus, and olfactory bulb (Harrison et al., 1998). FKN mRNA was also detected in unstimulated astrocytes (more) and microglia (less) (Mizuno et al., 2003). Interestingly, FKN immunoreactivity and mRNA was observed also in the rat SC and dorsal root ganglia neurons, but not in glia, and levels were not enhanced by neuropathic conditions (Verge et al., 2004). FKN, or CX3CL1, exists as a membrane-bound and soluble protein (sFKN) allowing both adhesive and chemoattractive properties (Bazan et al., 1997). In fact, the sFKN has potent chemoattractant activity, recruiting CXCR1-expressing T cells, monocytes, and microglia to the injured neurons, as well as in regulating the phagocytic capacity of microglia (Cardona et al., 2006). sFKN was shown to increase upon stimulation of glutamate (Chapman et al., 2000) and to induce proliferation of human microglia (Hatori et al., 2002). The constitutive expression of FKN and its receptor CX3CR1 in microglia indicates its involvement in fundamental processes of communication between neurons and microglia (Harrison et al., 1998).

Under stimulation, such as an excitotoxic stimulus (Chapman et al., 2000), the membrane-bound form of FKN is rapidly cleaved from cultured neurons and significantly reduces neuronal NMDA-induced apoptosis (Deiva et al., 2004). Indeed attenuated glutamate-induced neuronal cell death was observed after treatment of primary neuron–microglia co-cultures with sFKN (Noda et al., 2011). Proteolytic cleavage of CX3CL1 is mediated by action of metalloproteinase ADAM10 (Gough et al., 2004), ADAM17 (Garton et al., 2001; Tsou et al., 2001), cathepsin S (CatS) (Clark et al., 2009), and MMP-2 (Bourd-Boittin et al., 2009). Thus, cleavage of FKN may be prevented by the inhibition of MMPs (Chapman et al., 2000). The source of CatS is the activated microglial cells that upon stimulation with LPS secrete sFKN to the extracellular media (Clark et al., 2009). Similarly, stromal cell-derived factor-1 (SDF-1) was indicated to stimulate the expression of ADAM17 and to increase sFKN, while up-regulating FKN expression (Cook et al., 2010).

In addition, FKN has a neuroprotective function by inhibiting the nitric oxide (NO) production and the expression of inducible NO synthase (iNOS) mRNA in activated microglia (Mizuno

Table 2 | Glial impairment and deregulated glia–motor neuron (MN) interconnectivity in amyotrophic lateral sclerosis (ALS).

Mutant SOD1 cells	Loss of supportive functions	Contribution to ALS disease and MN death	Reference
Astrocytes	Deficient astrocyte-specific glutamate transporter EAAT2 (GLT-1)	Increase in the excitatory amino acid glutamate	Valori et al. (2014)
	Increased release of D-serine	co-activator of the *N*-methyl-D-aspartate (NMDA) receptors, exacerbating glutamate toxicity on MNs	Valori et al. (2014)
	Mitochondrial dysfunction	Increased production of reactive oxygen species (ROS)	Valori et al. (2014)
	Release of interferon-γ and transforming growth factor-β (TGF-β)	Increased neuroinflammation	Valori et al. (2014)
	Ubiquitin- and active caspase-3-immunopositive	Degenerating astrocytes at the pre-symptomatic stage when MNs show axonal damage but are still alive	Valori et al. (2014)
	Increased nerve growth factor (NGF) and NO production	MN apoptosis	Pehar et al. (2004)
Astrocytes (aberrant)	Increased S100B and connexin-43 (Cx-43)	Decreased MN survival	Diaz-Amarilla et al. (2011)
Microglia (spinal cord – early stage)	Recruitment of peripheral monocytes to the CNS	Neuronal viability impairment	Butovsky et al. (2012)
Microglia (spinal cord – end stage)	Decreased expression of M1 and M2 markers	Decreased reactivity to stimuli	Nikodemova et al. (2013)
Microglia (M2) – early stage	High levels of anti-inflammatory cytokines and neurotrophins	Enhancement of MN survival (neuroprotection) at ALS early stage	Zhao et al. (2013)
Microglia (M1) – progressive stage	Increased release of reactive oxygen species (ROS), tumor necrosis factor-α (TNF-α) and interleukin (IL)-1β	Toxicity to MN (death) in the late rapid phase of ALS	Zhao et al. (2013)
Dystrophic microglia – end stage	Decreased migration and phagocytosis by aging (not yet confirmed in ALS)	Neuronal degeneration by failure of the senescent microglia response to stimuli	Luo and Chen (2012)
Oligodendrocytes	Loss of the monocarboxylate transporter 1 (MCT1)	Decreased delivery of the metabolic substrate lactate to MNs and axonal sufferance	Philips et al. (2013)
$NG2^+$ cells	Increased proliferation rate and degeneration of early-born oligodendrocytes	Gray matter demyelination	Kang et al. (2013)
Schwann cells	Signs of distress at the asymptomatic stage	Not known	Valori et al. (2014)

et al., 2003). Interestingly, it was observed that FKN expression was reduced in the brain of aged rats probably accounting for the increase in microglial activation in such condition. In fact, treatment with FKN has attenuated the age-related increase in microglial activation (Lyons et al., 2009).

Taken together, FKN seems to have both intrinsic and anti-inflammatory properties in the CNS and to act by interfering with toxic microglial–neuron interactions (Suzuki et al., 2011). Whether FKN may have a role in the development of ALS is still not known.

High-mobility group box 1 protein

High-mobility group box 1 (HMGB1) protein, also known as amphoterin, is an inflammatory factor that can be released by astrocytes, microglia, and neurons, mainly when cells are dying (for review, see Fang et al., 2012). HMGB1 has dual activities depending on whether is alone (probably promoting inflammation resolution and tissue regeneration) or forming complexes with several proinflammatory mediators (potentiating inflammation and promoting innate immune cell activation) (Bianchi, 2009). Nuclear HMGB1 regulates transcription of

different sets of genes, including proinflammatory genes (Bianchi and Manfredi, 2009; Park et al., 2009; Wong, 2013). Beneficial effects were observed in early CNS development but increased levels of HMGB1 were shown to be correlated with apoptosis and degeneration of neurons (Liu et al., 2009; Kawabata et al., 2010). When neurons are injured, secretion of HMBG1 activates microglia through receptor for advanced glycation end products (RAGE), Toll-like receptors (TLRs) 2, 4, and 9, as well as Mac1 receptors (Kim et al., 2006a; Park et al., 2006; Neusch et al., 2007), as depicted in **Figure 1**. Release of inflammatory mediators by activated microglia further induces neuronal necrosis. HMGB1 was indicated to decrease in neurons and to increase in astrocytes with aging (Enokido et al., 2008). When expression and localization of HMBG1 was evaluated in the lumbar SC of SOD1G93A transgenic mice, although intense reactivity was found, no differences were obtained between controls and the SOD1 mice (Lo Coco et al., 2007). However, since HMBG1 was identified in the cytoplasm of astrocytes and microglia in SC samples from ALS patients (Casula et al., 2011) it may trigger TLR signaling pathways. The finding was observed at the late ALS phase and, thus, it will be important to follow the TLR/RAGE cascade in animal models at different stages of the disease. In contrast, a progressive reduction of HMGB1 immunopositive MNs was found at advanced stages and may reflect the loss of MNs, reduced synthesis or enhanced released of the cytokine (Lo Coco et al., 2007). Additional studies are needed to investigate the causative hypothesis indicated for the decreased HMGB1 immunoreactivity in MNs, inasmuch since it can also have beneficial effects on neuroregeneration (Fang et al., 2012).

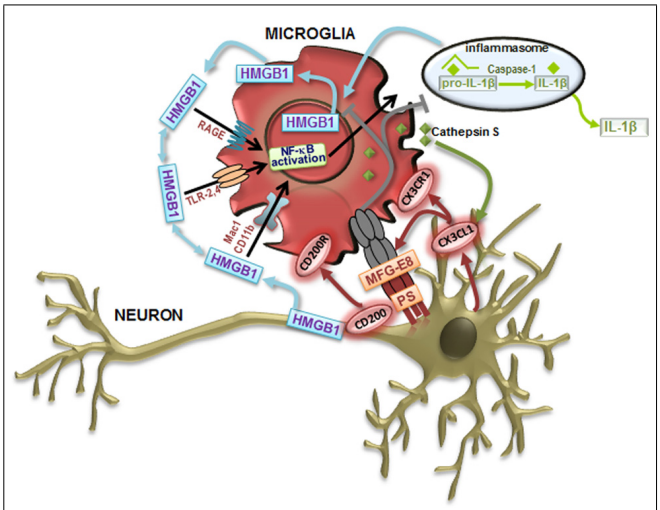

FIGURE 1 | Neuron–microglia communication signaling pathways that modulate microglia cell phenotypes. Toll-like receptor (TLR) signaling contributes to classically activated microglia (M1) in response to damage-associated molecular patterns (DAMPs). Following recognition of DAMPs, TLRs activate downstream signaling cascades, activate nuclear factor-κB (NF-κB) inducing the transcription of inflammatory mediators associated with the M1-like microglial cell phenotype, such as the proinflammatory cytokines interleukin (IL)-1β and tumor necrosis factor (TNF)-α. The production of IL-1β can be achieved by the inflammasome activation by DAMPs, leading to caspase-1 activation that orchestrates the cleavage of pro-IL-1β to form active IL-1β, which leaves and binds to the IL-1 receptor, resulting in inflammation. Following activation, microglial cells augment the immune response by releasing metalloproteinases (MMPs) and increasing proinflammatory cytokines. High-mobility group box 1 (HMGB1) is an alarmin that signals cell damage in response to injury and are associated to all the described signaling events. HMGB1 released by activated microglia and damaged neurons concur for a vicious cycle mediating chronic, progressive neurodegeneration associated with neuroinflammation. HMGB1 can interact with receptors that include RAGE (receptor for advanced glycation end products), TLR-2, TLR-4, Mac-1 also known as CD11b, and possibly others. Alternatively activated microglia (M2) functions include phagocytosis. Milk fat globule factor-E8 (MFG-E8) produced by microglia recognizes phosphatidylserine (PS) as "eat me" signals expressed on the surface of apoptotic neurons, triggering a signaling cascade that stimulates phagocytosis to engulf the dying cell. In order to maintain a quiescent microglia phenotype (M0) under steady-state conditions, neurons suppress the activation of microglia through cell–cell contact (CD200–CD200R) and by the release of the chemokine ligand 1 (CX3CL1) mediated by the cathepsin S that binds to its receptor CX3CR1 on microglia.

CCL21

CCL21 was shown to be implicated in signaling neuronal injury to microglia through the receptor CXCR3. CCL21 expression was demonstrated to increase in cortical neurons, *in vitro*, 2 h after excitotoxic stimulus (de Jong et al., 2005). Intriguingly, these authors demonstrated its location within vesicles that are transported along the neuronal process till presynaptic structures. This chemokine is considered a chemotactic agent important to drive microglia to the site of lesion. Astrocytes were also indicated to have the receptor CXCR3, but unless high levels of CCL21 are produced, no relevant changes can be observed, indicating separate functions from microglia (van Weering et al., 2010). CCL21 expression in CNS revealed to induce a massive brain inflammation, but not lymphocytic infiltration in transgenic mice expressing the chemokine (Chen et al., 2002). Interestingly, CCL21/CXCR3 signaling axis was never explored in ALS.

CD200

The membrane glycoprotein CD200 is expressed in neurons and in endothelial cells and its receptor CD200R is restricted to cells of myeloid origin including macrophages and microglia. CD200 was also evidenced to be induced by kainic acid in microglia (Yi et al., 2012) and the authors have suggested that microglia are maintained in an activated state with autocrine signaling by interactions between microglial CD200 and CD200R, as well as in a surveillant/quiescent state by interactions between neuronal CD200 and microglial CD200R. In addition, they also demonstrated that IL-4 leads to an increased expression of CD200 what may be the mechanism down-regulating microglia activation whenever IL-4 is produced. In conclusion, deficits in CD200–CD200R system exacerbate microglia activation and the release of proinflammatory cytokines (for review, see Jurgens and Johnson, 2012). Curiously, multivariate analyses of gene expression have previously identified alterations on the CD200R expression in presymptomatic SOD1 mice model (Chen et al., 2004). Thus, more studies on the role of CD200/CD200R in ALS are needed to evaluate whether its disruption is implicated in ALS.

Neuregulins

Neuregulins have been showing to be implicated in a wide range of neurological and psychiatric disorders including multiple sclerosis, schizophrenia, and AD. Neuregulin precursors are expressed predominantly in cortical neurons, but also accumulate at the surface of white matter astrocytes. CSF neuregulin was found reduced in ALS and increased in AD (Pankonin et al., 2009). Neuregulin-1 (NRG1) is a growth and differentiation factor that binds to erbB receptors in microglia. NRG1–erbB signaling is activated after peripheral nerve injury and contributes to microgliosis and neuropathic pain (Calvo et al., 2010). Recently, it was suggested that NRG1 released from damaged neurons and other cells in the SC triggers microglial activation leading to progressive MN degeneration in ALS (Song et al., 2012). Indeed, it was observed that in the early stage of ALS there was activation of NRG1 receptors on microglia. Later on, a reduced membrane-bound NRG1 and an increased mRNA expression were noticed in the SC. More studies are, however, needed to sustain the NRG1 contribution to ALS pathogenesis.

β2-Microglobulin

Major histocompatibility complex (MHC) class I and class II molecules present fragments of peptide antigens to $CD8^+$ or $CD4^+$ T cells, respectively, and cells lacking such molecules are unable to interact with immunocompetent T cells (Parnes, 1989). Presentation of antigenic peptides to $CD8^+$ cells is mediated by β2-microglobulin, which is non-covalently bound to MHC class I. Despite being recently observed during hippocampal neurodevelopment (Leinster et al., 2013), adult neurons do not constitutively express MHC class I (Arthur-Farraj et al., 2012). In contrast, spinal MNs were shown to display a high constitutive expression of both MHC class I and β2-microglobulin mRNAs (Thams et al., 2009). A latest paper describes a strong up-regulation of β2-microglobulin in MNs during disease progression in the SOD1G93A mice model, which revealed to be important for the ALS mouse survival (Staats et al., 2013). Therefore, β2-microglobulin target driven therapies may be of help in strategies counteracting ALS.

GLIAL CELL RESPONSES

It is believed that non-neuronal cells expressing mSOD1 may secrete toxic mediators or fail to secrete trophic factors, or both, resulting in either reduced function or survival of MNs. Indeed, several SFs were shown to drive axonal degeneration and regional-specific microglia activation expressing SODG93A was suggested to be implicated (Kim et al., 2006b). This calls our attention for the existence of multiple factors, and MNs and glial cells interplay in contributing to ALS onset and progression. Microglia activation and dysfunction have lately been related with the onset, but mostly with the progression, of several neurodegenerative diseases. Particularly, in ALS, it has been suggested that besides MNs, also glia and muscles are implicated in the disease.

Prominent neuroinflammation is a pathological hallmark in human ALS and mouse models of the disease. Gliosis and accumulation of large numbers of activated microglia and astrocytes can be observed in CNS and in SC areas. Active contributions of glial cells in ALS pathology have recently been reviewed (Lasiene and Yamanaka, 2011; Zhao et al., 2013). Conflicting results were published on whether the expression of mSOD1 in astrocytes and microglia really contributes to the progression of ALS disease. Some argues against (Yoshii et al., 2011) and others in favor (for review, see Boillée et al., 2006b). Overall, it will be important to identify the molecules released by glia, as well as the ones secreted by dysfunctional neurons targeting microglia or even acting retrogradely.

Toll-like receptor activation

Glial cells (astrocytes, microglia, oligodendrocytes, and Schwann cells), as well as neurons are known to express different members of the TLR family (Lee et al., 2013b). Increasing evidence indicates that, in the absence of pathogens, TLR signaling can be activated by molecules released by the injured tissue, namely the HMGB1 protein, one of the damage-associated molecular patterns (DAMPs) molecules (Bianchi and Manfredi, 2009). Recent findings have underlined the activation of TLRs and RAGE signaling pathways in ALS (Casula et al., 2011). TLR2, TLR4, and RAGE expression was increased in reactive glial cells in both gray (ventral horn) and white matter in SC. TLR2 was predominantly detected in cells of the microglia/macrophage lineage, whereas TLR4 and RAGE were strongly expressed in astrocytes. Activated macrophages (He et al., 2012) and microglia (personal communication) release HMBG1 that promotes the secretion of IL-1β and IL-18 further inducing the necrosis of neighboring neurons and the amount of extracellular HMBG1, which binds to microglial Mac1 leading to the activation of nuclear factor-κB (NF-κB) pathway (Gao et al., 2011) and inflammasome (Lu et al., 2013), forming a vicious circle that sustains progressive neurodegeneration (**Figure 1**). Recent data have demonstrated that NF-κB activation in wt microglia causes MN death providing a therapeutic target for ALS (Frakes et al., 2014).

When evaluating whether extracellular mSOD1 caused a direct or indirect injury to MNs, it was observed that neurodegeneration was mediated by microglia and concerted activation of CD14/TLR pathway involving both TLR2 and TLR4 (Zhao et al., 2010). In addition, granulocyte macrophage-colony stimulating factor (GM-CSF), a pleiotropic cytokine predominantly released by astrocytes that up-regulates TLR4 and CD14 expression in microglia, may function to exacerbate TLR signaling in ALS disease (Parajuli et al., 2012). Indeed, when GM-CSF was blocked it was observed a delayed onset and increased life span in the ALS mice (Turner and Talbot, 2008). The activation of these pathways may contribute to the progression of inflammation and to a further injury to MNs.

NG2 Cells, oligodendrocytes, and Schwann cells

Oligodendrocytes in CNS, and Schwann cells in the peripheral nervous system (PNS), are responsible for the myelin sheaths surrounding neurons which provide electrical insulation essential for rapid signal conduction. Schwann cells also participate in the clearance of debris and in guiding the axon after neuron damage (Ilieva et al., 2009). In the SC injury, reactive gliosis emerges in the lesion accompanied by the up-regulation of chondroitin sulfate proteoglycans (CSPGs) in oligodendrocytes and Schwann cells.

Considering the regenerative capacity of NG2 cells, one of the first cells responding to any alteration in CNS environment, it will

be important to clarify their role in ALS since they can be mobilized to originate cells or to release factors. Nevertheless, NG2 cells were shown to remain committed to an oligodendrocyte lineage in both controls and mutant ALS mice, indicating their scarce participation (Kang et al., 2010). However, increased proliferation rate of NG2 revealed to mediate an elevated number of early-born oligodendrocytes that degenerate, resulting in gray matter demyelination in ALS mice and human CNS (Kang et al., 2013).

Although only a few studies have examined whether oligodendrocytes or myelin sheaths have a role in ALS, the myelin abnormalities consisting in loss of compact myelin and lamellae detachment in the SC of pre-symptomatic SOD1 transgenic rats and aggravated at symptomatic stages (Lasiene and Yamanaka, 2011) suggest that it may be an interesting target for further study. Biochemical examinations of the myelin structural components revealed a decrease in the phospholipid content along disease progression, as well as in cholesterol (already in the early presymptomatic stage) and cerebrosides (in the paralyzed animals) (Niebroj-Dobosz et al., 2007). Degeneration of oligodendrocytes was observed in human patients and mice models of ALS prior to the disease (Kim et al., 2013). Cells that were lost were replaced by newly differentiated oligodendrocyte precursor cells, which, however, evidenced reduced myelin basic protein. This finding is suggestive that they may contribute to MN degeneration in ALS (Philips et al., 2013). Moreover, these cells evidenced a loss of the monocarboxylate transporter 1 (MCT1), thus compromising the supply of lactate to MNs (**Table 2**).

Little is known about Schwann cell involvement in ALS pathology. Schwann cells are located near the MN axons and are known to bridge between denervated and reinnervated endplates, and to guide axonal sprouts (Magrane et al., 2009). Expression of mSOD1 in perisynaptic Schwann cells was suggested to interfere with the trophic maintenance of normal or regenerating motor axons (Inoue et al., 2003). *In vivo* evidence suggests that glial fibrillary acidic protein (GFAP) up-regulation in the stressed/proliferating Schwann cells may be the underlying pathological events (Keller et al., 2009). Conversely, different results were obtained in SOD1G73R mice where elimination of mutant SOD1G37R from Schwann cells failed to slow disease progression (Lobsiger et al., 2009). Nevertheless, Wang et al. (2012) found that knockdown of mSOD1 in Schwann cells of SODG85R transgenic mice delayed disease onset and extended survival indicating that SOD1G85R expression is neurotoxic. These results imply that diverse mutations confer different outcomes to cell toxicity and, in the case of Schwann cells, oxidative damage seems to be an important feature in the context of ALS.

Astrocyte reactivity

Astrocytes are a potential source of both pro- and anti-inflammatory cytokines and are ideally placed in close proximity to BBB and BSCB, thus translating signals from the periphery to the CNS. Although not being immune cells, they can also contribute to the immune response. Astroglial activation, or astrogliosis, is characterized by hyperplasia, hypertrophy of cell bodies and cytoplasmic processes, up-regulation of intermediate filament proteins, namely GFAP and vimentin, mediating a histologically apparent glial scar at the lesion site in the damaged SC (Sofroniew, 2009). Reactive astrogliosis in ALS was first revealed in 1990s by increased GFAP staining in the subcortical white matter (Kushner et al., 1991), and later on similarly observed in the ventral and dorsal horns of the SC, as well as in the transition between gray matter and anterior and lateral funiculi, where the dystrophy of neuritis exists (Schiffer et al., 1996). SC astrocytes were shown to assume a neurotoxic phenotype in response to extracellular ATP. In SOD1G93A astrocytes this activation is mediated through P2X7 receptor signaling (Gandelman et al., 2010). Reactive astrocytes surround degenerating MNs in patients and transgenic animal models of ALS, and in particular those localized in the ventral SC of SOD1G93A mice, are a source of nerve growth factor (NGF) and NO, which are both required for MN apoptosis (**Table 2**) (Pehar et al., 2004). Interestingly, the NO-induced MN death was shown to be mediated by astrocytes expressing SOD1 and TDP-43 mutations (Rojas et al., 2014). Ferraiuolo et al. (2011) have shown that the increase in total NGF is due to the secreted pro-NGF fraction before cleavage to the mature form, achieving a twofold increased ratio in the SOD1G93 mice as compared to control conditions. Such finding may derive from the increased ALS-associated MMP-9 (Niebroj-Dobosz et al., 2010), one of the enzymes that degrade mature NGF in the extracellular space. In this scenario, neuroprotection of MNs could be achieved by promoting pro-NGF cleavage.

Recently, it was demonstrated that loss of GFAP did not affect disease onset and marginally shorten the SOD1H46R mice survival, indicating that GFAP only plays modulatory effects (Yoshii et al., 2011), at least in this mice. On the contrary, astrogliosis was observed in the SOD1G93A mice in either symptomatic or presymptomatic phase, preceding microglial activation (Yang et al., 2011). Indeed, depending on the ALS animal model used, astrocyte activation was observed to occur earlier or later and to be rather complex, increasing and decreasing in waves at different times throughout the disease (for review, see Evans et al., 2013). Despite no differences in the astrocyte number, increased GFAP labeling in the ventral horn of lumbar SC from SOD1G93A mice was observed before MN loss (Gerber et al., 2012). Astrocytes derived from such mice evidenced to uptake glutamate less efficiently and showed a reduced trophic response to activation, deficiently protecting MNs (Benkler et al., 2013). Attesting this low astrocyte efficiency, the mainly expressed astroglial S100B protein was found to be decreased in CSF (Sussmuth et al., 2003) and serum (Otto et al., 1998) from ALS patients. Interestingly, exposure of primary cultures of astrocytes from ALS patients to CSF evidenced to enhance GFAP and S100B expression (Shobha et al., 2010). Similarly to neurons, astrocytes have demonstrated constitutive and regulated expression of FKN, which may control the migration and function of the microglia (Sunnemark et al., 2005). Additional contribution of astrocytes to MN death involves the release of ROS mediated by mitochondrial dysfunction and of D-serine that is a co-activator of the NMDARs, thus exacerbating glutamate excitotoxicity, among other factors as indicated in **Table 2** and reviewed in Valori et al. (2014).

A recent publication has characterized a specific astrocytic phenotype (aberrant astrocytes) obtained from primary SC cultures of SOD1G93A symptomatic rats (Diaz-Amarilla et al., 2011). These

aberrant astrocytes isolated on the basis of marked proliferative capacity and lack of replicative senescence, lack GLT-1 and the NG2 marker and were shown to release toxic factors accounting for a MN hostile environment. This specific astrocyte phenotype expressing increased S100B and connexin-43 (Cx-43) is abundant in the symptomatic phase of the disease and seems to be located close to MNs, representing a new potential target for delaying ALS progression.

Data in *postmortem* tissues of ALS patients revealed changes in the morphology of astrocytes together with elevated GFAP and aldehyde dehydrogenase family 1, member L1 (ALDH1L1) (Philips and Robberecht, 2011), corroborating findings in animal models of ALS. Indeed, mSOD1 gene excision from microglia and selective reduction in astrocytes significantly slowed disease progression (Yamanaka et al., 2008b). However, there is still some controversy on whether astrogliosis is detrimental or beneficial, what surely will depend from spectrum intensity and toxicity potential of the aberrant phenotype.

Microglia activation

Microglia are the immune resident cells of the CNS with both a supporting role to neurons and astrocytes, and immunological properties with either neuroprotective or neurotoxic potential. Though microglia activation was indicated to precede astrocyte reactivity (Alexianu et al., 2001), reduced neuroprotective behavior of mSOD1 microglia in a resting-state (Sargsyan et al., 2011), and at the disease end-stage (Nikodemova et al., 2013) was documented. If confirmed, boosting of microglia with stimulatory factors may reveal to be clinically useful.

In a recent paper, Roberts et al. (2013) verified that incubation of microglia with aggregated SOD1 first drives its location at the membrane level, and later within the cell. Moreover, supernatants of these SOD1 activated microglia caused a significant decrease in MN viability, which was not related with tumor necrosis factor-α (TNF-α) secretion, NO, or superoxide anion radical. The toxic factors involved are currently unknown, although it was shown that expression of mSOD1 increases TNF-α secretion (Liu et al., 2009), which may induce neurotoxicity by increasing the glutamate release by microglia in an autocrine manner (Takeuchi et al., 2006). Therefore, a very early stage of the disease may rely in this link between protein aggregation and microglial activation. Indeed, neurotoxic potential of mutant microglia in MN degeneration in ALS is a very well documented issue: (1) mSOD1 acting on microglia is required to cause the disease (Clement et al., 2003); (2) limiting mutant damage to microglia slows progression (Boillée et al., 2006b); replacement of mSOD1 microglia for wt microglia delays disease and prolongs mice survival (Lee et al., 2012).

Microgliosis at sites of MN injury is a neuropathological hallmark of ALS and recent therapeutic interventions are looking into factors capable of skewing microglia neurotoxic potential into a neuroprotective phenotype. However, a trophic role for activated microglia has been suggested at early stages of the disease (for review, see Lewis et al., 2012). In the SOD1G93A mice, it was observed a decrease in microglia number in the entire SC at the pre-symptomatic age (Gerber et al., 2012). The authors identified two diverse microglia subpopulations (low and high Iba1 expression cells), suggesting a distinct microglia involvement at pre- and early-symptomatic ALS stages. Interestingly, microglia heterogeneity observed between cervical and lumbar SC regions in ALS mice may derive from different environmental specificities determined by local MN/astrocyte/lymphocyte disposition and activation (Beers et al., 2011b). While cortical microglia appear unaffected by the disease, additional studies evidenced an increased microglial number in the lumbar SC at symptom onset, and neither the typical inflammatory nor the anti-inflammatory phenotypes were identified at end-stage (Nikodemova et al., 2013). In addition, elevated TNF-α gene expression and immunoreactivity were observed in lumbar SC of mSOD1 mice and related with invading microglia (Yoshihara et al., 2002). Early microglia activation in ALS may be further explored *in vivo* by positron emission tomography (PET). The possibilities of PET suggest its valuable contribution to monitor the progression of the disease and the efficacy of the therapy in use (Corcia et al., 2012).

Context-dependent neuroprotective and neurotoxic properties. An important neuroprotective role of microglia is phagocytosis. Following signaling by neuronal FKN, milk-fat globule EGF factor-8 protein (MFG-E8, SED1) is up-regulated and serve as a bridge via specific integrins between apoptotic neurons and microglia (**Figure 1**) (Leonardi-Essmann et al., 2005). The authors hypothesize that MFG-E8 is assembled on the surface of exosomes and apoptotic neurons so microglia can recognize their target cells. MFG-E8 seems to be essential for microglia engulfment and removal of the dying neurons. An interesting concept is that inflammatory microglia can also phagocyte viable neurons (Fricker et al., 2012a) through MFG-E8 mediation (Fricker et al., 2012b) accounting for a reduced number of neurons. Phagocytic ability may, however, be lost if the cell is continuously stressed by a neurotoxic stimulus.

Our recent data with the neurotoxic unconjugated bilirubin have evidenced that following the phagocytic ability at eliminating cell debris microglia changes to a more inflammatory phenotype (Silva et al., 2010) and even to a senescent-like cell morphology and death if the duration of exposure is prolonged. However, microglia behavior in the presence of unconjugated bilirubin is modulated by the presence of both astrocytes and neurons (Silva et al., 2011). Also to consider is that during the inflammatory phenotype, microglia may intervene in the glutamate homeostasis, but may also contribute to neurite degeneration through the release of NO (Silva et al., 2012). Interestingly, microglia phagocytic features were shown to occur early in ALS disease. Microglia were revealed to aggregate, proliferate, and phagocyte in the lumbar SC of pre-symptomatic mutant SOD1H46R transgenic mice (Sanagi et al., 2010). However, in other studies microglia have shown to contribute to MN death (Dibaj et al., 2011; Brettschneider et al., 2012) and to decrease in number within disease progression (Butovsky et al., 2012), thus contributing to the disease propagation. By using *in vivo* imaging by two-photon laser-scanning microscopy and axonal transection, it was observed different phases of microglia-mediated inflammation in the ALS mice model. Indeed, a first phase (preclinical)

with highly reactive microglia was followed by another (clinical stage) with morphologically transformed microglia presenting reduced surveillance activity and reactivity (Dibaj et al., 2011). Regional, temporal, and immune environmental differences may contribute to changes in microglia phenotypes and response heterogeneity, thus requiring differentiated immunomodulatory or even combinatory therapeutic approaches along ALS disease progression.

Microglia phenotypes. Activation of microglia may be observed through the up-regulation of CD11b, Iba1, and CD68 markers. Primary microglia differ from other blood macrophages in the expression of CD11b/CD45$^{low/high}$ and CD68$^{low/high}$ (for review, see Hinze and Stolzing, 2011), although not specific for microglia in a pathologic brain (Matsumoto et al., 2007). These cells show a round morphology with an enlarged cell body and smaller and thicker processes in the resting/quiescent state (M0; **Figure 2**). When activated in response to an insult or injury, microglia are capable of acquiring diverse and complex phenotypes, allowing them to participate in the cytotoxic response, immune regulation, and injury resolution. Microglia may then favor the entrance of inflammatory T cells with which the cells seem to interact. Recent work have classified and characterized M1 and M2 phenotypes (**Table 2**) (for review, see Evans et al., 2013). The first is cytotoxic, characterized by the release of proinflammatory cytokines and influenced by T helper cell type 1 (Th1) that release GM-CSF and interferon-γ (IFN-γ), triggering M1 proliferation. Cytotoxic M1 markers include IL-1β, IL-6, TNF-α, iNOS, COX2, and CX3CL1, and known inducers are the TLR4 agonist LPS and IFN-γ (Chhor et al., 2013).

The M2, promoted by the cytokines IL-4 and IL-13 released by Th2, contribute to neuroprotection once they also secrete anti-inflammatory cytokines, such as IL-4 and IL-10, and growth factors such as insulin-like growth factor 1 (IGF-1). Indeed, IL-4 was shown to protect MNs from the injury produced by LPS-activated microglia (Zhao et al., 2006). Thus, M2 polarization may be desirable, although excessive or prolonged M2 polarization may become prejudicial in allowing unwanted fibrotic responses and scarring, not facilitating axonal growth. However, there are three M2 phenotypes: the M2a or alternate activation repair/regeneration/remodeling phenotype, the M2b immunoregulatory and the M2c acquired-deactivating (**Figure 2**). Reliable markers for M2a stimulated by IL-4 and IL-13 are high IL-1 receptor antagonist (IL-1Ra) and high arginase (Arg1). M2b is stimulated by immune complexes, TLR agonists and IL-1R ligands. Useful markers are IL-1Ra and SOCS3. Characterization of M2c stimulated by IL-10, transforming growth factor-β (TGF-β) and glucocorticoids is obtained through the increased levels of anti-inflammatory cytokines (IL-10, TGF-β), low levels of pro-inflammatory cytokines and enhanced IL-4Rα, Arg1, SOCS3, and CD206 (David and Kroner, 2011; Wilcock, 2012; Chhor et al., 2013). While LPS, IL-1β, TNF-α, and IFN-γ lead to cytotoxic M1 and immunomodulatory M2b activation states, IL-4 mainly triggers M2a phenotype (Chhor et al., 2013).

Therefore, balance between M1 and M2 phenotypes may be a desirable therapeutic goal. Interestingly, mSOD1 microglia isolated from ALS mice at disease onset showed higher levels of Ym1, CD163, and brain-derived neurotrophic factor (BDNF) (M2 markers) and lower levels of Nox2 mRNA (M1 marker) as compared to the end-stage disease (Liao et al., 2012). Interestingly, when co-cultured with wt MNs the first microglia phenotype exerted neuroprotection while the M1 phenotype was neurotoxic, supporting the pathoprogression-related changes in microglia. We may probably consider that microglia display the M2 phenotype at an early stage of the disease switching to the M1 phenotype during the late rapid phase (reviewed in Zhao et al., 2013). However, we should also consider that microglia may become functionally impaired at the end-stage, as recently observed in the SOD1G93A SC microglia (Nikodemova et al., 2013). Different roles of microglia depending on the neurological disease context was recently suggested by Chiu et al. (2013) based on the observation by FACS-transcriptome comparisons that SOD1G93A microglia show a unique phenotype that differs from M1 or M2 macrophages, and from activation with LPS. Robust up-regulation of MMP-12, IGF-1 and osteopontin were pointed as hallmarks. Transcriptomic technology has been carried out to examine the gene expression changes of ALS tissue as compared to controls (reviewed in Heath et al., 2013) and in the future may provide insights into the microglia phenotype profiling prior to disease onset and along ALS progression to assist in the development of new treatments for ALS at different stages.

Inflammatory microRNA profiling. MicroRNAs (miRNAs), small, non-coding RNAs, have been recently pointed to mediate cell-to-cell communication (Xu et al., 2013). Inflammatory phenotypes are miR-155, miR-21, miR-146a/b, and miR-124 (Quinn and O'Neill, 2011). Recent studies demonstrated the miR-124 involvement in promoting microglia quiescence by skewing their polarization from an M1 to an M2 phenotype (Willemen et al., 2012) and that miR-155 together with miR-124 are likely to be directly related to M1 and M2 phenotypes, respectively (Cardoso et al., 2012; Ponomarev et al., 2013). Up-regulation of miR-155 is induced by TNF-α and Il-1β (Pottier et al., 2009), as well as by HMGB1 through the activation of the TLR2/MyD88/miR-155 pathway (Wen et al., 2013), while it is down-regulated by TGF-β (Pottier et al., 2009). Increase of miR-146a expression was shown to occur in the aged mice (Rodier and Campisi, 2011), as well as after LPS stimulation (Jiang et al., 2012), and is related with the microglia phagocytic potential (Saba et al., 2012). Moreover, up-regulation of miR-21 in murine models of SC injury was pointed as a modulator of the pro-reactive effects of inflammatory signaling cascades (Nieto-Diaz et al., 2014). Dysregulation of miRNAs expression was lately found in the SC of ALS patients and in microglia isolated from the hSOD1G93A mice. Promising candidates that were found to be altered in patients were miR-146a*, miR-524-5p, and miR-582-3p (Campos-Melo et al., 2013), while those up-regulated in the mice model were miR-155, miR-146b, miR-22, miR-365, and miR-125b (Parisi et al., 2013), which need to be further investigated for their relevance as ALS biomarkers and therapeutic targets. Actually, it was recently discovered that inhibition of miR-155 prolongs survival in the mSOD1 mice (Koval et al., 2013).

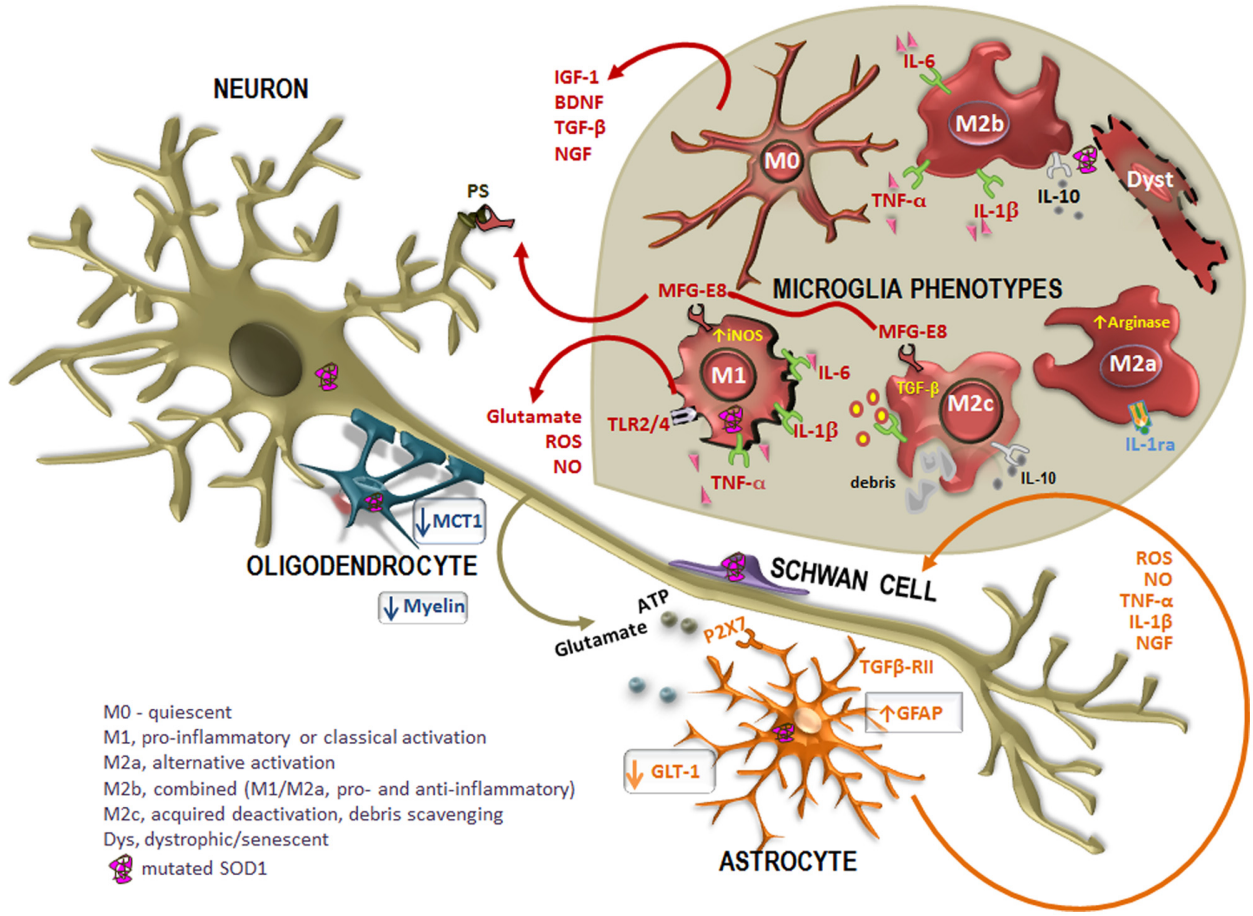

FIGURE 2 | Altered cross-talk between glial cells and motor neurons (MNs) in ALS disease. Many studies report the intervention of mutated superoxide dismutase-1 (SOD1)-expressing non-neuronal cells in the pathogenesis of the disease. In case of injury, astrocytes become activated in a process called astrogliosis, characterized by the up-regulation of intermediate filament glial fibrillary acidic protein (GFAP) and increased release of toxic products into the extracellular media, such as pro-inflammatory cytokines [tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β)] oxidative stressors [reactive oxygen species (ROS), nitric oxide (NO)], as well as glutamate and ATP mediated by the receptor P2X7. In addition, astrocytes also evidence a reduced expression of the glutamate transporter GLT-1, which additionally contribute to neuronal excitotoxicity. However, reactive astrocytes up-regulate nerve growth factor (NGF), which can modulate neuronal survival. Disorganization and destruction of the myelin sheath with a progressive loss of phospholipids and cholesterol is also observed. Moreover, oligodendrocytes also evidence a loss of the of the monocarboxylate transporter 1 (MCT1), thus compromising the energy supply to MNs. On the other hand, increased mutated SOD1 expression in Schwann cells may have intricate ways contributing to slow ALS progression. When mutated SOD1 accumulates within microglia, there is a pattern of activation. Microglia acquire different activation phenotypes (M0, M1, M2a, M2b, M2c, and dystrophic/senescent) and, consequently, produce a diversity of substances that may be either beneficial [insulin-like growth factor 1 (IGF-1), transforming growth factor-β (TGF-β), brain-derived neurotrophic factor (BDNF), and nerve growth factor (NGF)] or toxic (glutamate, ROS, NO) to other cells. Phagocytosis is mediated by the release of milk factor globule-8 (MFG-E8) from microglia M1 and M2c phenotypes that recognizes phosphatidylserine (PS) in apoptotic neurons. Reliable markers for M2a are high IL-1 receptor antagonist (IL-1Ra) and high arginase, for M2b are IL-10, TNF-α, IL-6, and IL-1β, for M2c are TGF-β and IL-10 and for M1 are TNF-α, IL-6, and IL-1β, increased inducible nitric oxide synthase (iNOS) and toll-like receptors (TLR)2 and 4.

Autotaxin. Autotaxin (ATX) is a secreted lysophospholipase D that converts lysophosphatidylcholine (LPC) to lysophosphatidic acid (LPA), a phospholipid growth factor that activates several transduction pathways, and is involved in migration, proliferation, and survival of various cells (Mori et al., 2011; Furukawa et al., 2013). LPA acts as an autocrine and/or paracrine signaling molecule mediating a broad range of intracellular signaling cascades, especially the RHOA pathway (reviewed in Willier et al., 2011). LPA receptors are expressed by astrocytes, oligodendrocytes, and microglia (Lorenzl et al., 2006; Shi et al., 2010a; He et al., 2013).

Data suggest that ATX levels increase within reactive astrocytes following neurotrauma (Savaskan et al., 2007), but other studies have obtained a down-regulation instead (Shi et al., 2010b). Most fascinating, it was observed that overexpression of ATX inhibits microglial activation and protects the cell against oxidative stress (Awada et al., 2012). Thus, reduced expression of ATX in microglia may contribute to a sustained neuroinflammation condition. Since ATX is also a motility driver that stimulates cell invasion (Sau et al., 2007; Hoelzinger et al., 2008; Magrane and Manfredi, 2009) it can be involved in microglia migration, a property not exploited till

now. Although ATX is considered an inflammatory mediator we were not able to find studies demonstrating its relevance or not in ALS.

NEUROVASCULAR CHANGES

BBB and BSCB are dynamic and complex interfaces between the blood and the CNS. Endothelial cells and tight junctional complexes physically limit solute exchanges between the blood and the brain. These cells together with pericytes, astrocytes, neurons, microglia, and oligodendrocytes and the basement membrane form the neurovascular unit (for review, see Cardoso et al., 2010; Sá-Pereira et al., 2012). The interaction between all these components provides a sustainable environment for neural function while restricting permeability and transport. Alterations in barrier properties and dynamics were observed in transgenic SOD1 rats (Andjus et al., 2009; Nicaise et al., 2009). In particular, it was noticed a reduction of endothelial tight junctions (Zhong et al., 2008), together with the disruption of the neurovascular unit and up-regulation of MMP-9 (Miyazaki et al., 2011) prior to MN degeneration. MMP-9 activation was observed in blood vessel-like structures and microglia. Increased microvascular microglia, expressing CX3CR1 and weakly labeling Iba1, were detected in the SCs from the ALS mice model and suggested to have a bone marrow origin (Lewis et al., 2009). In addition, it was recently demonstrated that SC microglia in the mSOD1 mice promote the recruitment of inflammatory monocytes into the CNS well before the onset of the disease, triggering microglia apoptosis (**Table 2**) (Butovsky et al., 2012). Actually, the BSCB has been shown to be disrupted in different mSOD1 mice before MN degeneration, thus favoring blood monocytes infiltration (Barbeito et al., 2010). To that it may account the 54% reduction in pericytes at the BSCB level in patients with ALS (Winkler et al., 2013). In fact, some authors consider ALS as a neurovascular disease (Garbuzova-Davis et al., 2012) with evidence indicating impairment of all neurovascular unit components, including the BBB and the BSCB in both ALS patients and animal models. Disruption of BBB and BSCB was observed in the G93A mice by electron microscopy in places of MN degeneration at both early and late disease stages (Garbuzova-Davis et al., 2007). The damage of barriers besides allowing the entrance of T lymphocytes, such as $CD4^+$ (helper/inducer) and $CD8^+$ (cytotoxic) into the brain parenchyma, may also permit the entrance of harmful substances that will contribute to disrupt neuronal homeostasis and accelerate MN degeneration. The impact of microvascular damage in ALS pathology is a novel and promising research topic in that interactions with glia may determine neuroprotective or cytotoxic cell phenotypes with consequences on MN survival.

INFLAMMATORY COMPONENTS IN ALS AND PATHOLOGICAL CELL–CELL COMMUNICATION

Communication between neurons and microglia is essential for maintaining homeostasis in the CNS and altered cross-talk is implicated in the pathogenesis of ALS and other MN diseases (Appel et al., 2011). Dysregulated neuron–neuron signaling and neuron–microglia cross-talk in ALS, and other neurodegenerative diseases, may derive from: (i) secretion of toxic mediators or fail to secrete trophic factors, or both, resulting in reduced function and survival of MNs; (ii) SFs released by neurons and their action on microglia receptors; (iii) changes in direct cellular interactions.

Reduced expression of trophic factors such as BDNF, fibroblast growth factor 2 (FGF2), and IGF-1 was found in the SC of the newborn rat after intrathecal administration of CSF from ALS patients (Deepa et al., 2011). To note, that the release of mSOD1 was shown to trigger microgliosis and neuronal death (Urushitani et al., 2006) and increased levels of ROS/RNS, mainly of NO, were shown to play a critical role in the earliest stages of neuronal dysfunction in ALS (Drechsel et al., 2012). NO mainly produced by glia through the activation of iNOS can react with superoxide producing the potent oxidant peroxynitrite (Beckman et al., 1990), which mediates apoptosis or necrosis, depending on the concentration (Bonfoco et al., 1995). Peroxynitrite also induces toxic glial phenotypes further propagating oxidative damage and cellular dysfunction. Interestingly, the same concentrations of NO may either promote the survival of MNs healthy conditions or induce apoptosis and glia reactivity when in the presence of stressors. Up-regulation of neuronal NO synthase (nNOS) was shown to occur in MNs before iNOS in either pre-symptomatic ALS animal models or in patients and to be associated with MN loss (Moreno-Lopez et al., 2011; Drechsel et al., 2012).

Activated microglia and astrocytes amplify the initial damage to the MNs by activating AP-1 and NF-κB through production of proinflammatory cytokines and apoptosis-triggering molecules such as TNF-α and Fas ligand (FASL). TNF-α and IL-1β exert neurotoxic effects *in vitro*, but deletion of the individual genes seems to not affect the course of the disease. In addition, dying MNs release ATP that can further activate glia through the purinergic receptor P2X7 expressed by both microglia and astrocytes (**Figure 2**) (Gandelman et al., 2010; Glass et al., 2010). Classical microglia activation results in upregulation of MHC class II proteins that are involved in presentation of antigens to T lymphocytes. Microglia activation is mediated by the release of SFs and/or expression of surface receptors by neurons and astrocytes, such as CX3CL1, CD200, and CCL21. Microglia also express a diverse set of pattern recognition receptors (PRRs) for pathogen-associated molecular patterns (PAMPs) and endogenous ligands derived from injury that include TLRs and inflammasome. In addition, emerging evidence suggests that members of the nuclear receptor (NR) family of transcription factors, many of which are ligand-dependent, control the activation of microglia under physiological and pathological conditions (for review, see Saijo et al., 2013).

Neuroinflammation involves the activation and proliferation of microglia and the infiltration of T cells into the brain and SC. In these conditions, astrocytes and microglia release IL-1β, TNF-α, and IL-6 (**Figure 2**). Cytokines can then up-regulate oxidative stress by NO and superoxide (O_2-) generation (for review, see Philips and Robberecht, 2011). Repair and limitation of the damage is promoted by the release of trophic and anti-inflammatory factors. Deleterious microglia M1 and benign M2 phenotypes are influenced by astrocytes and T cell subsets. In ALS, it is still currently unknown the precise function of microglia and astrocytes and how they mediate neuroinflammation and contribute to the pathology. Identification of the factors driving microglia

phenotype and consequent functional changes, once known, may advance our knowledge on their role and on ways to modulate these cells. In addition, it will be important to clarify whether inflammation contributes to ALS pathogenesis or in opposite is a protective response, and if different individual immune responses to the disease are also implicated, so that immunomodulatory therapies can be pursued.

MICROGLIA–T CELL CROSS-TALK

Evidence for autoimmunity in ALS was proposed in 1990s (for review, see Appel et al., 1993). Inflammation is not a resultant from MN degeneration since it regulates the balance between neuroprotection and neurotoxicity (Henkel et al., 2009). Initially, the diverse populations and phenotypes of $CD4^+$ T cells that cross-talk with microglia can slow disease progression, but later they may contribute to the acceleration of the disease. The inflammatory process involves infiltration of T cell subpopulations at sites of neuronal injury in the brain parenchyma. T cells may damage MNs by cell–cell contact or cytokine secretion through the activation of microglia (Holmoy, 2008). $CD8^+$ cytotoxic T cells and natural killer T (NKT) cells were found significantly increased in patients with ALS, while regulatory T (Treg) cells were decreased (Rentzos et al., 2012). It was suggested that $CD4^+$ T cells may trigger oxidative phosphorylation in microglia and $CD8^+$ may stimulate phagocytosis accordingly to data obtained by FACS-transcriptome comparisons in cells isolated from the SC of SOD1G93A mice (Chiu et al., 2013).

The naive T cells, or Th0 cells, expand and differentiate into at least four functionally distinct subsets upon stimulation: Th1, Th2, Treg, and Th17 cells. Th1 cells secret IFN-γ and turn resting microglia into M1 phenotype; in contrast Th2 and Treg cells release IL-4 which induces activation of resting microglia into M2 phenotype (for review, see Lewis et al., 2012). Th1 and Th17 are $CD4^+$ T cells that produce proinflammatory cytokines, causing damage while $CD4^+CD25^{high}$ T lymphocytes (Treg) suppress Th1 cell effector function (Dittel, 2008).

Interestingly, it was demonstrated that mSOD1 mice lacking $CD4^+$ T cells evidence a faster disease progression and decreased microglia reactivity and as so, future therapeutic interventions should consider the benefits that T cells may have (Beers et al., 2008; Chiu et al., 2008; Zhao et al., 2013). Compromise of Treg lymphocytes with disease progression diminishes the secretion of IL-4 and fails in suppressing the toxic properties of microglia (Beers et al., 2011a). Indeed, mSOD1 Treg suppress immune toxicity by inhibiting microglial activation, $CD4^+CD25^-$ (T effector cells) proliferation, and the accompanying cytotoxicity, thus providing MN protection in ALS (Zhao et al., 2012). As disease progresses, the supportive Treg/Th2/M2 changes to an injurious Th1/M1 response triggering increased TNF-α secretion that was shown to induce the dysfunction of Tregs (Zhao et al., 2013). Therefore, elevation of Tregs in patients with ALS may trigger a longer life expectancy.

MN–ASTROCYTE CROSS-TALK

It was before considered that the mutant protein was the responsible for the disease onset in MNs with microglia and astrocytes only determining disease progression and extent. However, expression of mSOD1 in microglia and astrocytes is now being related with the disease onset and early stage disease, while healthy SOD1 in those cells was shown to delay ALS progression.

Several studies evidenced the demise of MNs in the presence of astrocytes harboring SOD1 mutations (Yamanaka et al., 2008b), attesting the non-cell-autonomous pathology in ALS, i.e., degeneration of MNs requires mSOD1 expression in other cells additionally to neurons. This finding was similarly obtained for astrocytes derived from ALS patients, which have shown to cause MN death (Haidet-Phillips et al., 2011). Deregulation between astrocytes and MNs communication seems to involve TGF-β signaling pathways (Phatnani et al., 2013) and miR-124a mediated regulation (Morel et al., 2013). Evidence for the role of miRNAs in MN diseases is substantiated by the relevance that the proteins TDP-43 and FUS/TLS, responsible for the processing of miRNAs, RNA maturation and splicing (Kiernan et al., 2011), have recently acquired in ALS (Lagier-Tourenne and Cleveland, 2009; Haramati et al., 2010). A major challenge would be to establish circulating miRNAs as particularly accessible biomarkers to monitor ALS.

Activated astrocytes (see Astrocyte Reactivity for more details) may also interfere with MNs function due to the reduced secretion of trophic factors such as BDNF, glial cell line-derived neurotrophic factor (GDNF) and vascular endothelial growth factor (VEGF), but this is not a clarified issue yet (for review, see Evans et al., 2013).

REGULATION OF ASTROCYTE–MICROGLIA INTERCONNECTIVITY ACTIVATION

Both *in vitro* and *in vivo* experiments have shown that astrocytes and microglia containing mSOD1 exert deleterious effects on MNs, by releasing proinflammatory factors (Phani et al., 2012). Microglia may respond earlier than astrocytes to injury and stress by first activating NF-κB and mitogen-activated protein kinase (MAPK) signaling pathways, thus leading to a faster release of TNF-α and IL-1β (Brites, 2012). In that way one may believe that the activation of microglia precedes the reactivity of astrocytes and depends from the factors and cytokines that microglia release. Curiously, TNF-α and IL-1β released from activated microglia were reported to produce an inhibitory effect on Cx-43 expression, the main constitutive protein of gap junctions. Therefore, blockage of communication between astrocytes by the activated microglia can contribute to decrease the neuroprotective role of astrocytes (Meme et al., 2006). When considering the p25-mediated neuroinflammation, astrogliosis was shown to precede microglia activation, and apparently mediate the production of LPC, which as a chemoattractant for T cells may recruit peripheral cells into the brain (Sundaram et al., 2012). Recently, it was shown that astrocytes depend on functional microglia for response to LPS and to TLR2, 3, and 4 ligation. In their absence, astrocytes did not respond to TLR4 ligation and only weakly responded to TLR2 and 3 ligation (Holm et al., 2012). Thus, activation of astrocytes may be modulated by the proinflammatory cytokines released from microglia in an attempt to diminish the extent of excitotoxicity (Tilleux et al., 2007), but they can modulate microglia activation, as well (Welser and Milner, 2012). Which cells between microglia and astrocytes are first activated by the aggregated mSOD1 is a very controversial issue since different studies have obtained diverse

profiles of glial activation in ALS models along disease progression. Some studies point out that microglia activation precede astrocyte reactivity (Alexianu et al., 2001). Others that astrogliosis is initiated in the symptomatic phase, while prominent microgliosis is only evident later at the moribund phase (Yang et al., 2011). However, since a close interaction/communication of both cells occurs in ALS, a better understanding of the benefits and risks of astrocyte and microglia activation in ALS will help to determine whether therapeutic strategies should envisage enhancing or impairing the actions of glial cells in ALS.

MN–MICROGLIA SIGNALING

Communication between MNs and microglia is essential for maintaining local homeostasis during both physiological and inflammatory conditions. Neuroprotective signaling from MN to microglia involve FKN and CD200, as previously mentioned. In addition, microglia "calming" effects during neuroinflammation are mediated through ATX (Awada et al., 2012). Insights into the complex intercellular perturbations and influence of alarming and calming factors underlying neurodegeneration will enhance our efforts toward target-driven directed therapeutic strategies in ALS.

CX3CR1 deficiency

The neuroprotective/neurotoxic role of CX3CL1/CX3CR1 signaling is still a matter of debate once it seems to depend upon the CNS insult (for review, see Desforges et al., 2012). Deletion of CX3CR1 in a transgenic model of ALS mice was shown to extend neuronal cell loss, suggesting that CX3CL1/CX3CR1 signaling limits microglial toxicity in ALS (Cardona et al., 2006). Interestingly, it was also shown that treatment with LPS down-regulated the expression of CX3CR1, thus suppressing the functional response to FKN (Boddeke et al., 1999) and potentiating LPS neurotoxic effects (Zujovic et al., 2000). Accordingly, in ALS, the disruption of CX3CL1/CX3CR1 signaling evidenced to promote neurodegeneration following LPS administration (Cardona et al., 2006).

Cathepsin S influence

Although it has been proposed that cathepsins compensate for each other because of their overlapping substrate specificities, there is increasing evidence that disturbance of the normal balance and extralysosomal localization of cathepsins contribute to age-related diseases (for review, see Nakanishi, 2003). CatS that is a lysosomal/endosomal cysteine protease that degrades extracellular matrix proteins, even at neutral pH, was shown to be increasingly expressed by microglia upon LPS surcharge (Petanceska et al., 1996). However, some authors indicate that a further challenge with ATP is required to observe CatS release (Clark and Malcangio, 2012). This protease is preferentially expressed by antigen-presenting cells, including microglia.

Microglial CatS seems to be responsible for the liberation of neuronal FKN (**Figure 1**), which via the specific receptor CX3CR1 in microglia activate p38 MAPK pathway leading to the release of mediators that interact with neurons (Clark et al., 2007). Several inhibitors have been tried to regulate the immune modulatory effects of CatS (Clark and Malcangio, 2012) and a shift from a Th1/Th17 type response to a Th2 type of response was obtained (Baugh et al., 2011).

The up-regulation of cathepsins appears as a general transcriptional event in ALS (Gonzalez de Aguilar et al., 2008; Offen et al., 2009; Boutahar et al., 2011). However, reference to CatS and microglia activation was not until now indicated in the context of ALS. Therefore, if CX3CL1/CX3CR1 demonstrates to have a role in any stage of ALS progression, inhibition of CatS may constitute a therapeutic approach for ALS.

Exosomes and disease spread

Exosomes are secretory vesicles deriving from late endosomes and multivesicular bodies that mediate neuron–glia communication, have 50–100 nm in size and carry specific protein and RNA cargo (Fitzner et al., 2011; Fruhbeis et al., 2012; El Andaloussi et al., 2013). Exosomes contain both miRNAs and mRNAs that may be delivered and be functional in another cell (Valadi et al., 2007). Selectivity for miRNA incorporation into exosomes is proposed based on the fact that some are exclusive in exosomes derived from immature dendritic cells while others only exist in those from mature dendritic cells (Stoorvogel, 2012). Indeed, exosomes and miRNAs have been found to participate in cellular senescence and contribute to aging (Xu and Tahara, 2013). In addition senescent cells produce high levels of exosomes thus interacting and inducing the senescence of neighboring cells. As so, exosomal miRNAs might become useful biomarkers of disease. Recently, it was suggested that exosomes may be important candidates to deliver siRNA (El Andaloussi et al., 2013) and specific drugs, based on the perceived advantages of nanoparticle size and non-cytotoxicity (Sun et al., 2013), thus constituting a therapeutic platform.

Microglia was shown to internalize oligodendroglial exosomes, thus participating in the degradation of oligodendroglial membrane (Fitzner et al., 2011). However, microglia also release exosomes (Potolicchio et al., 2005; Hooper et al., 2012), which generate IL-1β to the extracellular environment propagating inflammation (Turola et al., 2012). Increased exosome discharge by microglia was observed after stimulation with α-synuclein and such activated exosomes revealed an increased membrane content in TNF-α (Chang et al., 2013). Interestingly, it was shown that mouse MN-like NSC-34 cells overexpressing hSOD1G93A secrete SOD1 via exosomes probably accounting to cell–cell-mediated mutant toxicity in ALS pathogenesis (Gomes et al., 2007). Therefore, exosomes from microglia may spread pathogenic factors, such as SOD1 and promote inflammation while influencing neuronal survival (Chang et al., 2013). In line with this, a latest study reports that cell-to-cell transmission of SOD1 misfolding is mediated by two non-exclusive mechanisms: through the release of protein aggregates that are taken up by macropinocytosis or via exosomes secreted from living cells (Grad et al., 2014).

CHALLENGES TO NERVE REGENERATION IN ALS

Reconstruction of neural network implies the restoration of tissue architecture and cell functionality. Cell replacement-based repair strategies have been tested both *in vitro* and *in vivo* and a major challenge resides in maintaining the cell function when transplanted to a broken parenchyma homeostasis. Confront with

excessive neuroinflammation and cell senescence may compromise the success of the strategy. In particular, reactive astrocytes exacerbate inflammation and by forming glial scars impede regenerating axons from traversing the lesions, while myelin debris prevent axon growth and microglia lose the ability to migrate, phagocyte or sustain inflammation from spread. Therefore, the rewiring of the CNS to foster a permissive environment for neuroregeneration is the key to a successful functional integration and repair (Xu et al., 2011; Kim et al., 2012).

CELL SENESCENCE

Redox changes within neurons (Olivieri et al., 2001), ER stress condition (Di Virgilio, 2000), and mitochondria dysfunction (Konnecke and Bechmann, 2013) are accelerated by aging and are emerging as common features relevant to the pathogenesis of neurological disorders, including ALS. Actually, the variation in SOD1 activity in aging ALS patients, when compared to younger ones, point to an increased oxidative misbalance vulnerability (Fiszman et al., 1999). Nevertheless, it was recently indicated that the ALS incidence decline in the elderly (Schoser and Blottner, 1999; Demestre et al., 2005), suggesting that the disease is not merely the result of aging. However, markers of senescence were found increased in satellite cells from ALS muscle biopsies suggesting a vulnerability to muscle atrophy (Gottschall and Deb, 1996). In addition, autophagic dysfunction and mitochondrial DNA damage in the CNS are prominently found in microglia with aging and may lead to a defective turnover of mitochondria and accumulation of hypergenerated ROS (reviewed in Nakanishi and Wu, 2009). Interestingly, dysfunctional and senescent microglia may even release compounds that inhibit neuronal autophagy (Alirezaei et al., 2008) and neurogenesis (reviewed in Wong, 2013). Given the important and necessary functions of microglia in the CNS homeostasis it is of major relevance to understand the multiple stage-related microglia phenotypes in ALS, including the increased vulnerability to a senescent cell with the disease progression, as observed in other neurodegenerative and age-related CNS disorders (Luo et al., 2010; Kaplan et al., 2014).

Microglia degeneration

Recent data obtained in the mSOD1 mouse model suggest a dominant neuroinflammatory response in the CNS, with a reactive microglia in preclinical stages that turns into an irresponsive cell during disease progression, and a degenerative process in the PNS (Dibaj et al., 2011). Earlier studies evidenced that mutated SOD1 microglia have an age-dependent cytotoxic potential which reveals upon a stimulatory effect (Weydt et al., 2004). Interestingly, extracellular SOD1G93A mediates the activation of CD14–TLR2 pathway with the consequent release of TNF-α and IL-1β, thus propagating the proinflammatory stimuli (Bowerman et al., 2013). However, since immunosuppressive strategies have not proven consistent efficacy (Appel et al., 1993, 2011; Baugh et al., 2011), one may believe that microglia function may change along disease progression with consequent differential effects on MNs. Late data evidenced that SC microglia proliferate and that TNF-α mRNA expression decreases during disease progression in SOD1G93A rats (Nikodemova et al., 2013). Indeed, microglia revealed to not be polarized to M1 or M2 phenotypes at any disease stage and CNS region evaluated. In addition, SC microglia evidenced to be irresponsive to experimental systemic inflammation at ALS end-stage. Diminished glial neuroprotection by senescent and/or dysfunctional microglia has been suggested to play a role in neurodegenerative diseases, mainly in the late stage (Streit, 2006). In such circumstances the aged microglia evidenced a "dystrophic" morphology with the loss of finely branched cytoplasmic processes, cytoplasmic beading/spheroid formation, and cytoplasmic fragmentation (cytorrhexis; **Figure 2**) (Streit et al., 2004). Such severe abnormalities in microglia, including cell fusion (multinucleated giant cells) at the symptomatic stage and cytorrhexis at the end stage are indicative of microglial aberrant activation and degeneration, respectively, and were observed in SOD1G93A transgenic rats (Fendrick et al., 2007). Although prevalent in older human subjects this dystrophic cells may also in rare instances be observed in the young brain (Luo and Chen, 2012). Pathogenic miRNAs, such as miR-155, miR-146a, and miR-124 in microglia may be associated with the acquisition of a senescent phenotype (Saba et al., 2012; Ponomarev et al., 2013). We recently observed that aged-cultured microglia exhibit lower phagocytic ability and higher expression levels of miR-146a than younger cells (Caldeira et al., 2013). Whether these findings are related with a dysfunctional microglia at ALS end-stages deserve to be further investigated.

Demyelination progression

Although new oligodendrocytes were observed in the SC of SOD1G93A mice, they do not fully maturate, resulting in progressive demyelination and accelerated disease (Liu et al., 2002). The authors also found similar myelination defects in postmortem samples taken from SC and motor cortex from ALS patients. Actually, there are many potent inhibitors of axonal regeneration in the injured CNS including myelin-associated proteins, fibrinogen, and axonal guidance molecules, where epidermal growth factor receptor (EGFR) and eukaryotic ribosome biogenesis protein 1 (Erb1) may have a special role in reducing the effects of multiple inhibitors of axonal regeneration (Arthur-Farraj et al., 2012; Leinster et al., 2013). However, the role of EGFR on the protection of MN synapses and survival extension in SOD1G93A mice is still a matter of debate; hence, it was reported that EGFR inhibitors failed to extend ALS mouse survival although influencing disease progression (Le Pichon et al., 2013).

Another important point to be considered is the decrease in the phagocytic clearance by the dysfunctional microglia that results in the accumulation of myelin debris, leading to oligodendrocyte differentiation arrest and decreased recruitment of oligodendrocyte precursor cells (Walter and Neumann, 2009). Activated microglia were also shown to attenuate the proliferation of the oligodendrocyte precursor cells, thus concurring for demyelination progression (Taylor et al., 2010), and reinforcing the recovery of healthy microglia as a potential therapeutic target in ALS. Indeed, studies on the global gene expression during demyelination and remyelination by microarray analysis reinforced that the primary function of microglia is the tolerance induction and support to regeneration (Olah et al., 2012).

CELL REPLACEMENT THERAPY

Cell replacement therapy has been suggested as a promising strategy for MN disease. The use of combined strategies to restore both the healthy state of MNs and glial cells, such as microglia, and their correct cross-talk to face persistent neurotoxic insults may even provide better benefits for this devastating disease.

Mesenchymal stem cells (MSCs) isolated from the bone marrow of ALS patients did not show morphological or functional differences from those obtained from donors and seem to be useful for cell-based therapy for ALS patients (Hadass et al., 2013). In a few well-monitored ALS patients the autologous transplantation of such MSCs into the SC evidenced to be well tolerated and to promote some clinical improvement (Min et al., 2012).

Another proposed strategy is the olfactory ensheathing cell (OEC) transplantation that although evidencing to slow the rate of ALS progression in a short period (Inoue et al., 2003) and to improve pulmonary function (Guegan et al., 2001) has been a matter of debate. Indeed, prominent glial and inflammatory reaction around the brain delivery track was observed in postmortem samples (Reyes et al., 2010).

Efficacy of transplantation-based astrocyte replacement was also evidenced as a promising therapy for slowing focal MN loss associated with ALS while also reducing microgliosis in the hSOD1G93A rodents (Lepore et al., 2008). Effects may derive from the release of growth factors that are decreased in ALS and already evidenced to increase mSOD1 mice survival (Kaspar et al., 2003; Park et al., 2009). However, limited efficacy was obtained in a later study (Lepore et al., 2011). The use of induced pluripotent stem cell (iPSC) technologies may allow in the future the autologous cell transplantation in ALS patients, including MNs (Papadeas and Maragakis, 2009).

More recently, depletion of microglia cells expressing mSOD1 with clodronate liposomes and subsequent transplantation with bone marrow cells (BMCs) expressing wtSOD1 was shown to trigger microglia replacement and to slow ALS disease progression in the SOD1G93A mice model (Lee et al., 2012). The method seems to afford better therapeutic effects than the one by Ohnishi et al. (2009) using BMCs transplantation since microglia renewal is better achieved by tissue-resident microglia rather than by BMCs (Davoust et al., 2008). Overall, the mechanisms and functional implications of microglia replacement require further elucidation, inasmuch because myeloid-derived infiltrating cells (monocyte-derived macrophages) revealed to be functionally distinct from the resident microglia assisting them (Jung and Schwartz, 2012; London et al., 2013). Clearly, additional research is required to address these issues and contribute to develop strategies able to stop, or at least delay, ALS progression.

CONCLUSION

Amyotrophic lateral sclerosis is a fatal neurodegenerative disorder with limited identified targets, biomarkers, and therapeutic options. Therefore, a better comprehension of the underlying molecular mechanisms is necessary to develop novel etiological therapeutic strategies. Multiple studies suggest that a complex pathological interplay between MNs and glial cells, involving neuroinflammation and microglia physiopathological changes drive the performance of these glial cells before the ALS onset and during disease progression till late and end stages. In this context, we here summarized the growing body of evidence supporting the key role of microglia in the deregulated motor-neuron interconnectivity and in the dreadful chain of events leading to MN degeneration in ALS. As such, insights into the complex intercellular perturbations underlying ALS disease and centered on microglia phenotypic changes, and associated detrimental functions, will help on our efforts to develop effective therapeutic approaches for recovering adequate microglia function in initiation and progression phases of ALS. In conclusion, the role of microglia in keeping brain homeostasis leads to consider that healthy microglia may be used to replace the senescent or irresponsive cell. In addition, dysfunctional microglia may be differently modulated, either directly or indirectly, to be transformed in a less reactive cell to challenges in excessive and chronic neuroinflammation, or alternatively rejuvenated to enhance their capacity to fight the insult and improve disease outcomes. Considerably more research is necessary to realize the feasibility and usefulness of such strategies before their potential in clinic can be realized.

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Can we talk about microglia without neurons? A discussion of microglial cell autonomous properties in culture

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MICROGLIAL ORIGIN AND FUNCTION

Microglial cells originate from precursor cells located in the yolk sac that migrate into the developing central nervous system (CNS) around E8.5-10 in mice. Considering their origin, microglial cells could be regarded as invaders of the CNS in charge of debris clearance, just active during brain injury, infection or degeneration. In adulthood and under certain conditions, monocytes may penetrate the blood brain barrier, reach the CNS and become non-resident brain macrophages. CNS infiltrating-macrophages are difficult to distinguish from resident microglial cells and it is not clear the grade of functional similarity between these two types of cells (Prinz and Mildner, 2011; Gomez Perdiguero et al., 2013). Interestingly, microglial cells are able to self-renew independently of circulating monocytes or bone marrow hematopoietic stem cells (Ajami et al., 2007; Elmore et al., 2014), and show a specific molecular signature (Butovsky et al., 2014). Moreover, during the last decade microglial cells have been demonstrated to be involved in normal brain development and function while maintaining a, previously assumed, "resting" state (Pont-Lezica et al., 2011; Tremblay et al., 2011; Valero et al., 2012). Thus, microglial cells cannot be considered just as macrophages.

THE ACTIVELY "RESTING" MICROGLIA AND THEIR INTERACTION WITH NEURONS

The healthy brain is continuously under the active surveillance of a dynamic network of microglial processes. These processes have been shown to permanently protrude and retract, and to physically interact with neuronal synapses to modulate synaptic plasticity. Microglia phagocytic control of synaptic pruning is mediated by the complement pathway (Schafer et al., 2012) and fractalkine signaling (Paolicelli et al., 2011). Furthermore, microglia also regulate synaptic plasticity by the release of diffusible molecules like the brain-derived neurotrophic factor (Parkhurst et al., 2013) or the coactivator of synaptic NMDA receptors D-serine (Scianni et al., 2013). Therefore, microglial cells are able to modulate neuronal function in several ways. Importantly, this is not a unidirectional path of communication, but a dialog between microglia and neurons (Saijo and Glass, 2011; Eyo and Wu, 2013). As a clear example, the chemokine fractalkine (constitutively expressed by neurons), and its receptor (CX3CR1, mainly present in microglial cells), participate in the modulation of hippocampal synaptic pruning and function (Paolicelli et al., 2011; Scianni et al., 2013; Zhan et al., 2014). Fractalkine/CX3CR1 axis is better known to be involved in keeping microglia in their "resting" surveillance state (Sheridan and Murphy, 2013; Wolf et al., 2013). Thus, fractlakine/CX3CR1 system represents a clear example of mutual functional regulation between neurons and microglia. Moreover, there are other pairs of neuronal ligands and microglial receptors which mediate communication between these two elements of the CNS: the neuronal surface proteins CD200, CD47, and CD22 that bind to the microglial receptors CD200R, CD172, and CD45, respectively (Biber et al., 2007).

Microglial cells show regional differences in the brain in terms of density, molecular characteristics, morphology, and responsiveness (Olah et al., 2011; Butovsky et al., 2014). All microglial cells express the colony-stimulating factor receptor (CSF1R), which is required for their development. Interleukine-34, a ligand of CSF1R, is mainly expressed by neurons in specific regions of the brain (cortex, hippocampus, striatum, and anterior olfactory nucleus) where it promotes microglial survival (Greter et al., 2012; Wang et al., 2012). Similarly, high levels of fractalkine expression have been found in neurons of the amygdala, striatum, globus pallidus, thalamus, olfactory bulb, hippocampus, and cerebral cortex (Tarozzo et al., 2003; Kim et al., 2011). Fractalkine is also expressed by astrocytes at lower levels (Hulshof et al., 2003; Sunnemark et al., 2005). Interestingly, the specific molecular signature of adult microglia is dependent on the presence of the transforming growth factor-β (TGF-β) which is expressed at low levels by neurons and glial cells (Butovsky et al., 2014). Therefore, neurons, by producing different levels of the aforementioned molecules, may be responsible of the regional characteristics and heterogeneity of microglial cells.

MICROGLIAL CELLS IN THE ABSENCE OF NEURONS

Taking into account previous data, it seems clear that neurons are active modulators of microglial functional state and could be responsible for microglial regional

differences. Considering that, under normal conditions, neurons shape microglial cells and that the main role of microglia is the functional modulation and maintenance of neurons, an important question emerges: does it make sense to talk about microglia in the absence of neurons? Indeed, this question can be re-formulated as: do microglial cells retain their identity in the absence of neurons? If we consider that the interaction with neurons is crucial for the definition of microglia, the answer seems to be clear. Our opinion paper aims to provoke reflection and is in no way intended to despise the value of prior studies that, using isolated microglial cells or cell lines, have contributed to the current knowledge of microglial cell biology.

It is important to note that isolated microglia do not display, in cell cultures, the highly ramified structure that is typically observed in the normal, healthy CNS. *Ex vivo* microglial cells analyzed immediately upon isolation resemble reactive amoeboid microglia, probably due to the isolation process itself. Nevertheless, this activation seems to wane with time and cell passages, reaching an approximation to the typical "resting" morphology. Immortalized microglial cell lines are an alternative to the use of primary microglial cell cultures, which are costly and time consuming. Several microglial cell lines derived from rat (HAPI), mouse (BV2, N9, EOC), and also human (HMO6), have been used. These cell lines exhibit characteristic microglial/macrophage cell markers and behaviors (cytokine release, migration, and phagocytosis) in the presence of endotoxins such as the gram-negative bacteria lipopolysaccharide (LPS, **Figure 1**). However, the immortalization renders these cells different from primary microglia; in terms of molecular expression (Butovsky et al., 2014), morphology, proliferation, and adhesion (reviewed in Stansley et al., 2012).

FILLING THE GAP BETWEEN *IN VITRO* AND *IN VIVO* MICROGLIA

It still remains to be elucidated whether isolated microglial cells of *in vitro* systems behave as proper microglia. The importance of this question probably depends on the parameters to be evaluated, e.g., in the absence of direct contact with neurons these cells will fail to develop some specific characteristics of microglial cells. The obvious solution for the aforementioned problem is the use of co-culture systems in which microglial cells and neurons co-exist. Again, the regional origin of microglial and neuronal cells may influence their interaction and should be taking into account when inferring general mechanisms. Nevertheless, some studies require the use of isolated microglia. As previously mentioned, neurons release several factors that regulate microglial state. The use of the adequate combination of these factors in cell culture could contribute to the reduction of the gap between *in vivo* and *in vitro* systems, even in the absence of neurons. On this respect, we have investigated the effects of treating N9 microglial cells with soluble fractalkine in basal conditions or after activation with LPS. We observed that N9 cells expressed CX3CR1 mRNA and protein using quantitative real time PCR (not shown), immunofluorescence (**Figure 1A**) and western blotting (**Figure 1B**). Interestingly, in basal and LPS conditions fractalkine induced N9 cells to acquire a more ramified morphology (**Figure 1C**). Fractalkine treatment also increased the number of phagocyting (**Figures 1D–F**) and migrating N9 cells (**Figures 1G,H**). Furthermore, fractalkine was also able to reduce the expression of interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) mRNAs induced by LPS treatment (**Figures 1I,J**) while maintaining elevated levels of phagocytic and migratory activity in N9 cells. Fractalkine has a clear role in shaping microglial cells, evident at the morphological, functional and molecular levels. This role could be carried out by the membrane bound or even the constitutively cleaved fractalkine *in vivo*, keeping the cells in an alerted but relatively latent state (Sheridan and Murphy, 2013; Wolf et al., 2013). Thus, the lack of fractalkine in isolated microglial cultures is a possible reason for the lack of "surveying"-like microglial phenotype observed in these systems. Our data suggest that supplementation of microglial culture media with fractalkine may serve to shorten the gap between the typical morphology and behavior of microglia that is observed *in vivo* versus displayed in most *in vitro* models. Nevertheless, the addition of fractalkine to cell cultures is far from being a definitive solution. Therefore, this idea could be extended to the use of the adequate combination of microglial modulating factors released by neurons and/or macroglial cells. Butovsky et al. (2014) observed that adult microglia cultured in the presence of MCSF (macrophage colony-stimulating factor) and TGF-β1 showed a molecular expression pattern similar to freshly sorted adult microglia. Nevertheless, treatment of N9 and BV2 cells with these factors did not induce the expression of such microglial molecular pattern (Butovsky et al., 2014), indicating the limitations of microglial cell lines. Thus, further research should be done to identify and define the individual contribution and combined effect of different factors to maintain the functional and molecular characteristics of microglial cells.

We must assume that the perfect *in vitro* system is just a utopia. By definition, *in vitro* microglia systems will never reach the complexity of *in vivo* ones, but in turn will allow the study of some aspects of microglial biology that are masked by surrounding factors (e.g. by the presence of neurons and/or macroglial cells). Thus, some intrinsic characteristics of microglia can be easier analyzed *in vitro*, like the molecular mechanisms involved in phagocytosis, cell migration, transcriptional control, and metabolic functioning. In the other way around, the analysis of complex systems can mask particular aspects of its individual components (microglial cells), but lead to the discovery of new mechanisms and functionalities that emerge from the interaction of the parts (e.g., microglial regulation of synaptogenesis). However, even *in vivo* experimental designs, due to the need of controlling as many variables as possible and to the use of experimental manipulations, are normally far from the complexity of natural systems. Thus, we should be cautious when trying to predict, from our particular experimental systems, the features and behavior of microglial cells in their natural milieu (a CNS integrated into a full alive organism, which is also influenced by its surrounding environment).

CONCLUSIONS

Our initial question could be seen just as a mere language issue related to how

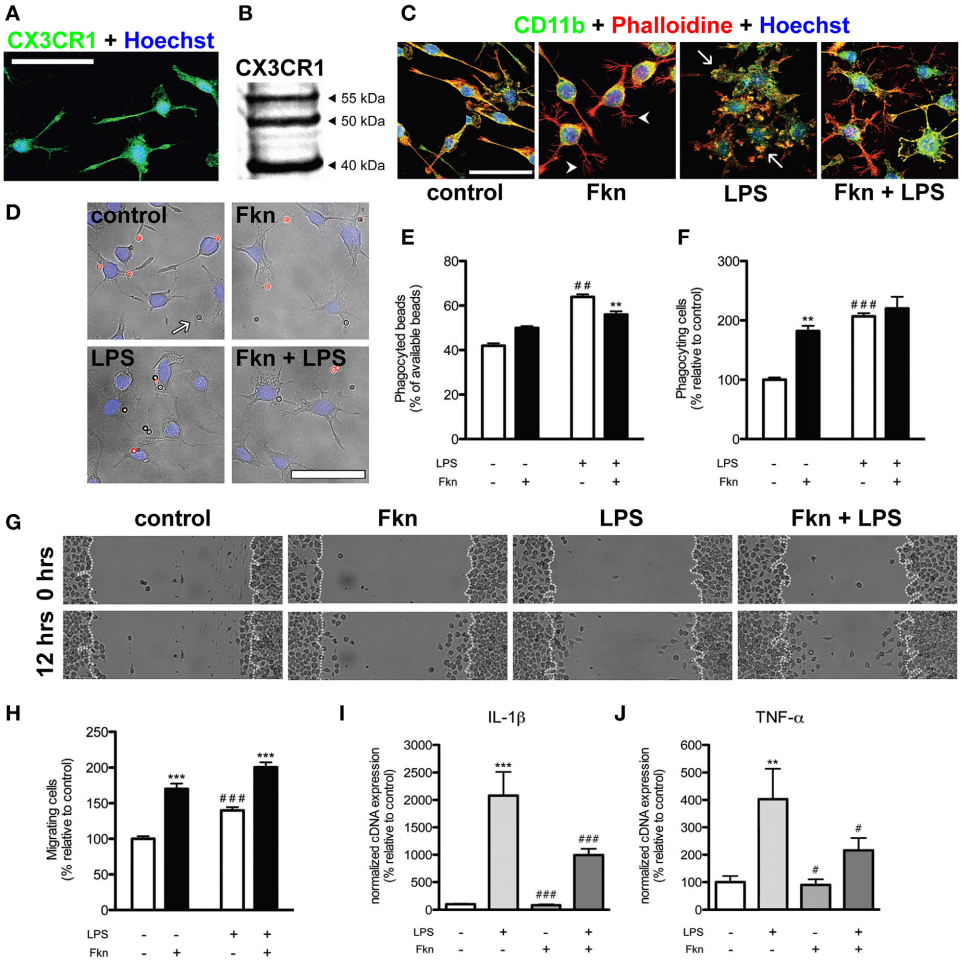

FIGURE 1 | Soluble fractalkine changes morphology and behavior of N9 microglial cells. (A) Confocal microscopy image of N9 microglial cells expressing CX3CR1 (green, rabbit anti-CX3CR1, eBioscience). Cell nuclei were stained with Hoechst 33342 (blue, Life Technologies). Almost all N9 cells in basal culture conditions showed expression of CX3CR1. **(B)** Western blotting of 50 μg whole cell extracts from N9 cells showing the previously described pattern (Yang et al., 2007). **(C)** Representative confocal images of N9 microglial cells stained for CD11b (green, rat anti-CD11b, AbD Serotec), filamentous actin (red, phalloidin-Alexa Fluor® 594 conjugate, Life Technologies) and Hoechst 33342 (blue). In basal (control) conditions N9 microglial cells showed different morphologies (from bipolar to ramified). Treatment of N9 cells with fractalkine chemokine domain (Fkn, 6 h, 200 ng/ml, Sigma-Aldrich) induced the adoption of a ramified morphology by N9 cells characterized by thin filopodia-like structures (arrowheads). As expected, lipopolysaccharide treatment (LPS, 6 h, 100 ng/ml, from *Escherichia coli*, Sigma-Aldrich) induced swelling, loss of ramified morphology and the appearance of thick membrane protrusions ("ruffles," arrows) in N9 microglia. In the presence of both LPS and Fkn, N9 cells displayed an intermediate morphology showing "ruffles" and filopodia-like structures. **(D)** Representative epifluorescence/phase contrast images of N9 cells (cell nuclei stained with Hoechst 33342, blue) in the presence of beads (2×10^6 beads/well, Sigma-Aldrich) coated with IgG from rabbit serum (Sigma-Aldrich). Due to the protocol used (for details check: Ferreira et al., 2011) phagocyted beads were not stained (arrows) while non-phagocyted beads showed red fluorescence (Alexa Fluor® 594 donkey anti-rabbit IgG, Life Technologies). **(E)** LPS treatment (6 h) led to an increase in the total number of phagocyted beads. Incubation with Fkn (6 h) just slightly reduced the percentage of beads phagocyted by LPS treated N9 cells. **(F)** LPS and Fkn treatments (6 h) increased the proportion of phagocyting cells. **(G,H)** A scratch wound assay was carried out to analyse N9 migratory activity (for details see: Ferreira et al., 2012) based on the mean number of cells that moved into the wound after 12 h. **(G)** Phase-contrast representative images of the scratch wound assay. **(H)** Co-incubation of N9 cells with LPS and Fkn resulted in a greater migratory induction than that elicited by LPS treatment alone. **(I)** IL-1β and **(J)** TNF-α mRNA expression in N9 cells was increased in the presence of LPS (4 h). Importantly Fkn treatment downregulated the increase in IL-1β and TNF-α mRNAs induced by LPS. All scale bars = 50 μm. Data are presented as mean ± s.e.m. **(E,F,H)**: $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. respective condition without Fkn, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ vs. control condition (2-Way ANOVA and Bonferroni's *post-hoc* test). **(I,J)**: $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. control condition, $^{\#}p < 0.05$ and $^{\#\#\#}p < 0.001$ vs. LPS (Pair Wise Fixed Reallocation Randomization Test©, Pfaffl et al., 2002).

we define "microglia": (1) based on their intrinsic properties or (2) based on the specific characteristics that emerge through their interaction with other elements of the CNS. We consider that microglia are defined by these two aspects of their biology and that one influences the other. As an example, the constitutive expression of CX3CR1 by microglial cells, an intrinsic property shared with other macrophages, allows them to be shaped by neuronal fractalkine. As previously

mentioned, the binding of fractalkine to its receptor controls microglial surveillance state but also mediates their role on the modulation of synaptic function, a specific characteristic that emerges through their interaction with neurons. Therefore, the study of intrinsic features (maybe shared with other cell types) and specific characteristics of microglia that emerge through their interaction with other components of the CNS are equally important to understand the nature of these cells. Obviously, these two ways of studying microglia will benefit in different grades from distinct experimental approaches, ranging from the examination of isolated cells *in vitro* to their analysis in their natural environment *in vivo*. Again, we will just need to be careful when generalizing or directly translating our observations from one to another level of biological complexity. These considerations will be specially relevant for the development of strategies aimed to use microglial cells as cell therapeutic agents.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: Ismael Neiva, João O. Malva, and Jorge Valero. Performed the experiments: Ismael Neiva, Analyzed the data: Ismael Neiva. Prepared figures: Ismael Neiva and Jorge Valero. Wrote the paper: Ismael Neiva, João O. Malva, and Jorge Valero.

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Histamine: a new immunomodulatory player in the neuron-glia crosstalk

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Histamine is an amine acting as a major peripheral inflammatory mediator. In the brain, histamine was initially viewed as a neurotransmitter, but new evidences support its involvement in the modulation of innate immune responses. Recently, we showed that histamine modulates microglial migration and cytokine release. Its pleiotropic actions, ranging from neurotransmission to inflammation, highlight histamine as a key player in a vast array of brain physiologic activities and also in the pathogenesis of several neurodegenerative diseases. Herein, we emphasize the role of histamine as a modulator of brain immune reactions, either by acting on invading peripheral immune cells and/or on resident microglial cells. We also unveil the putative involvement of histamine in the microglial-neuronal communication. We first show that histamine modulates the release of inflammatory mediators, namely nitric oxide, by microglia cells. Consequently, the microglia secretome released upon histamine stimulation fosters dopaminergic neuronal death. These data may reveal important new pharmacological applications on the use histamine and antihistamines, particularly in the context of Parkinson's disease.

Keywords: microglia, histamine, nitric oxide, substantia nigra, neuroinflammation, Parkinson's disease

HISTAMINE: GENERAL ASPECTS

Histamine is an endogenous biogenic amine synthesized from L-histidine through the catalytic activity of the enzyme histidine decarboxylase. In peripheral tissues, mast cells and basophils are the main sources of histamine. Other sources of histamine include gastric enterochromaffin-like cells, platelets and neutrophils. In the adult brain, histamine is produced by neurons, mast and microglia cells (Katoh et al., 2001; Haas et al., 2008). Histamine exerts its functions through the activation of four distinct receptors belonging to the rhodopsin-like family of G protein-coupled receptors (GPCRs): H_1 receptor (H_1R), H_2 receptor (H_2R), H_3 receptor (H_3R) and H_4 receptor (H_4R). These functions range from the modulation of the allergic reactions and behavioral state and reinforcement (H_1R); regulation of heart and gastric acid secretion, learning and memory (H_2R); neurotransmitter release, cognition and memory (H_3R); and chemotactic effects (H_4R), among others (Haas et al., 2008; Molina-Hernández et al., 2012). Three (H_1R, H_2R and H_3R) out of the four histamine receptors are widely expressed in the nervous system (Haas et al., 2008; Leurs et al., 2009). However, the expression of H_4R has remained controversial. Several groups could not detect H_4R mRNA, while other labs reported their expression in the amygdala, cerebellum, hippocampus, caudate nucleus, substantia nigra (SN), thalamus and hypothalamus (Strakhova et al., 2009). Recently, we found that all four types of histamine receptors are expressed by N9 microglia cell line and cortical-derived microglial cells (Ferreira et al., 2012). The levels of histamine and its metabolites have been evaluated in physiologic and pathologic brain conditions. The concentrations of histamine found in the cerebrospinal fluid (CSF) and parenchyma of the intact brain are very low, at the nanomolar range (Croyal et al., 2011). Importantly, circulating levels of histamine and histaminergic innervations are increased following brain injury and degeneration or infection, suggesting that histamine may have an important role in modulating neuronal survival (Anichtchik et al., 2000a; **Figure 1**).

HISTAMINE: A PERIPHERAL IMMUNE MEDIATOR

Several brain injuries and inflammatory conditions are associated with increased levels of circulating histamine, both in the blood and in the CSF, leading to blood brain barrier permeability (BBB). Histamine may thus modulate the expression of several inflammatory molecules by peripheral immune cells, including, macrophages, monocytes, T and B lymphocytes (Jutel et al., 2006). In these conditions, activated peripheral immune cells may cross the BBB and exert its immunomodulatory activities in the brain parenchyma (**Figure 1**).

Histamine may play a dual role in the inflammatory response driven by macrophages. In fact, it was shown that histamine induced interleukin (IL)-6 release by macrophages via H_1R activation (Marone et al., 2001), whereas inhibited chemotaxis, phagocytosis, Tumor Necrosis Factor (TNF)-α, IL-12 and superoxide anion production via H_2R (Azuma et al., 2001). Histamine can also stimulate monocytes, but not macrophages, to express monocyte chemoattractant protein (MCP)-1 and its receptors CCR2-A and -B via H_2R receptor activation (Kimura et al., 2004). Gschwandtner et al. showed that histamine down-regulated IL-27 production by human monocytes through H_2R and H_4R, but did

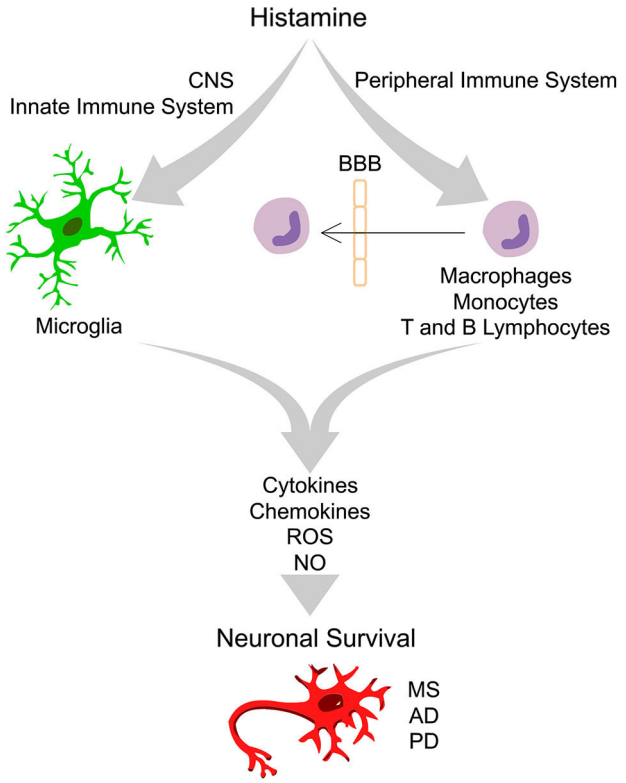

FIGURE 1 | Integrative scheme of the effects driven by histamine in peripheral and innate immune cells that ultimately may interfere with neuronal survival. In the healthy brain the "bulk" concentration of histamine is very low. Upon brain injury, degeneration or infection, the inflammatory response may trigger degranulation of mast cells, leading to a massive release of histamine in the blood and in the cerebrospinal fluid, leading to an increase of blood brain barrier (BBB) permeability. In this context, peripheral immune cells may cross the BBB and invade the brain parenchyma. Increased levels of histamine may activate distinct histamine receptors at peripheral (macrophages, monocytes, T and B lymphocytes) and innate immune cells (microglia) leading to the release of pro- or anti-inflammatory cytokines, chemokines, reactive oxygen species (ROS) and nitric oxide (NO). Histamine may thus have a dual role in the modulation of neuronal survival in the context of several neurodegenerative diseases, including Multiple Sclerosis (MS), Alzheimer's disease (AD) and Parkinson's disease (PD), depending on the type of histamine receptor activated, signaling pathways involved and factors released.

not influence IL-6, IL-10 and TNF-α production (Gschwandtner et al., 2012). Lymphocytes are predominantly involved in adaptive immunity and play a key role in the pathogenesis of brain disorders associated with inflammation, such as multiple sclerosis (MS). T cells express histamine receptors being thus responsive to histamine stimulation. For instance, histamine enhances T_H1-type activity through H_1R, whereas both T_H1- and T_H2-type responses are negatively regulated by H_2R (Jutel et al., 2001). The impact of histamine on immunoglobulin secretion by B cells depends on the requirement of T cells (Ferstl et al., 2012).

In conclusion, the effects of histamine on peripheral immune cells are often contradictory leading either to the stimulation or inhibition of inflammatory processes. This can be due to the type, affinity and abundance of each receptor subtype and consequently to the involvement of distinct downstream regulatory pathways, to the levels of histamine and to the cell types used in each study. Even if comprehension regarding the regulatory pathways activated by each histamine receptor is warranted, there are already several evidences that strengthen the idea that histamine and its receptor agonists/antagonists are promising targets to modulate inflammatory conditions.

HISTAMINE: NEW MODULATOR OF MICROGLIAL ACTIVITY

Microglial cells, the resident immune cells in the brain, have the ability to patrol and protect the parenchyma against brain injuries and/or infections. In a healthy environment, resting microglia display low expression levels of inflammatory molecules, but when activated, microglial cells abandon their ramified surveilling morphology, become ameboid, phagocytic, migrate to the injured site and release inflammatory molecules (Polazzi and Monti, 2010). The density and phenotype of microglial cells are region-specific. In particular, a higher density of microglia in the substantia nigra pars compact (SNpc) lead several authors to suggest that microglia play an important role in the pathogenesis of Parkinson's disease (PD; Collins et al., 2012). In accordance, higher numbers of activated microglia were found in the vicinity of degenerating SNpc dopaminergic neurons of post-mortem PD brains (Long-Smith et al., 2009). A major unanswered question is whether microglia activation is a consequence or a cause of nigra dopaminergic cell loss. Recent studies suggest that neuronal loss leads to the extracellular release of protein aggregates from neurons causing microglia activation (Hafner-Bratkovič et al., 2012). On the other hand, a large body of literature also supports the idea that microglia potentially increase the risk of development and exacerbation of the neurodegenerative pathology (Phani et al., 2012).

Microglia release a huge number of inflammatory mediators, including histamine (Katoh et al., 2001). We have recently shown, for the first time, that histamine modulates microglial migration and cytokine release (Ferreira et al., 2012). Histamine, acting via H_4R, increased microglial motility through the involvement of α5β1 integrins and the p38 MAPK and Akt signaling pathways. Additionally, histamine also modulated IL-1β release by microglia (Ferreira et al., 2012). In that sense, histamine may have an impact in the pathogenesis of brain diseases which are associated with inflammatory conditions (**Figure 1**). Our previous study was performed using a N9 microglia cell line, but there is a lack of information regarding the effects of histamine in microglia cells derived from the SN, a brain region enriched with microglia and highly susceptible to dopaminergic neuronal loss. Thus, we evaluated the effects of histamine on the production of an inflammatory mediator, nitric oxide (NO), by microglial cells derived from the SN of neonatal rats. We measured the amount of nitrite (a stable metabolite of NO) released by primary microglial cells after 24 h of treatment with different histamine concentrations (1 μM; 10 μM and 100 μM). Lipopolysaccharide (LPS; 100 ng/mL), a potent stimulator of microglia activation that causes the release of various inflammatory factors and free radicals, was used as a positive control. Microglial cells, isolated from

the ventral midbrain of postnatal (P2-3) Wistar rats, were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% Fetal Bovine Serum and 100 U/mL penicillin plus 100 μg/mL streptomycin, as described previously (Saura et al., 2003). The Griess Assay was used to measure NO production and values were expressed as percentage of increase with respect to the untreated cultures (controls set to 100%). We found that histamine significantly increased NO release ($mean_{H1}$ = 138.5 ± 11.7; $mean_{H10}$ = 125.0 ± 9.4; $mean_{H100}$ = 140.2 ± 5.1, n = 3–15), as compared to control (**Figure 2A**). Histamine did not interfere with microglia cell death or proliferation at all concentrations tested (data not shown). As expected, 100 ng/ml LPS increased NO production ($mean_{LPS}$ = 178.4 ± 12.2, n = 16; **Figure 2A**). Based on these results and on prior studies reported by our group (Agasse et al., 2008; Bernardino et al., 2008; Grade et al., 2010; Rosa et al., 2010) and by others (Wang et al., 1997; Hernández-Angeles et al., 2001; Nicolson et al., 2002; Tran et al., 2004; Molina-Hernández and Velasco, 2008; Nemeth et al., 2012), we then used 100 μM histamine in further experiments, a concentration of pathophysiological relevance.

NO is produced from L-arginine by different isoforms of NOS and takes part in many normal physiological functions, such as promoting vasodilation of blood vessels and mediating cell communication within the brain. In addition to its physiological actions, the free radical activity of NO may cause cellular damage through a phenomenon known as nitrosative stress (Knott and Bossy-Wetzel, 2009). Since the main inducible enzyme responsible for NO synthesis in microglia cells is inducible nitric oxide synthase (iNOS), we then hypothesized that iNOS expression was also upregulated by histamine. To test this hypothesis, microglial cells were treated for 24 h with 100 ng/mL LPS or 100 μM histamine, fixed and stained against iNOS (polyclonal rabbit anti-iNOS; 1:100; BD Transduction Laboratories). Fluorescent images were acquired using a Zeiss inverted microscope (Axiobserver Z1, Zeiss) and the fluorescence intensity was measured through ImageJ software (60 cells *per* condition). The background fluorescence intensity was always subtracted in order to quantify the corrected intensity of the iNOS fluorescence in each condition. The same confocal image acquisition settings were used in all experiments. As shown the **Figures 2B** and **2C**, both histamine and LPS significantly increased the expression of iNOS in microglial cells ($mean_{H100}$ = 232.0 ± 31.8; $mean_{LPS}$ = 316.6 ± 36; n = 3). This suggests that histamine could act as a NO-regulating factor by inducing iNOS expression. Others also showed that histamine stimulates endothelial NO production and iNOS expression via H_1R and nuclear factor (NF)-kappaB signaling pathway in intimal smooth muscle cells (Tanimoto et al., 2007). NO has been shown to modify protein function by nitrosylation and nitrotyrosination, to contribute to glutamate excitotoxicity, inhibit mitochondrial respiratory complexes, participate in organelle fragmentation, and mobilize zinc from internal stores (Knott and Bossy-Wetzel, 2009). NO can react with superoxide radicals to form peroxynitrite radicals that are short-lived oxidants and highly damaging to neurons. Mitochondrial injury is prevented by treatment with an iNOS

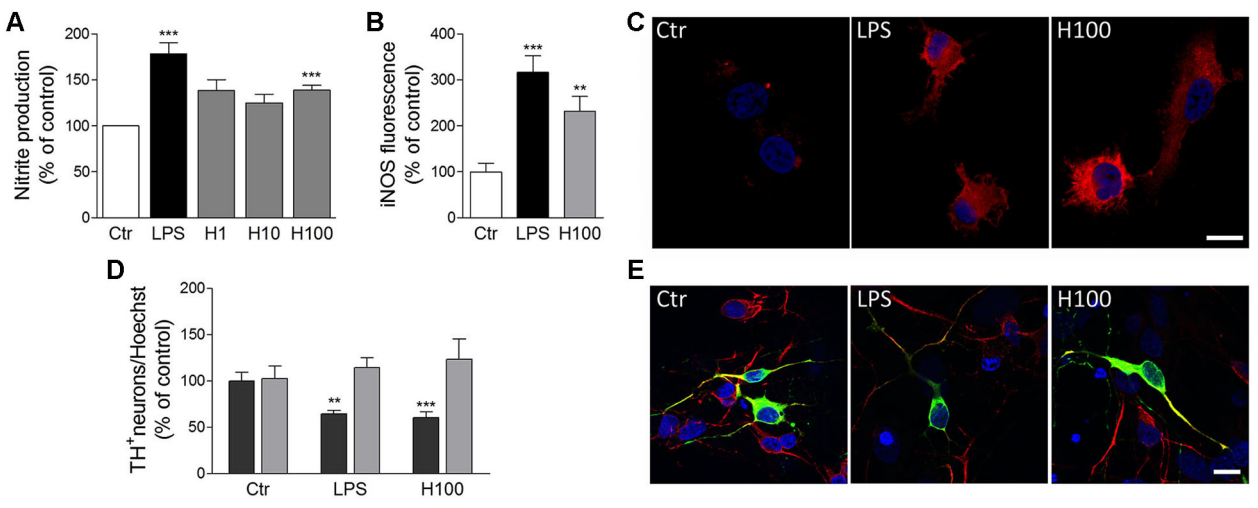

FIGURE 2 | Histamine induced NO release by microglial cells and subsequent dopaminergic neuronal death. (A) Histamine at 1 μM (H1), 10 μM (H10) and 100 μM (H100) triggered an increase of NO release by microglial cells. LPS (100 ng/ml) was used as a positive control. **(B)** Bargram shows that histamine increased inducible nitric oxide synthase (iNOS) expression by microglial cells. **(C)** Representative fluorescent digital images of microglial cell cultures treated with 100 μM histamine or 100 ng/ml LPS and stained against iNOS (red staining). For nuclear labeling, cells preparations were counterstained with Hoechst (2 μg/mL; blue staining). **(D)** The conditioned medium derived from microglial cells pre-treated with 100 μM histamine or 100 ng/mL LPS decreased the numbers of TH^+-neurons (dark gray bars). The conditioned medium pre-treated solely with histamine or LPS (devoid of microglia-induced soluble factors) did not affect dopaminergic neuronal survival (light gray bars). Results are expressed as the mean value of TH^+ cells in relation to all nuclei stained with Hoechst. **(E)** Representative fluorescence digital images of midbrain neuronal-glial co-cultures treated with microglia-derived conditioned medium. Green staining: TH^+ neurons; red staining: MAP-2 positive neurons; blue staining: nuclei. Scale bar = 10 μm. Ctr: control; LPS: 100 ng/mL LPS; H100: 100 μM histamine. Data are expressed as mean ± SEM. Statistical analysis was performed using one-way ANOVA with Dunnett's correction. ** $P < 0.01$ and *** $P < 0.001$ as compared with the untreated control—set to 100%.

inhibitor, suggesting that iNOS-derived NO is also associated with the mitochondrial impairment (Choi et al., 2009). NO inhibits cytochrome c oxidase in competition with oxygen, resulting in glutamate release and excitotoxicity (Brown and Neher, 2010). However, the molecular mechanisms involved on the modulatory effect of histamine on NO production are unknown. Nevertheless, it is known that activation of extracellular signal-regulated kinases, c-Jun N-terminal kinases and p38 MAPK leads to activation of transcription factors, such as NF-kappaB and activator protein 1 (AP-1), that are involved in expression of the iNOS gene, which is followed by the sustained production of NO by activated microglia (Jung et al., 2008). Since LPS-induced NO production by microglia involves all these intracellular signaling pathways, we hypothesize that histamine may also mediate NO production by some of these pathways. The use of selective inhibitors of these pathways as well as the evaluation of their expression and activation may be helpful to disclose the signaling pathways involved in NO-mediated histamine effects.

HISTAMINE: DUAL ROLE IN NEUROPROTECTION/NEURODEGENERATION

The neuronal histaminergic system is involved in many physiological functions and consequently severely affected in age-related neurodegenerative diseases. The production of neuronal histamine shows diurnal fluctuations in control patients who had no neuropsychiatric disorders, while this fluctuation was strongly altered in patients with neurodegenerative diseases (Shan et al., 2013). Moreover, altered levels of histamine found in the diseased brain and in the peripheral system may modulate both the innate and adaptive immune responses ultimately affecting neuronal survival. Brain diseases in which the histaminergic system may be involved include MS, PD, Alzheimer's disease (AD), among others (**Figure 1**).

MS is characterized by focal lymphocytic infiltrations to the brain leading to damage of myelin and axons. Initially, the inflammatory response is transient and remyelination occurs, but over time widespread microglial activation ensues along with extensive and chronic neurodegeneration (Passani and Ballerini, 2012). The observation that histamine may be implicated in MS dates back to the early 1980s when Tuomisto et al. reported that patients with remitting or progressive disease showed histamine levels about 60% higher than controls, suggesting an altered histamine metabolism in MS (Tuomisto et al., 1983). However, another clinical study did not show elevated concentrations of histamine and its metabolite methylhistamine in MS patients when compared, in this case, with individuals affected by other neurological diseases (Rozniecki et al., 1995). More recently, gene-microarray analysis has shown that H_1R expression is upregulated in MS lesions (Lock et al., 2002), and epidemiological studies suggest a protective effect of brain penetrating H_1R antagonists (Alonso et al., 2006). Furthermore, in a small pilot study, a cohort of MS patients treated with an H_1R antagonist showed signs of neurological amelioration (Logothetis et al., 2005). The H_1R has long been associated with inflammatory responses and H_1R antagonists may be used in brain repair therapies.

The role histamine in AD is also well documented. However, contradictory data exists regarding the quantification of histamine levels in brain compartments of AD patients, making difficult a direct correlation between histaminergic neurotransmission and AD pathology (Brioni et al., 2011). Increased histamine levels have been reported not only in the frontal cortex, basal ganglia and hippocampus, but also, together with its metabolites, in the CSF and serum of AD patients (Fernandez-Novoa and Cacabelos, 2001). On the contrary to the above findings, several other studies show decline in histamine levels in AD brains. Recent studies show that H_3R antagonists may be efficient in AD therapy. Nathan et al. demonstrated that GSK239512, a selective H_3R antagonist, displayed a satisfactory level of tolerability in AD patients with evidence of positive effects on attention and memory (Nathan et al., 2013). Others studies also support that H_3R antagonists/inverse agonists are potentially novel therapeutic targets for other diseases, such as vigilance and sleep-wake disorders (Parmentier et al., 2007) and schizophrenia (Vohora and Bhowmik, 2012).

Several abnormalities in the histaminergic system were also found in PD patients. In post-mortem brain of PD patients, it has been reported a dramatic increase of histaminergic innervations, enlarged axonal innervations (Anichtchik et al., 2000a) and increased histamine levels (Nuutinen and Panula, 2011), while animal studies showed that increased endogenous histamine levels may accelerate degeneration of SN dopaminergic neurons in 6-hydroxydopamine (OHDA) lesioned rats (Liu et al., 2007). In addition, increased mRNA levels of histamine methyltransferase (HMT), a key enzyme involved in histamine metabolism, were found in the SN and in the putamen of PD patients (Shan et al., 2012). This increase may act as a protective mechanism by metabolizing enhanced histamine levels in these brain regions. Moreover, a Thr105Ile polymorphism of HMT was shown to be associated with PD (Palada et al., 2012). Regarding the expression of histamine receptors, it has been shown that H_3R mRNA was significantly decreased in the SN, while H_4R mRNA expression showed a significant increase in caudate nucleus and putamen in PD patients (Shan et al., 2012). Vizuete et al. showed that the SN dopaminergic neurons are highly sensitive to histamine-induced neurotoxicity (Vizuete et al., 2000). Altogether, these data suggest that histamine and its receptors may play an important role in PD pathogenesis. However, it is still unclear how microglia activation induced by histamine may contribute to dopaminergic neuronal survival. Thus, we aimed to uncover whether soluble factors released by microglia previously stimulated with histamine or LPS could modulate dopaminergic neuronal survival. LPS *per se* does not seem to have a direct effect on neuronal viability making it an excellent tool to study inflammation-mediated dopaminergic neurodegeneration (Block et al., 2007). In our study, dopaminergic neuronal viability was assessed by counting the numbers of tyrosine hydroxylase (TH^+)-immunoreactive neurons (using monoclonal mouse anti-TH; 1:100 dilution; Transduction Laboratories), counterstained with MAP2 (using polyclonal rabbit anti-MAP2; 1:200 dilution; Santa Cruz Biotechnologies), on neuron-astrocyte midbrain co-cultures from neonatal rats. The midbrain cultures were obtained

from Wistar pregnant females with 15–16 gestational days and were grown in Neurobasal medium supplemented with 2% B27, 25 μM/mL glutamate, 0.5/mL glutamine and 120 μg/mL gentamicine for 5–6 days as described in Campos et al. (2012). Conditioned medium derived from untreated microglial cells were considered as the control condition and the resulting values were set to 100%. As shown in **Figures 2D** and **2E**, the conditioned media derived from microglial cells pre-treated for 24 h with 100 μM histamine or 100 ng/mL LPS induced a 30% reduction in the number of TH^+ cells ($mean_{H100}$ = 69.2 ± 4.3; $mean_{LPS}$ = 68.5 ± 1.5; n = 4; dark gray bars). To ensure that this effect was solely dependent on soluble factors released by microglial cells, we then incubated midbrain neuronal cultures with culture media pre-treated for 24 h with LPS or histamine, at 37°C, but in the absence of microglial cells. We found that the dopaminergic neuronal survival was not affected when midbrain neuronal cultures were treated with conditioned medium devoid of microglia-released factors ($mean_{H100}$ = 123.2 ± 28.5; $mean_{LPS}$ = 115.5 ± 18.4; n = 3; **Figure 2D**; light gray bars). Herein we showed, for the first time, that histamine promotes the release of toxic inflammatory factors, including NO, by microglial cells, which can be capable of damaging dopaminergic neurons. With this work we open a new perspective for the therapeutic use of histamine and histamine receptor antagonists to treat or ameliorate inflammation-associated processes of neurodegenerative diseases, like those seen in PD.

FUTURE PERSPECTIVES

Accumulating clinical and experimental evidences show that changes in the histaminergic system may be associated with the pathogenesis and progression of several neurodegenerative diseases, including PD. We showed that histamine boosts neuroinflammation by promoting microglia migration and the release of cytokines and NO. We also showed that production of neurotoxic and inflammatory mediators by microglial cells upon histamine stimulation leads to dopaminergic neurodegeneration. Based on these data we may infer that microglia activation induced by histamine may contribute to PD pathology, and may thus provide a rationale for possible novel therapeutic strategies. We suggest that the therapeutic use of histamine receptors antagonists may be of great value to treat or ameliorate CNS pathologies or neurodegenerative disorders which are commonly accompanied by inflammation. Therefore, the following steps urge a better understanding of the involvement of histamine and its receptors in the modulation of microglial activation and subsequent neuronal survival.

AUTHOR CONTRIBUTIONS

Sandra M. Rocha: Provision of study material; Collection and assembly of data; Data analysis and interpretation; Manuscript writing.

Joel Pires: Provision of study material; Collection and assembly of data; Data analysis and interpretation.

Marta Esteves: Provision of study material; Data analysis and interpretation.

Graça Baltazar: Conception and design; Provision of study material; Critical reading of manuscript.

Liliana Bernardino: Conception and design; Provision of study material; Data analysis and interpretation; Administrative support; Financial support; Manuscript writing; Final approval of manuscript.

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Microglia change from a reactive to an age-like phenotype with the time in culture

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Age-related neurodegenerative diseases have been associated with chronic neuroinflammation and microglia activation. However, cumulative evidence supports that inflammation only occurs at an early stage once microglia change the endogenous characteristics with aging and switch to irresponsive/senescent and dystrophic phenotypes with disease progression. Thus, it will be important to have the means to assess the role of reactive and aged microglia when studying advanced brain neurodegeneration processes and age-associated related disorders. Yet, most studies are done with microglia from neonates since there are no adequate means to isolate degenerating microglia for experimentation. Indeed, only a few studies report microglia isolation from aged animals, using either short-term cultures or high concentrations of mitogens in the medium, which trigger microglia reactivity. The purpose of this study was to develop an experimental process to naturally age microglia after isolation from neonatal mice and to characterize the cultured cells at 2 days *in vitro* (DIV), 10 DIV, and 16 DIV. We found that 2 DIV (young) microglia had predominant amoeboid morphology and markers of stressed/reactive phenotype. In contrast, 16 DIV (aged) microglia evidenced ramified morphology and increased matrix metalloproteinase (MMP)-2 activation, as well as reduced MMP-9, glutamate release and nuclear factor kappa-B activation, in parallel with decreased expression of Toll-like receptor (TLR)-2 and TLR-4, capacity to migrate and phagocytose. These findings together with the reduced expression of microRNA (miR)-124, and miR-155, decreased autophagy, enhanced senescence associated beta-galactosidase activity and elevated miR-146a expression, are suggestive that 16 DIV cells mainly correspond to irresponsive/senescent microglia. Data indicate that the model represent an opportunity to understand and control microglial aging, as well as to explore strategies to recover microglia surveillance function.

Keywords: autophagic capacity, *in vitro* cell aging, microglia, microRNAs, migration, phagocytosis, reactivity, senescence

INTRODUCTION

Microglia are the first line of defense against brain injury. In the healthy brain, microglia actively survey surrounding parenchyma via dynamic movement of processes (Nimmerjahn et al., 2005) and are kept in a relatively quiescent state, in part due to specific signals derived from neurons and astrocytes (Cardona et al., 2006; Lyons et al., 2007). Upon brain injury or changes of central nervous system (CNS) homeostasis, microglia are capable of acquiring diverse and complex phenotypes, allowing them to participate in the cytotoxic response, immune regulation, and injury resolution. The classical pro-inflammatory M1 phenotype is cytotoxic and release pro-inflammatory cytokines while the M2 polarization contributes to neuroprotection through the release of anti-inflammatory cytokines and growth factors (Chhor et al., 2013; Evans et al., 2013). These transitional phenotypes may exert beneficial or destructive effects depending on the stimuli, their duration and the environment they encounter (Schwartz et al., 2006). Thus, balance between M1 and M2 phenotypes can be considered a desirable therapeutic goal.

Age-related CNS disorders have been related with chronic and progressive neuronal loss but also with chronic mild neuroinflammation involving activated/primed microglia (Maezawa et al., 2011; Williamson et al., 2011). These cells showed to switch from M2 to M1 phenotype with age and disease progression (Solito and Sastre, 2012; Varnum and Ikezu, 2012). However, other studies claim that neuroinflammation is only present in the early stages of Ahlzheimer's disease (AD), once lately disappears (Wojtera et al., 2012) and that, instead, microglia become senescent/dystrophic (Graeber and Streit, 2010) and less responsive to stimulation with age (Njie et al., 2012; Streit and Xue, 2012). The dysmorphic characteristics of aged microglia suggested that, rather than maintaining an overactivated state, microglia may display decreased ability to mount a normal response to injury. Indeed, reduced migration (Damani et al., 2011), clearance (Li, 2013) and production of neurotrophic factors (Ma et al.,

2013), as well as inability to shift from a pro-inflammatory to an anti-inflammatory state to regulate injury and repair have been observed in aged microglia (Norden and Godbout, 2013) and related with senescence (Streit and Xue, 2012). These changes in microglia potentially contribute to an increased susceptibility and neurodegeneration as a function of age. Accordingly, non-steroidal anti-inflammatory drugs (NSAIDs) were only successful when administered before the development of neurodegeneration (Weggen et al., 2001). If administered in later stages of disease they showed to be detrimental (Martin et al., 2008), reinforcing that microglia may switch from a reactive to an irresponsive phenotype along the progression of AD and other age-associated CNS disabilities. Restraining of aged microglia may weak even more the already decreased neuroprotective properties of the cell in removing extracellular protein aggregates. These changes in microglia neuroprotective properties will potentially contribute to enhance neurodegeneration and susceptibilities with aging and reveal the need of adequate experimental models to follow the changes in microglia performance accordingly to cell senescence.

Most of the work intended to evaluate the neurodegenerative network associated with aging has used cultures of microglia derived from early postnatal brains, which differ from adult or aged ones (Harry, 2013). Recently, a few studies compared behavior of microglia isolated from animals at different ages. In these studies young and aged microglia were isolated using a Percol-based method (von Bernhardi et al., 2011; Njie et al., 2012) or distinctly isolated using a mild-trypsinization method for embryonic/neonatal microglia and Percol-based method for adult and aged microglia (Lai et al., 2013). In addition, these cells were analyzed either 24–48 h after isolation (Njie et al., 2012; Lai et al., 2013) or following trypsinization when kept in culture for several weeks in the presence of conditioned medium containing increased levels of mitogens (von Bernhardi et al., 2011). Such methods may promote microglia activation and bias the translation of culture findings, since it has been suggested that microglia may need some time in culture to recover its quiescent state (Cristóvão et al., 2010). Moreover, there are no means to isolate degenerating microglia for experimentation (Njie et al., 2012) once only the more resistant ones will survive to the isolation procedure. Nevertheless, the hypothesis of microglia senescence during aging and related neurodegenerative diseases emerged as a key determinant (Luo et al., 2010). *In vitro* aging of astrocytes and neurons has demonstrated to be associated with different cell response to stimuli, with the younger cells evidencing an increased reactivity when compared to the older ones (Falcão et al., 2005, 2006). In addition, it was shown that the repeated stimulation of the microglia cell line BV2 with lipopolysaccharide (LPS) lead to cell senescence corroborating the idea that sustained neuroinflammation may ultimately contribute to a microglia senescent phenotype (Yu et al., 2012). Therefore, we decided to isolate microglia from neonatal mice and culture cells from 2 days *in vitro* (DIV) until 16 DIV, similarly to what we previously did with neurons and astrocytes, and to explore aging-related differences in functional response characteristics associated to "young" and "aged" microglia phenotypes. We assessed changes in microglia morphology, nuclear factor kappa-B (NF-κB) signaling pathway activation, Toll-like receptor (TLR) expression, phagocytic ability and migration capacity, as well as cell death, inflammatory microRNA (miRNA) profiling, autophagy and senescence-associated β-galactosidase (SA-β-gal) in mice primary cortical cell cultures maintained up to 16 DIV.

MATERIALS AND METHODS

ANIMALS

Animal care followed the recommendations of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Council Directive 86/609/EEC) and National Law 1005/92 (rules for protection of experimental animals). All animal procedures were approved by the Institutional animal care and use committee. Every effort was made to minimize the number of animals used and their suffering.

PRIMARY CULTURE OF MICROGLIA

Mixed glial cultures were prepared from 1-to-2 day-old CD1 mice as previously described (McCarthy and de Vellis, 1980), with minor modifications (Gordo et al., 2006). Cells (4×10^5 cells/cm^2) were plated on uncoated 12-well tissue culture plates (with 18 mm coverslips) or 75-cm^2 culture flasks in culture medium [DMEM-Ham's F-12 medium supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, non-essential amino acids 1×, 10% fetal bovine serum (FBS), and 1% antibiotic-antimycotic solution] and maintained at 37°C in a humidified atmosphere of 5% CO_2.

Microglia were isolated as previously described by Saura et al. (2003). Briefly, after 21 days in mixed culture, microglia were obtained by mild trypsinization with a trypsin-EDTA solution diluted 1:3 in DMEM-Ham's F12 for 45–60 min. The trypsinization resulted in detachment of an upper layer of cells containing all the astrocytes, whereas the microglia remained attached to the bottom of the well. The medium containing detached cells was removed and the initial mixed glial-conditioned medium was added.

Mixed cultures were maintained in culture for 21 days to achieve the maximal yield and purity of the cultures. In fact, contamination by astrocyte and neurons was less than 2 and 0%, respectively, as assessed by immunocytochemical staining with a primary antibody against GFAP and MAP-2, respectively, followed by a species-specific fluorescent-labeled secondary antibody (Silva et al., 2010).

CHARACTERIZATION OF MICROGLIA ALONG THE DAYS IN CULTURE

After mild trypsinization, attached cells on uncoated 18-mm coverslips were maintained in culture until reaching 2, 10, or 16 DIV for characterization, with medium replaced every 4 days. Microglia characterization was first performed considering cell morphology and NF-κB activation, at these three time-points, and thereafter only at 2 and 16 DIV for additional properties related with migration ability, phagocytic capacity, differential cell reactive ability and markers of cell senescence.

CELL MORPHOLOGICAL ANALYSIS

For morphological analysis, cells were fixed for 20 min with freshly prepared 4% (w/v) paraformaldehyde in phosphate-buffer saline (PBS) and a standard immunocytochemical technique was performed using a primary antibody raised against Iba-1 (rabbit,

1:250; Wako Pure Chemical Industries Ltd, Osaka, Japan), and a secondary Alexa Fluor 594 goat anti-rabbit (1:1000; Invitrogen Corporation, Carlsbad, CA, USA). To identify the total number of cells, microglial nuclei were stained with Hoechst 33258 dye. Fluorescence was visualized using an AxioCam HRm camera adapted to an AxioSkope® microscope (Zeiss). Pairs of U.V. and red-fluorescence images of ten random microscopic fields (original magnification: 400×) were acquired per sample. To quantitatively characterize microglia morphology we used the particle measurement feature in ImageJ (1.47v, USA) to automatically measure the 2D area, perimeter, and Feret's diameter of single microglia cells. Feret's (maximum) diameter, a measure of cell length, is the greatest distance between any two points along the cell perimeter. We also evaluated the transformation index, first defined by Fujita et al. (1996) as [perimeter of cell (μm)]^2/4π [cell area (μm^2)], which categorizes microglia ramification status. A cell with long processes and a small soma exhibits a large index that is dependent on cell shape but independent of cell size.

DETECTION OF NF-κB ACTIVATION

For immunofluorescence detection of NF-κB nuclear translocation, cells were fixed as above and a standard indirect immunocytochemical technique was carried out using a polyclonal rabbit anti-p65 NF-κB subunit antibody (1:200; Santa Cruz Biotechnology®, CA, USA) as the primary antibody, and an anti-rabbit Cy2 as the secondary antibody (1:1000; GE Healthcare, Chalfont St. Giles, UK). Microglial nuclei were stained with Hoechst 33258 dye. Fluorescence was visualized and acquired as above. NF-κB positive nuclei were identified by localization of the NF-κB p65 subunit staining exclusively at the nucleus and total cells were counted to determine the percentage of NF-κB-positive nuclei at each cell DIV group.

DETERMINATION OF CELL DEATH

We used phycoerythrin-conjugated annexin V (annexin V-PE) and 7-amino-actinomycin D (7-AAD; Guava Nexin® Reagent, #4500-0450, Millipore) to determine the percentage of viable, early-apoptotic and late-apoptotic/necrotic cells by flow cytometry. After incubation adherent microglia were collected by trypsinization and added to the cells present in the incubation media. After centrifugation cells were resuspended in PBS containing 1% bovine serum albumin, stained with annexin V-PE and 7-AAD, following manufacturer's instructions, and analyzed on a Guava easyCyte 5HT flow cytometer (Guava Nexin® Software module, Millipore), as previously described (Barateiro et al., 2012). Three populations of cells can be distinguished by this assay: viable cells (annexin V-PE and 7-AAD negative), early apoptotic cells (annexin V-PE positive and 7-AAD negative), and late stages of apoptosis or dead cells (annexin V-PE and 7-AAD positive).

ASSESSMENT OF MICROGLIA MIGRATION

Cell migration assays were performed in a 48-well microchemotaxis Boyden chamber (Neuro Probe, Gaithersburg, MD, USA), as previously described (Miller and Stella, 2009), with minor modifications. The bottom wells, filled with ATP (10 μM), a known chemoattractant for microglia migration, served as positive controls. The 8 μm diameter polycarbonate membranes with polyvinylpyrrolidone (PVP) surface treatment was equilibrated in control medium and after chamber set up, 50 μl of cell suspension containing 2×10^4 cells was added to each top well. After 6 h incubation in a CO_2 incubator at 37°C for microglial migration, membrane was fixed with cold methanol and cells stained with 10% Giemsa in PBS. Non-migrated cells on the upper side of the membrane were wiped off with a filter wiper. The rate of migration was determined by counting cells on the lower membrane surface in 10 microscopic fields to cover all the well, acquired using a Leica DFC490 camera adapted to an AxioSkope HBO50 microscope. For each experiment, at least three wells per condition were analyzed.

EVALUATION OF PHAGOCYTIC PROPERTIES OF MICROGLIA

To evaluate the phagocytic capacity of the primary microglial cultures, cells collected at 2 and 16 DIV were incubated with 0.0025% (w/w) 1 μm fluorescent latex beads (Sigma Chemical Co., St. Louis, MO, USA) for 75 min at 37°C and fixed with freshly prepared 4% (w/v) paraformaldehyde in PBS. Microglia were stained for Iba-1, nuclei counterstained with Hoechst dye, and fluorescence was visualized and acquired as above. The number of ingested beads per cell was counted. Results are presented as mean number of ingested beads per cell and as the percentage of cells that phagocytosed <5, 5–10, or >10 beads.

DETERMINATION OF SUPPLEMENTARY FEATURES OF MICROGLIA REACTIVE ABILITY

We used several markers to assess microglia reactive ability, such as the concentration of glutamate and the activation of matrix metalloproteinase (MMP)-2 and MMP-9 in the extracellular media, together with the expression of TLR-2, TLR-4, miR-124 and miR-155.

Glutamate content in the media derived from microglial cultures was determined as described before (Silva et al., 2012) by an adaptation of the L-glutamic acid kit (Roche), using a 10-fold volume reduction. The reaction was performed in a 96-well microplate and the absorbance measured at 490 nm. A calibration curve was used for each assay. All samples and standards were analyzed in duplicate and the mean value was used.

Detection of MMPs activity was performed as previously mentioned (Silva et al., 2010). Aliquots of culture supernatant were analyzed by SDS-PAGE zymography in 0.1% gelatine-10% acrylamide gels under non-reducing conditions. After electrophoresis, gels were washed for 1 h with 2.5% Triton X-100 (in 50 mM Tris pH7.4; 5 mM $CaCl_2$; 1 μM $ZnCl_2$) to remove SDS and renature the MMP species in the gel. Then the gels were incubated in the developing buffer (50 mM Tris pH7.4; 5 mM $CaCl_2$; 1 μM $ZnCl_2$) overnight to induce gelatine lysis. For enzyme activity analysis, the gels were stained with 0.5% Coomassie Brilliant Blue R-250 and destained in 30% ethanol/10% acetic acid/H_2O. Gelatinase activity, detected as a white band on a blue background, was quantified by computerized image analysis and normalized with total cellular protein.

Determination of TLR-2 and TLR-4 mRNA expression was performed by RealTime PCR as usual in our laboratory (Barateiro et al., 2013). Total RNA was extracted from

microglia using TRIzol® (LifeTechnologies), according to manufacturer's instructions. Total RNA was quantified using Nanodrop ND-100 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Aliquots of 0.5 μg of total RNA were treated with DNase I and then reverse transcribed into cDNA using oligo-dT primers and SuperScript II Reverse Transcriptase under the recommended conditions. Quantitative RealTime-PCR (qRT-PCR) was performed using β-actin as an endogenous control to normalize the expression level of TLR-2 and TLR-4 transcription factors. The following sequences were used as primers: TLR-2 sense 5′-TGCTTTCCTGCTGAAGATTT-3′ and anti-sense 5′-TGTACCGCAACAGCTTCAGG-3′; TLR-4 sense 5′-ACCTGGCTGGTTTACACGTC-3′ and anti-sense 5′-GTGCCAGAGACATTGCAGAA-3′; β-actin sense 5′-GCTCCGG-CATGTGCAA-3′ and anti-sense 5′-AGGATCTTCATGAGGTAGT-3′. qRT-PCR was performed on a 7300 Real time PCR System (Applied Biosystems) using a SYBR Green qPCR Master Mix (Thermo Scientific). The PCR was performed in 96 well plates with each sample performed in triplicate, and no-template control was included for each amplificate. qRT-PCR was performed under optimized conditions: 94°C at 3 min followed by 40 cycles at 94°C for 0.15 min, 62°C for 0.2 min and 72°C for 0.15 min. In order to verify the specificity of the amplification, a melt-curve analysis was performed, immediately after the amplification protocol. Non-specific products of PCR were not found in any case. Relative mRNA concentrations were calculated using the Pfaffl modification of the ΔΔCT equation (CT, cycle number at which fluorescence passes the threshold level of detection), taking into account the efficiencies of individual genes. The results were normalized to β-actin in the same sample and the initial amount of the template of each sample was determined as relative expression by the formula 2-ΔΔCT. ΔCT is a value obtained, for each sample, by the difference between the mean CT value of each gene and the mean CT value of β-actin. ΔΔCT of one sample is the difference between its ΔCT value and ΔCT of the sample chosen as reference, in our case the 2 DIV cells.

Expression of miR-124 and miR-155, which has been related with microglia activation phenotype, was performed by qRT-PCR. Total RNA was extracted from primary microglia cultures using the miRCURY^{TM} Isolation Kit – Cells (Exiqon), according to the manufacturer's recommendations for cultured cells. Briefly, after cell lysis, the total RNA was adsorbed to a silica matrix, washed with the recommended buffers an eluted with 35 μl RNase-free water by centrifugation. After RNA quantification, cDNA conversion for miRNA quantification was performed with the universal cDNA Synthesis Kit (Exiqon) using 5 ng total RNA according to the following protocol: 60 min at 42°C followed by heat-inactivation of the reverse transcriptase for 5 min at 95°C. qRT-PCR was performed using an Applied Biosystems 7300 Sequence Detection system and 96-well plates. For miRNA quantification the miRCURY LNA^{TM} Universal RT microRNA PCR system (Exiqon) was used in combination with pre-designed primers (Exiqon) for miR-124, miR-155 and SNORD110 (reference gene). The reaction conditions consisted of polymerase activation/denaturation and well-factor determination at 95°C for 10 min, followed by 50 amplification cycles at 95°C for 10 s and 60°C for 1 min (ramp-rate of 1.6°/s). The miRNA fold change with respect to 2 DIV cells was determined by the Pfaffl method, taking into consideration different amplification efficiencies of miRNAs in all experiments. The amplification efficiency for each target was determined according to the formula: $E = 10^{(-1/S)} - 1$, where S is the slope of the obtained standard curve.

ASSESSMENT OF MICROGLIA SENESCENCE

Microglia senescence was evaluated by determining the activity of SA-β-gal, expression of miR-146a and capacity to undergo autophagy. Microglial SA-β-gal activity was determined using the Cellular senescence assay kit (Millipore), according to the manufacturer instructions. Microglial nuclei were counterstained with hematoxylin. Brightfield microscopy images of 10 random microscopic fields were acquired per sample. The number of turquoise stained microglia (SA-β-gal-positive cells) was counted to determine the percentage of senescent cells.

To confirm the senescent status of microglia it was also assessed the expression of the senescence-related miR-146a by qRT-PCR. Total RNA was extracted and expression of miR-146a was assayed using pre-designed primers (Exiqon) for miR-146a and SNORD110 (reference gene) as described above.

Autophagy was determined by both immunocytochemistry of microtubule-associated-protein-light-chain-3 (LC3) punctate and Western Blot detection of LC3 and Beclin-1 bands. For immunocytochemistry, cells were fixed as above and standard immunocytochemical technique was performed using a primary antibody raised against LC3 protein (rabbit, 1:500; Cell Signaling Technology Inc., MA, USA), and a secondary Alexa Fluor 488 goat anti-rabbit antibody (1:1000; Invitrogen Corporation, CA, USA). To identify the total number of cells, microglial nuclei were stained with Hoechst 33258 dye. Fluorescence was visualized and images acquired as above. The method is based on the increased localization of LC3 autophagosomes when autophagy is induced. Thus, the punctate fluorescence produced by LC3 staining provides a sensitive and specific indicator of autophagy (Aoki et al., 2008). Microglial cells presenting LC3 punctate were counted and the percentage of LC3 punctate-positive cells relatively to total microglia was determined. Detection of LC3-II, which is associated with autophagic vesicles (Kabeya et al., 2000), and Beclin-1 bands was processed by Western Blot as usual in our laboratory (Barateiro et al., 2012). Cells were washed in ice-cold PBS, lysed in a buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na2EDTA, 1 mM ethylene glycoltetraacetic acid, 1% (v/v) Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 μg/mL leupeptine, and 1 mM phenylmethylsulfonyl fluoride, and sonicated for 20 s. The lysate was centrifuged at 14,000 *g* for 10 min at 4°C and the supernatants were collected and stored at −80°C. Protein concentrations were determined using BioRad protein assay (BioRad). Cell extracts containing equal amounts of protein (50 μg) were separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membranes were blocked with 5% non-fat milk, incubated with the primary antibody overnight at 4°C [rabbit

anti-LC3B (1:1000; #2775, Cell Signaling), mouse anti-Beclin-1 (1:500; #MABC34, MerckMillipore) or mouse anti-β-actin (1:5,000; Sigma)], and then with a horseradish peroxidase-labeled secondary antibody for 1 h at room temperature. After extensive washes, immunoreactive bands were detected by LumiGLO® (Cell Signaling, Beverly, MA, USA) and visualized by autoradiography with Hyperfilm ECL. Results were normalized to β-actin expression and expressed as fold vs. vehicle-treated cells.

STATISTICAL ANALYSIS

Significant differences between the parameters evaluated were determined by the two-tailed Student's *t*-test performed on the basis of equal and unequal variance, as appropriate. Comparison of more than two groups (microglia morphology, NF-κB activation) was done by ANOVA using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, USA) followed by multiple comparisons Bonferroni *post hoc* correction. p value less than 0.05 were considered statistically significant.

RESULTS

IN VITRO AGING CHANGES MICROGLIA MORPHOLOGY TO A MORE RAMIFIED CELL SHAPE

Phenotypic changes in microglia are often accompanied by a morphological transformation, which has been widely used to categorize different activation states. In general, ramified quiescent microglia changes to an activated state displaying larger somata and shorter, coarser cytoplasmic processes progressing to a full amoeboid morphology (Fujita et al., 1996; Kozlowski and Weimer, 2012). Interestingly, microglia isolated from adult and aged animals show a propensity to acquire a more ramified morphology with thicker and more extensive processes (Lai et al., 2013), indicative of a less activated phenotype with age. So, we started by characterizing microglia morphology at 2, 10, and 16 DIV, following immunollabeling with the cell-specific marker Iba-1. As shown in **Figure 1**, diverse morphological forms of microglia may be observed throughout cell culturing. The microglial cells at 2 DIV were almost exclusively amoeboid, most frequently evidencing an ovoid shape with a few cells presenting fusiform shape (**Figure 1A**). At 10 DIV, microglia evidence a more heterogeneous morphology with an increased number of cells showing a ramified morphology, bearing typically one or two large processes or a single large lamellipodia, together with larger amoeboid forms (**Figure 1B**). Microglia cultures at 16 DIV still exhibited distinct polarized populations showing rod-like microglia, bipolar microglia with shorter processes and the residual amoeboid cells (**Figure 1C**). To quantitatively evaluate the effect of age on microglia morphology we measured the area, perimeter, and Feret's maximum diameter of microglia (**Figures 1D–F**). Consistent with a transformation of amoeboid to microglia ramified forms, the area, perimeter and the Feret's maximum diameter significantly increased at 16 DIV (∼2.0-, ∼1.6-, and ∼1.6-fold, respectively, $p < 0.05$). Analysis of the transformation index value, a dimensionless number that reflects the degree of process extension, revealed a continuum of microglial phenotypes between the amoeboid and the ramified morphologies (**Figure 1G**). While younger cultures with a predominant amoeboid microglia shape present a low transformation index, older cultures with a more heterogeneous morphological repertoire, involving cells with amoeboid and ramified morphologies, displayed an increased transformation index (∼1.6-fold, $p < 0.05$).

IN VITRO AGING REDUCES MICROGLIA NF-κB ACTIVATION

Microglia play key immune-related duties, intervening through the production of anti-inflammatory compounds and trophic factors, by phagocytosing non-functional cells and debris, but also by releasing pro-inflammatory cytokines, depending on the stimuli. Production of several cytokines during microglial activation process is associated with the activation of the inducible transcription factor NF-κB (O'Neill and Kaltschmidt, 1997). To explore whether microglia morphological changes along the time in culture could be related with the cell activation state, we investigated NF-κB transactivation at the time points used to assess morphological alterations. Following microglia immunollabeling for p65 NF-κB subunit, we determined the number of NF-κB-positive nuclei as an indicator of its activation (**Figure 2**). Our results show that microglia express maximal NF-κB activation at 2 DIV decreasing significantly thereafter and reaching minimal levels at 16 DIV (∼0.4-fold vs. 2 DIV, $p < 0.01$). These results corroborate the previous data in cell morphology and reinforce that microglia are highly reactive at 2 DIV but reduce their activation profile to a minimum state at 16 DIV. Thus, to settle that microglia at these *in vitro* stages may be associated to activated (2 DIV) and to age-like irresponsive cells (16 DIV), we additionally explored several markers that have been linked with age-related alterations in the dynamic behavior of microglia.

AGED MICROGLIA SHOW A RESIDUAL MIGRATION ABILITY

Microglia directed migration towards regions of injury, also known as chemotaxis, is a property that seems to be more related to the classically (M1) and alternatively activated microglia (M2a; Lively and Schlichter, 2013). The release of chemotactic molecules upon brain damage, such as ATP, was indicated to participate in the recruitment of microglia toward lesion sites (Miller and Stella, 2009; Kettenmann et al., 2011). Nevertheless, it was reported that microglia respond to ATP regardless of their activation state (Lively and Schlichter, 2013). Hence, we evaluated the ability of 2 and 16 DIV microglia to migrate towards 10 μM ATP. As shown in **Figure 3**, 16 DIV microglia revealed a poor ability to migrate to ATP when compared to 2 DIV cells (∼0.1-fold, $p < 0.01$). This finding points to a 2 DIV population of reactive microglia with capacity to migrate to local brain injury in contrast to the aged cells that lose invasion capacity property.

AGED MICROGLIA SHOW REDUCED PHAGOCYTIC ABILITY

Microglia are considered the professional phagocytes of the CNS, a function that is crucial along brain development, as well as in pathology and regeneration (Kettenmann et al., 2011). Therefore, and based on the previous results, we hypothesized that aging in culture could also have adverse effects on microglia phagocytic properties. As expected, 16 DIV microglia showed reduced engulfment ability when compared to 2 DIV cells (**Figure 4A**). Indeed,

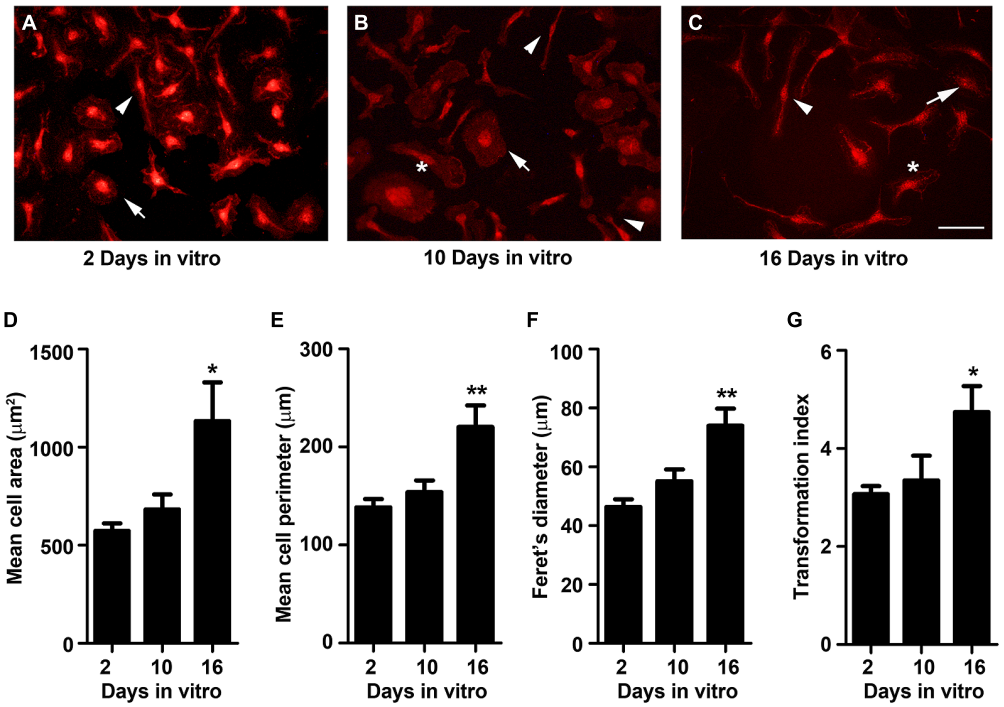

FIGURE 1 | Microglia morphology changes from amoeboid to a more ramified shape with cell aging in culture. Microglial cells were kept in culture for 2, 10, and 16 days *in vitro* (DIV), immunostained for Iba-1 and their morphology analyzed. **(A)** At 2 DIV, microglia were amoeboid with ovoid shape (arrow) and only a few showed a ramified bipolar morphology (arrowhead). **(B)** At 10 DIV, microglia became more heterogeneous with more cells presenting a ramified morphology (arrowhead), bearing a single large lamellipodia (*) and some a larger amoeboid shape (arrowhead). **(C)** At 16 DIV cells exhibited distinct polarized populations including ramified rod-like microglia (arrowhead), bipolar microglia with shorter processes (*) and residual amoeboid cells (arrow). Microglia area **(D)**, perimeter **(E)**, and Feret's diameter **(F)** values were measured using the computer program ImageJ; transformation index values **(G)** were calculated as [perimeter of cell (μm)]^2/4π [cell area (μm^2)]. Cultures, $n = 4$ per group. *Post hoc* Bonferroni test, $^*p < 0.05$ and $^{**}p < 0.01$ vs. 2 DIV cells. Each value represents the mean ± SEM. Scale bar equals 50 μm.

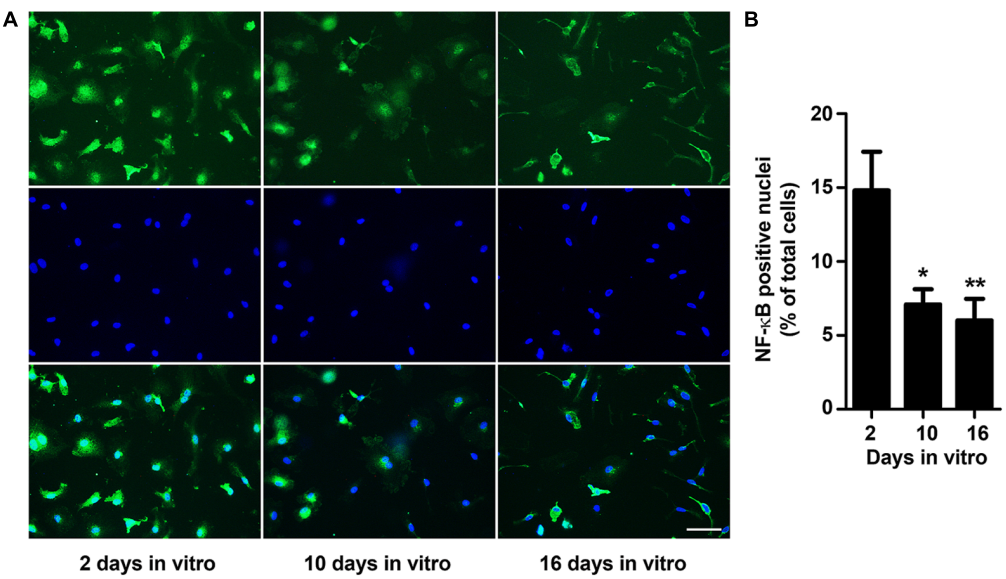

FIGURE 2 | NF-κB activation decreases with microglia aging in culture. Microglial cells were kept in culture for 2, 10, and 16 days *in vitro* (DIV), immunostained for nuclear factor kappa-B (NF-κB; green) and their nuclei stained with Hoechst dye (blue). **(A)** Representative images at 2, 10, and 16 DIV. **(B)** Cells bearing a NF-κB-positive nuclei were counted and results expressed in graph bars as mean ± SEM. Cultures, $n = 4$ per group. *Post hoc* Bonferroni test, $^*p < 0.05$ and $^{**}p < 0.01$ vs. 2 DIV cells. Scale bar equals 50 μm.

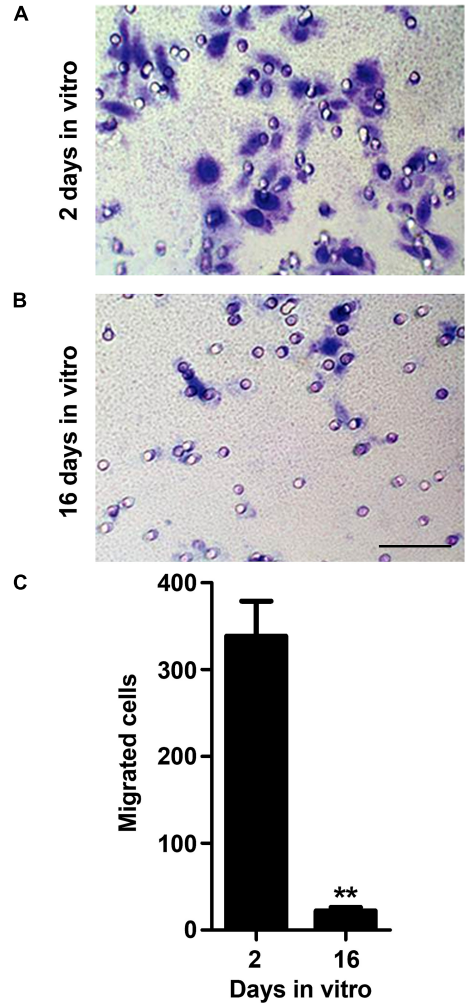

FIGURE 3 | Microglia migration ability decreases with cell aging in culture. Microglial cells were kept in culture for 2 and 16 days *in vitro* (DIV) and then cellular chemotactic migration to 10 μM ATP was evaluated using the Boyden chamber method. Representative images of 2 **(A)** and 16 **(B)** DIV microglia that migrated towards ATP were visualized by Giemsa staining. Number of migrated cells was counted and results expressed in graph bars as mean ± SEM **(C)**. Cultures, $n = 4$ per group. *t*-test, $^{**}p < 0.01$ vs. 2 DIV cells. Scale bar equals 50 μm.

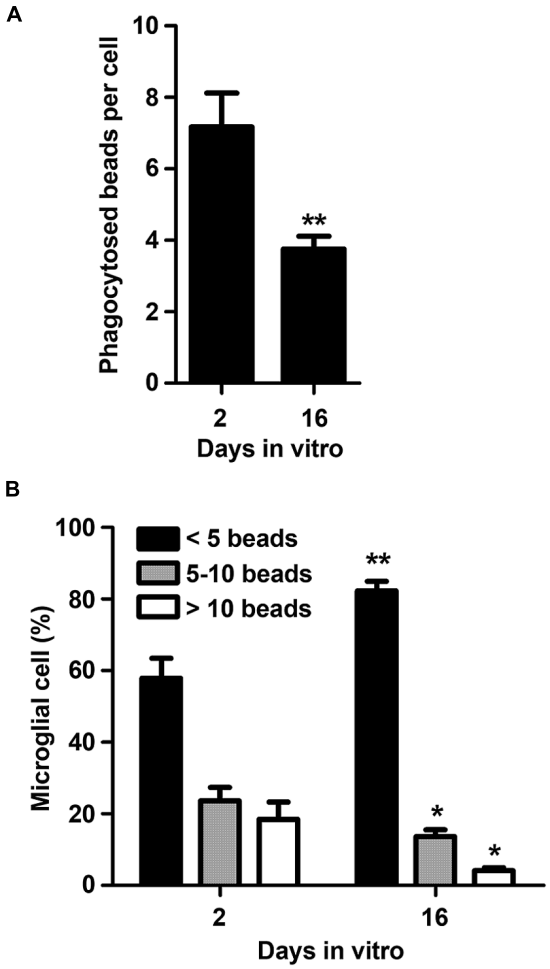

FIGURE 4 | Microglia phagocytic ability decreases with cell aging in culture. Microglial cells were kept in culture for 2 and 16 days *in vitro* and then exposed to fluorescent beads to measure their phagocytic capacity. Number of phagocytized beads per cell **(A)** and the number of microglia phagocytosing <5, 5–10, and >10 beads **(B)** was counted and results expressed in graph bars as mean ± SEM. Cultures, $n = 4$ per group. *t*-test and *post hoc* Bonferroni test, $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. 2 DIV cells.

the average number of beads phagocytosed by each microglial cell was markedly reduced from 2 to 16 DIV (∼0.5-fold, $p < 0.01$). In addition, we observed that aged microglia function less effectively than the 2 DIV cells based on the increased number of cells that engulf a small number of beads ($p < 0.01$) together with a decreased ability to digest 5 or more beads ($p < 0.05$; **Figure 4B**). Altogether these data suggest that *in vitro* aging of microglia obtained from neonatal mice change their dynamic behavior to a more inert or irresponsive phenotype compatible with an irresponsive/senescent cell.

MICROGLIA RETAIN VIABILITY DURING *IN VITRO* AGING

Given our previous results we wondered whether the loss of microglia function by *in vitro* aging was a consequence of reduced cell viability. Therefore, we evaluated microglia cell death by flow cytometry following staining with annexin V-PE and 7-AAD, to differentiate the total amount of cells (adherent plus detached) into viable, early apoptotic and late apoptotic/necrotic cells. As shown in **Table 1**, we did not observe differences in cell death between the 2 and the 16 DIV microglia, confirming that changes in aged microglia response are not due to reduced viability but rather derive from a switch in cellular phenotype and in its properties.

SUPPLEMENTARY FEATURES OF MICROGLIA REACTIVE ABILITY ARE REDUCED IN AGED CELLS

Since 16 DIV microglia have shown decreased ability to respond to chemotactic signals and to phagocytose extracellular particles, features that were not related with loss of cell survival (**Table 1**), we next decided to evaluate whether microglia aged in

Table 1 | Viability of culturing microglia.

	Viable cells	Early-apoptotic cells	Late-apoptotic/necrotic cells
2 DIV	81.8 (±2.6)	9.7 (±0.3)	6.2 (±2.2)
16 DIV	81.7 (±3.0)	11.4 (±1.9)	7.4 (±1.6)

All results are means ± SEM from at least four independent experiments. Microglial were kept in culture for 2 and 16 DIV. The percentage of viable microglia and microglia in early- or late-apoptosis/necrosis was determined by flow cytometry with phycoerythrin-conjugated annexin V (annexin V-PE) and 7-amino-actinomycin D (7-AAD). The three populations were distinguished as follows: viable cells (annexin V-PE and 7-AAD negative), early apoptotic cells (annexin V-PE positive and 7-AAD negative), and cells in late stages of apoptosis or dead cells (annexin V-PE and 7-AAD positive).

culture would also present additional markers of reduced reactive ability. Glutamate was shown to be released by activated microglia (Noda et al., 1999; Barger et al., 2007; Takaki et al., 2012), reason why we decided to evaluate the extracellular content of glutamate. As depicted in **Figure 5A**, 16 DIV microglia showed to release lower levels of glutamate to the culture media than the 2 DIV cells (∼0.7-fold, $p < 0.01$). Interestingly, when evaluating MMP-2 and MMP-9 activation in the extracellular media we verified that the influence of aging was also notorious (**Figure 5B**). Indeed we observed a marked increase of MMP-2 (∼2.2-fold, $p < 0.05$) and a decrease of MMP-9 (∼0.6-fold, $p < 0.05$) in the aged microglia when compared to 2 DIV cells. Again, the expression of TLR-2 and TLR-4 that is associated with microglia activation (Banks and Robinson, 2010; Liu et al., 2012) very much decreased in the 16 DIV microglia (∼0.4-fold, $p < 0.01$, **Figure 5C**). Recently, immune regulation by miR-124 was indicated to downregulate microglia activation (Ponomarev et al., 2011) in contrast with miR-155 that was shown to have a pro-inflammatory role in microglia (Cardoso et al., 2012), to be related with the M1 phenotype (Ponomarev et al., 2013) and to be up-regulated upon activation (Lu et al., 2011). Corroborating previous findings, the decreased expression of both miR-124 and miR-155 in 16 DIV microglia as compared to 2 DIV cells (∼0.5- and 0.4-fold, respectively, $p < 0.01$, **Figure 5D**) further reinforce that the cells become irresponsive/senescent when maintained in culture.

16 DIV MICROGLIA SHOW COMMON MARKERS OF SENESCENCE

Senescent microglia have been described to become dysfunctional and less efficient in their neuroprotective effects during aging in the human brain and in AD (Streit and Xue, 2012; Krabbe et al., 2013). The main proposal of the present study was to obtain an experimental model able to reproduce irresponsive/senescent microglia that could be used for exploring detrimental effects by aging and associated-neurodegenerative diseases. As so, we decided to evaluate if the *in vitro* aged microglia displayed typical signs of cell senescence. The senescence phenotype has been associated with changes in cellular morphology, increased activity for SA-β-gal, permanent DNA damage, chromosomal instability and altered inflammatory secretome (Sikora et al., 2011). More recently, new biomarkers of age-associated senescence have been reported, including an increased expression of miR-146a in aged macrophages (Jiang et al., 2012) and a reduced capacity to undergo autophagy (Ma et al., 2011). Quantitative assay of SA-β-gal activity revealed that the percentage of positively stained cells markedly increased from 2 to 16 DIV (∼2.5-fold, $p < 0.01$), as evidenced in **Figures 6A,B**. Similarly, we noticed a significant elevation in the expression of miR-146a along the cell aging in culture (∼2.3-fold, $p < 0.05$, **Figure 6C**). Finally, we evaluated autophagic capacity by LC3 immunostaining. As it may be observed in **Figure 7A**, 2 DIV cells displayed an increased amount of LC3 punctates when compared to 16 DIV microglia. Counting of LC3 punctate-positive cells confirmed that a reduced number of 16 DIV cells were undergoing autophagy (∼0.7-fold, $p < 0.05$, **Figure 7B**). Next, we evaluated the expression of LC3-II that is formed through lipidation of LC3-I during autophagy (Kabeya et al., 2000) and we additionally determined the Beclin-1 protein, recognized to have a central role in such process (Kang et al., 2011), by Western Blot (**Figures 7C,D**). Our results clearly show that LC3-II and Beclin-1 are markedly reduced in 16 DIV microglia when compared to 2 DIV cells (∼0.4- and ∼0.3-fold, respectively, $p < 0.01$), confirming a reduced autophagy by the aged microglia.

Overall, our data indicate that primary microglia harvested from neonatal mouse pups first evidence an increased reactive ability changing to an irresponsive/senescent cell when maintained in culture. Aged cells evidence a reduced ability to become activated, to migrate and to phagocytose, in parallel with markers of cellular senescence. Therefore, this *in vitro* model can be very useful in the exploitation of microglia reactivity and irresponsiveness to stimuli, respectively. In addition, changes in microglia miRNA signature may constitute a precious help in evaluating the key role of microglia as a determinant in age-associated CNS disorders and in modulating microglia dynamic properties.

DISCUSSION

Experiments in this study were carried out to investigate age-specific differences in the dynamic functional profiles of neonatal microglia aged in culture, from 2 DIV up to 16 DIV. Here we show that microglia isolated from neonatal pups evidence markers of reactive ability at early time culture changing their phenotype along *in vitro* culture to less responsive cells that present senescence biomarkers and miRNA profiling characteristic of microglia deactivation. Collectively, our results indicate that microglia aging can be reproduced *in vitro* using long-term murine cultures, which may be used as a model to evaluate microglia performance in age-associated disorders, inasmuch due to the similar characteristics such mice cells evidence to human microglia (Torres-Platas et al., 2014).

Mouse primary neonatal microglial cultures have the advantage to more closely represent their in situ counterparts when compared to immortalized cells, although by growing in isolation lack the normal CNS microenvironment (Ni and Aschner, 2010). Indeed, primary cultured microglia are not oncogene immortalized and are differentiated in mixed glial cultures before isolation. The protocol here described originates microglial cultures that exceeds 97% purity and has been used as a model for activated CNS-resident microglia (Carson et al., 1998; Schmid et al.,

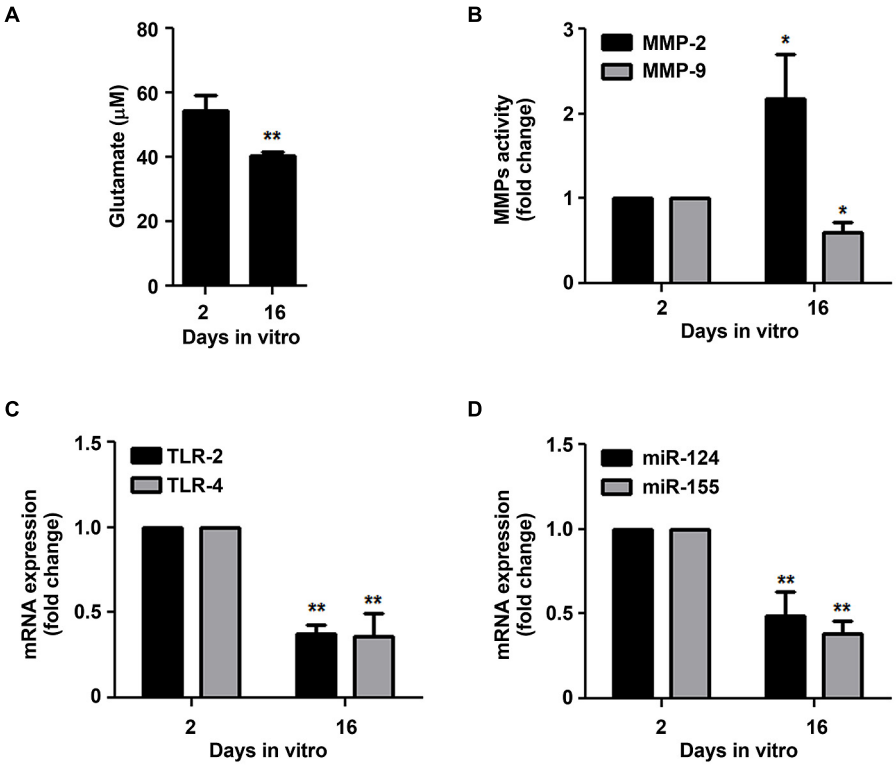

FIGURE 5 | Microglia supplementary features of reactive ability are reduced in aged cells. Microglial cells were kept in culture for 2 and 16 days *in vitro* (DIV). **(A)** Extracellular glutamate levels were determined using a commercial kit. **(B)** Matrix metalloproteinases (MMP)-2 and MMP-9 activities were assessed by gelatin zymography. Expression of Toll-like receptor (TLR)-2 and TLR-4 **(C)**, as well as of microRNA (miR)-124 and miR-155 **(D)** was evaluated by Real-Time PCR. Results are expressed in graph bars as mean ± SEM. Cultures, $n = 4$ per group. *t*-test, $*p < 0.05$ and $**p < 0.01$ vs. 2 DIV cells.

2009) and to prepare polarized M1 and M2 phenotypes (Jang et al., 2013). Indeed, it was previously suggested that the isolation process is a sufficient stimulus to induce microglia activation (Cristóvão et al., 2010). There is a high controversy on whether neonatal microglia are less (Moussaud and Draheim, 2010) or more reactive than adult (Christensen et al., 2014) and aged cells (Njie et al., 2012). Discrepancies also exist in the scientific community based on studies that consider microglia overactivation and increased release of pro-inflammatory cytokines with age and neurodegenerative diseases (for review see Wong, 2013; Mosher and Wyss-Coray, 2014), in contrast with others evidencing dystrophic microglia and senescence (Streit et al., 2004; Streit and Xue, 2013), decreased phagocytosis (Floden and Combs, 2011; Li, 2013), lower reactivity to stimulation (Damani et al., 2011; Njie et al., 2012), delayed response to exogenous ATP and decreased microglial process motility (Hefendehl et al., 2014). Such contradictory results may be caused by different experimental sets and conditions. Moreover, most of the data were derived from experimental models using LPS-induced microglia activation, when it is well known that only a small amount is able to enter the brain parenchyma (Banks and Robinson, 2010). Therefore, the effects of peripheral immunostimulation by intravenously administered LPS dose are indirect and some of them mediated by the cells that comprise the BBB. Another important aspect to consider is that NSAIDs were only successful when administered before the development of neurodegeneration (Weggen et al., 2001). When administered in later stages of disease they showed to be detrimental (Martin et al., 2008). These findings may underlie a first proinflammatory stage in neurodegenerative diseases associated to neuroinflammmation, later switching to dysfunctional neurodegeneration associated with a loss of microglia dynamic properties. Indeed, neither the typical inflammatory nor the anti-inflammatory phenotypes were identified at end-stage amyotrophic lateral sclerosis (Nikodemova et al., 2013) and microglial dystrophy associated with their senescence (Flanary, 2005), as well as to aged and AD brain (Lopes et al., 2008).

Lack of knowledge on the molecular mechanisms implicated in aged microglia dysfunction and how it is related to an increased individual vulnerability to neurodegenerative diseases has hindered the development of effective therapy for preventing or even halting the CNS network degenerative process. Major problems to investigate such mechanisms are determined by the current *in vitro* microglial models using cell lines, primary microglia isolated from neonatal murine animals and *ex vivo* isolation from adult and aged brain. First models are not suitable for the research of neurodegenerative diseases where aging is crucial since long-term culture experiments are critical, and the last one only provides specific microglia subsets that resist to the isolation procedure

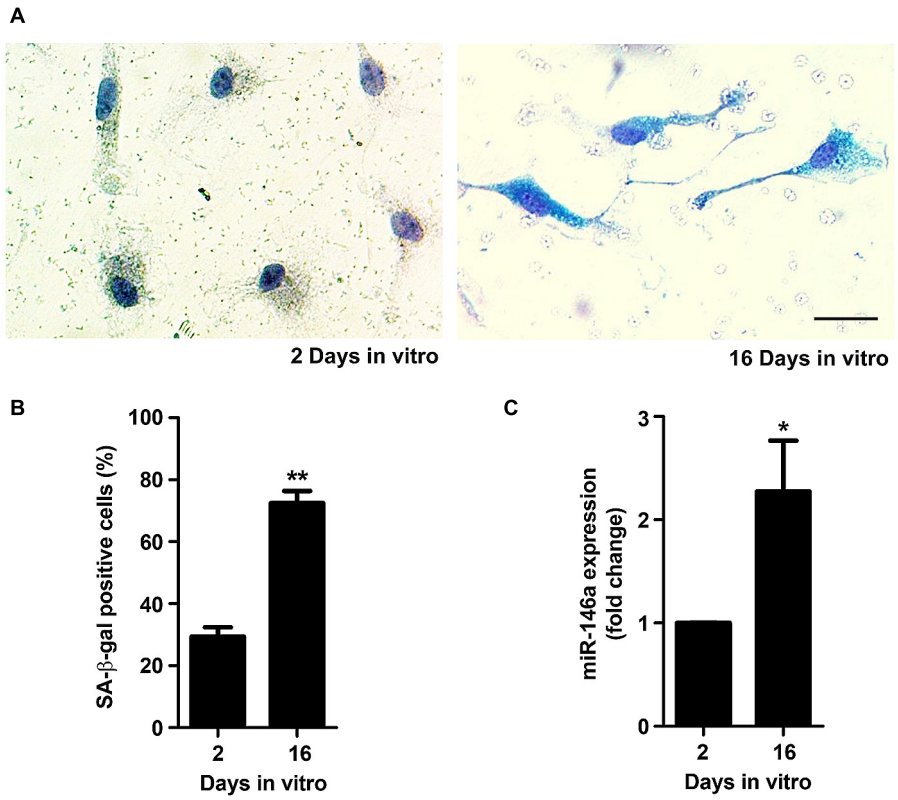

FIGURE 6 | Microglia aged in culture display signs of senescence, including increased senescent-associated β-galactosidase (SA-β-gal) activity and microRNA (miR)-146a expression. Microglial cells were kept in culture for 2 and 16 days *in vitro* (DIV). Activity of SA-β-gal was determined using a commercial kit. **(A)** Representative images of 2 and 16 DIV microglia showing SA-β-gal staining. **(B)** SA-β-gal-positive cells were counted and results expressed in graph bars as mean ± SEM. **(C)** miR-146a expression was evaluated by Real-Time PCR. Results are expressed in graph bars as mean ± SEM. Cultures, $n = 4$ per group. *t*-test, $*p < 0.05$ and $**p < 0.01$ vs. 2 DIV cells. Scale bar equals 20 μm.

(Moussaud and Draheim, 2010; von Bernhardi et al., 2011) or that are separated based on immunomagnetic cell sorting steps (Cardona et al., 2006). However, mixed microglial populations may coexist in the CNS and were also shown to be developed in culture (Szabo and Gulya, 2013; Gertig and Hanisch, 2014). In addition, microglia functionality from adult and aged animals is not well preserved, the yield is low and the cells undergo extensive cell death resulting in activation of the surviving population (von Bernhardi et al., 2011). The *in vitro* model we developed to obtain microglia senescence in primary culture has been likely used to identify aging-associated changes in fibroblasts at the molecular level (Chen et al., 2009). Finally, we have not used microglia culturing with astrocytes to avoid the complex interactions between these cells (Tanaka et al., 1999) that would be a disadvantage to assess natural microglia maturity and senescence. Therefore, establishment of well-defined stable *in vitro* cultures freshly isolated from neonatal mice and characterization of microglial phenotype with the time in culture may provide advantages over the other methods to determine aging microglial dynamics modifications and therapeutic approaches to recover microglial functionality.

Microglia morphology changed along *in vitro* maintenance from an almost exclusive round amoeboid shape to distinct polarized populations, including an increased number of ramified cells. In accordance, mixed primary glial cultures from embryonic rats have previously showed the existence of cells with an amoeboid morphology in the early stages of *in vitro* differentiation, which changed to mixed populations of amoeboid and ramified cell morphologies in older cultures (Szabo and Gulya, 2013). Interestingly, data from microglia isolated from different age animals also corroborate such findings with adult microglia presenting a more ramified morphology, in contrast with an amoeboid shape of embryonic and neonatal microglia (Lai et al., 2013). This is in line with *in vivo* data indicating that invading neonatal microglia have a predominant rounded morphology that differentiate with time into a surveying phenotype characterized by a small soma and highly branched processes (Hanisch and Kettenmann, 2007). Our aged microglia cultures besides exhibiting ramified and amoeboid cells also presented cells with a bipolar shape and shorter large processes. Morphological signs of microglia senescence with aging were observed *in vivo* and defined as abnormal morphological features, such as shortened, gnarled, beaded, or fragmented cytoplasmic processes, and loss of fine ramifications and formation of spheroidal swellings (Streit et al., 2004). Therefore, we hypothesize that such cells with shortened processes represent microglia with less ability to become reactive and should include a relevant population of senescent microglia.

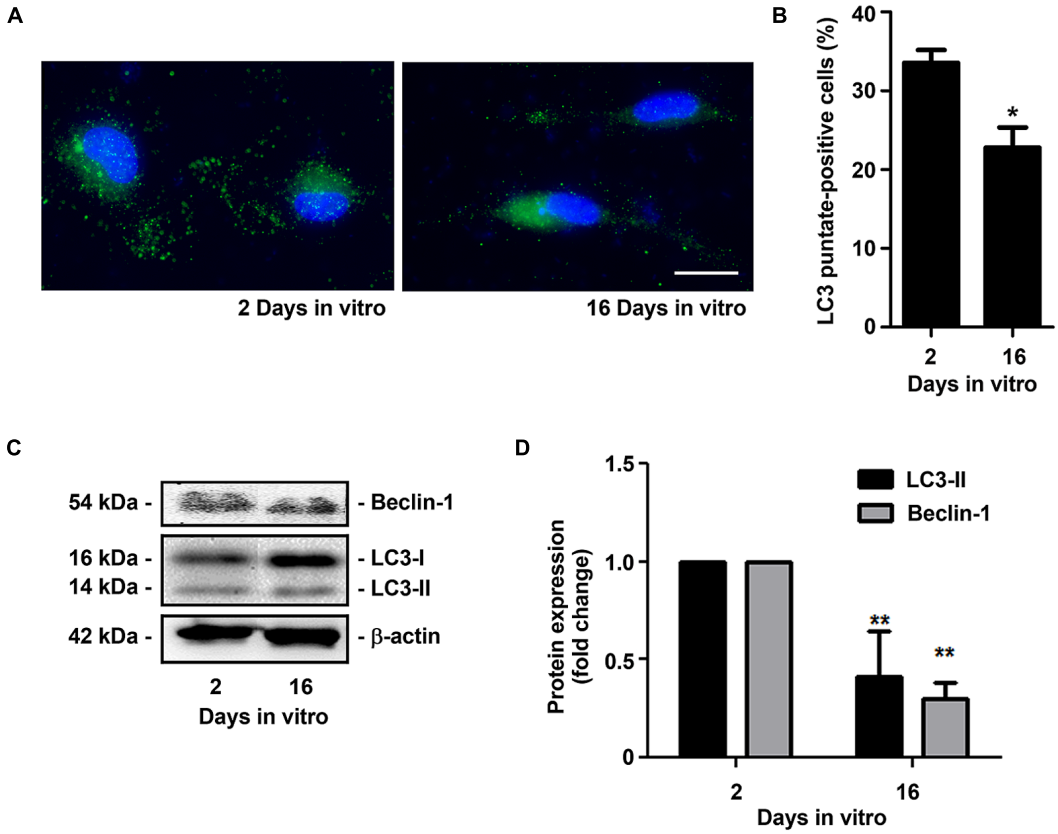

FIGURE 7 | Microglia aged in culture show reduced autophagic capacity. Microglial cells were kept in culture for 2 and 16 days *in vitro* (DIV), immunostained for LC3 and total cell lysates were analyzed for the presence of LC3-II and Beclin-1. **(A)** Representative images of 2 and 16 DIV microglia showing LC3 punctates. **(B)** Cells displaying LC3 punctates were counted and results expressed in graph bars as mean ± SEM. **(C)** LC3-II/LC3-I and Beclin-1 protein expression was assessed by Western Blot and **(D)** densitometric data analysis is represented in graph bars as mean ± SEM. Cultures, $n = 4$ per group. *t*-test, $*p < 0.05$ and $**p < 0.01$ vs. 2 DIV cells. Scale bar equals 20 μm.

The morphological changes of *in vitro* aging microglia occurred in parallel with a decrease in the transactivation of NF-κB. It is well known that this transcription factor is found throughout the cytoplasm, translocating to the nucleus upon activation triggering the transcription of target genes, such as the pro-inflammatory cytokines (O'Neill and Kaltschmidt, 1997). Therefore, maximal activation of NF-κB 2 days after isolation is consistent with an inflammatory phenotype that shifts to a deactivated microglia along with the time in culture. Intriguingly, although we showed a decreased NF-κB activation at 16 DIV, the activation of this transcription factor has been associated with the aging process. A recent report has shown that hypothalamic microglial NF-κB activation promoting a residual inflammatory status is required for systemic aging (Zhang et al., 2013). Nevertheless, a marked down-regulation of NF-κB was also observed in cultured senescent human WI-38 fibroblasts (Helenius et al., 1996). Considering that activators of the NF-κB signaling pathway are determinants of inflammation and aging process (Balistreri et al., 2013) and that CNS inflammation is present in the early stages of age-related disorders such as AD but disappears with disease progression (Streit et al., 2009), our *in vitro* aged microglia may represent a dystrophic and irresponsive phenotype whose functions have progressively declined as recently observed in mice with AD-like pathology (Krabbe et al., 2013).

The reduced migration observed for 16 DIV cells is in line with recent data showing that aged microglia become less dynamic with slower acute responses and lower rates of process motility (Damani et al., 2011). Here, we measured ATP-induced microglial chemotaxis, which occurs via P2X4R and P2Y12R purinergic receptors (Ohsawa et al., 2007). Interestingly, even considering that the expression of P2X4R in microglia is not age-dependent, the P2Y12R expression varies with animal age increasing to a maximum at 6–8 months and decreasing thereafter to extremely low levels at 13–15 months (Lai et al., 2013). Thus, it is possible that our aged microglia present reduced expression of purinergic receptors which may be in the origin of the reduced ability to migrate to ATP. Moreover, since it was demonstrated that monocyte chemoattractant protein-1 (MCP-1) produced downstream NF-κB activation is involved in the migration of microglia (Deng et al., 2009), based on the age-dependent reduction of NF-κB nuclear translocation we have observed it is reasonable to consider that the MCP-1-dependent migration pathway may also be affected.

Phagocytosis is crucial to maintain tissue homeostasis and innate immune balance, by ingesting both foreign pathogens and autologous apoptotic cells (Napoli and Neumann, 2009). Infectious pathogens are phagocytosed through TLRs or complement receptors to elicit the release of pro-inflammatory cytokines (Napoli and Neumann, 2009), while apoptotic cells or cellular debris are internalized through phosphatidylserine receptors, integrins or TREM2 to trigger immunosuppressive signaling with the release of anti-inflammatory cytokines (Li, 2012). During aging, clearance of both foreign pathogens and autologous apoptotic cells is diminished and has been associated with immunosenescence (Li, 2013). In accordance, microglia from aged mice also internalized less amyloid-β peptide (Aβ) than microglia from neonatal or young mice (Njie et al., 2012), corroborating our findings that 16 DIV microglia have a reduced ability to phagocytose possibly due to the manifestation of a senescent phenotype. Interestingly, microglial cells maintained in mixed primary neuronal-glial cocultures were shown to phagocytose more when amoeboid than in the ramified form, a property that decreased during culturing (Szabo and Gulya, 2013). In agreement, we observed a shift to a more ramified phenotype with cell aging, which paralleled a reduced phagocytic ability.

Activated microglia were shown to release increased levels of glutamate (Noda et al., 1999; Barger et al., 2007; Takaki et al., 2012). However, several studies have shown lower glutamate concentration in older subjects when compared to younger individuals (Kaiser et al., 2005; Sailasuta et al., 2008; Chang et al., 2009) and an age-dependent decline of glutamate release in mice (Minkeviciene et al., 2008). This finding is in line with the reduced glutamate levels we obtained in aged cell cultures. Similarly, the increased activation of MMP-2 we observed in 16 DIV microglia was identified in senescent cells (Liu and Hornsby, 2007; Lu et al., 2009; Malaquin et al., 2013). In what concerns MMP-9 there is some discrepancy between authors. Some indicate increased activity with age (Simpson et al., 2013) and others a decrease (Bonnema et al., 2007; Paczek et al., 2008), as we obtained. Furthermore, we think that the marked reduced expression we obtained at 16 DIV microglia for TLR-2 and TLR-4 (0.5- and 0.4-fold, respectively), as compared to 2 DIV cells, define with no doubt that 16 DIV microglia will be less able to respond to LPS immunostimulation. Actually, TLR-4 that is critical for the recognition of LPS, as well as TLR-2 that also recognizes some LPS species, are inducers of microglia activation leading to the production of proinflammatory cytokines (Banks and Robinson, 2010; Liu et al., 2012). Curiously, the TLR-4 downregulation-mediated supression of TNF-α and IL-1β expression revealed to also be accompanied by the suppression of NF-κB (Yao et al., 2013).

MicroRNAs are an abundant class of highly evolutionarily conserved small non-coding RNAs that are involved in posttranscriptional gene silencing, regulating diverse biological processes (Ambros, 2004). miR-146a was first associated with the innate immune response as a negative feedback regulator in TLR signaling (Taganov et al., 2006), and more recently implicated in age-related dysfunction of macrophages (Jiang et al., 2012). Our results clearly showed that aged microglia express increased levels of miR-146a, thus corroborating their senescent phenotype. Interestingly, expression of miR-146a that has been associated with several neurodegenerative disorders (Sinha et al., 2011; Jiang et al., 2013), was found elevated in the aged mouse (Jiang et al., 2012; Olivieri et al., 2013), in the cerebrospinal fluid of AD patients (Alexandrov et al., 2012), and to be induced in microglia upon Aβ and inflammatory challenge (Li et al., 2011). As so, our *in vitro* old microglia reproduce the aging-associated phenotype encountered in late-life common disorders. Moreover, decreased miR-124 and miR-155 that revealed a negative correlation with age (Fichtlscherer et al., 2010; Noren Hooten et al., 2010; Smith-Vikos and Slack, 2012), parallelled by the enhanced miR-146a expression, further reinforce that 16 DIV microglia mainly represent aged-like microglia. In addition the reduced miR-124 obtained in these cells, indicated as being associated to the M2a-alternatively activated state (Freilich et al., 2013) and to inhibit inflammation (Prinz and Priller, 2014), strengthen their dormant/senescent phenotype. In contrast, the predominant amoeboid morphology together with increased NF-κB activation, cell migration, phagocytosis and the higher levels of miR-155 expression in 2 DIV microglia, as compared with aged cells, are indicative of a major representation of cells with a stressed/reactive phenotype. Indeed, a strong up-regulation of miR-155 expression was shown to have a pro-inflammatory role in microglia (Cardoso et al., 2012) and to drive the M1 phenotype (Ponomarev et al., 2013) corroborating the stressful properties of 2 DIV cultured cells.

Nowadays, changed morphology and increased activity of SA-β-gal of permanently growth arrested cells are considered cellular senescence markers (Sikora et al., 2011). In accordance, 16 DIV microglia displayed a marked increase of SA-β-gal activity when compared to 2 DIV cells. The activity of SA-β-gal was also associated with senescence-unrelated settings, such as contact inhibition and serum starvation (Severino et al., 2000). Nevertheless, as observed by the Iba-1 pictures, our microglia culture did not reach confluence and was not cultured under serum starvation, attesting that the increase of SA-β-gal activity results from a senescent phenotype. Indeed, decreased microglia migration, phagocytic ability, NF-κB activation and increased SA-β-gal, as we here observed, have been indicated as hallmarks of microglial aging and cell senescence (Mosher and Wyss-Coray, 2014).

Several neurodegenerative diseases are characterized by the formation of intracellular protein aggregates in affected brain regions, indicating a failure of protein degradation system (McCray and Taylor, 2008). Autophagy is a stress-induced catabolic process responsible for the degradation of long-lived proteins and damaged organelles (Levine and Klionsky, 2004) that was shown to decline with aging (Bergamini, 2006) and to determine cell and individual lifespan (Juhasz et al., 2007). A study using the senescence accelerated mouse prone eight, a rodent model of aging and senile dementia, showed a reduced autophagic activity by aging with long-lasting autophagosomes and increased LC3 expression (Ma et al., 2011). In accordance, affected neurons with abnormal autophagosomes (Lee, 2009) and impaired autophagy (Komatsu et al., 2006) were seen in neurodegeneration. We showed that 16 DIV microglia display a reduced amount of LC3 punctates suggestive of a decreased

formation of autophagosomes. This finding was further corroborated by the decrease we also observed in the expression of Beclin-1 in the aged cells. Beclin-1 is known to intervene from autophagosome formation to autophagosome/endosome maturation but to also have other additional functions (Kang et al., 2011). Interestingly, Beclin-1 was recently considered to be required for efficient phagocytosis and to be reduced in microglia isolated from AD brains (Lucin et al., 2013), thus accounting to explain the reduced phagocytic ability in our 16 DIV cells and to such impairment in mice with AD-like pathology (Krabbe et al., 2013).

It is worth mentioning that the 2 and 16 DIV microglia differently react to some tested neurotoxins, as we antecipated. We used unconjugated bilirubin that has previously shown to induce the release of the pro-inflammtory cytokines TNF-α and Interleukin (IL)-1β from astrocytes and microglia in concentrations similar to those induced by 10 ng/ml LPS (Fernandes et al., 2004; Gordo et al., 2006; Brites et al., 2009), and Aβ at 50 nM, a concentration that was indicated to trigger microglia activation (Maezawa et al., 2011). The test was first directed to the expression of the high-mobility group box protein-1 (HMGB1) a mediator of inflammation directly correlated with NK-κB protein activation (Rovina et al., 2013). Both stimuli enhanced cellular HMGB1 expression in 2 DIV microglia (80 and 100% increase for bilirubin and Aβ, respectively; results not shown), whithout affecting the 16 DIV cells. Next, and similarly to what we have obtained for HMGB1, up-regulation of mRNA levels of IL-18 expression capable of more potently induce inflammatory response than IL-1β (Alboni et al., 2010) was again associated with the young/reactive microglia treated with bilirubin (60% increase over control) or Aβ (>100% increase over control) (data not shown), but no alterations were noticed in the aged cells.

Overall, we demonstrate that microglia isolated from neonatal mice and kept *in vitro* in long-term cultures switch from an activated/reactive phenotype to cells presenting aging-like alterations. Our results show that *in vitro* aged microglia change their morphology to a more ramified shape, with a reduced basal NF-κB activation, impaired migration and phagocytic abilities, low TLR-2 and TLR-4 expression, as well as reduced MMP-9 and glutamate efflux. This study is the first to provide the inflamma-miRNA signature for microglia aging in primary cultures. The cells evidenced decreased expression of miR-155 and miR-124, reduced autophagic capacity, and increased miR-146a expression and SA-β-gal activity, consistent with the existence of senescent cells at 16 DIV in culture. In conclusion, given the phenotypical changes observed for young/reactive and irresponsive/senescent microglia along the time in culture, the *in vitro* model of microglia aging could be of interest to assess how different signals may diversely modify cell functionality in separate microglia populations and to link increased age with risk for neurodegenerative diseases and other age-related phenomena.

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Microglia from neurogenic and non-neurogenic regions display differential proliferative potential and neuroblast support

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Microglia isolated from the neurogenic subependymal zone (SEZ) and hippocampus (HC) are capable of massive *in vitro* population expansion that is not possible with microglia isolated from non-neurogenic regions. We asked if this regional heterogeneity in microglial proliferative capacity is cell intrinsic, or is conferred by interaction with respective neurogenic or non-neurogenic niches. By combining SEZ and cerebral cortex (CTX) primary tissue dissociates to generate heterospatial cultures, we find that exposure to the SEZ environment does not enhance CTX microglia expansion; however, the CTX environment exerts a suppressive effect on SEZ microglia expansion. Furthermore, addition of purified donor SEZ microglia to either CTX- or SEZ-derived cultures suppresses the expansion of host microglia, while the addition of donor CTX microglia enhances the over-all microglia yield. These data suggest that SEZ and CTX microglia possess intrinsic, spatially restricted characteristics that are independent of their *in vitro* environment, and that they represent unique and functionally distinct populations. Finally, we determined that the repeated supplementation of neurogenic SEZ cultures with expanded SEZ microglia allows for sustained levels of inducible neurogenesis, provided that the ratio of microglia to total cells remains within a fairly narrow range.

Keywords: microglia, inducible neurogenesis, proliferation, functional heterogeneity, subependymal zone

INTRODUCTION

Microglia are the resident immune cells of the CNS, and are located diffusely throughout the parenchyma. Under normal conditions microglia are deemed to be in a state of interactive surveillance but are rapidly activated by trauma, ischemia, infection, neuronal death, neoplasia, and degeneration (reviewed by Streit, 2002). Activation results in increased microglia number as a consequence of both the proliferation of resident cells, and the recruitment of circulating hematopoietic cells that infiltrate the CNS (Ajami et al., 2007; Lambertsen et al., 2011). The primary role of microglia has classically been described as that of sentinels responsible for maintaining brain homeostasis in the face of neurological insult (Schwartz et al., 2006). Activated microglia phagocytose cellular debris, such as degenerating neurons (Thanos, 1991; Huizinga et al., 2012; Suzumura, 2013), and they express complement receptors and act as cytotoxic effector cells (Gehrmann et al., 1995).

In addition to the "reactive" or "defensive" roles for microglia, evidence continues to increase suggesting that microglia also play vital roles in modulating normal neural function. A number of published reports indicate that microglia regulate the migration, proliferation, and differentiation of neural stem/progenitor cells (Battista et al., 2006; Butovsky et al., 2006; Walton et al., 2006). Microglia are also implicated in regulating synaptic density both on regenerating motor neurons following peripheral nerve lesion (Blinzinger and Kreutzberg, 1968; Trapp et al., 2007) and during neural development (Paolicelli et al., 2011). There is also compelling evidence that microglia and associated immune factors modulate neurogenesis both *in vivo* and *in vitro*. For instance, using an environmental enrichment paradigm known to augment adult hippocampal neurogenesis, Ziv et al. (2006) showed microglia activation to be associated with increased neuronal production. These authors also reported that Severe Combined Immunodeficiency (SCID) mice display impaired levels of neurogenesis under normal conditions, and that these levels are not responsive to environmental enrichment. Using an adrenalectomy model, Battista et al. (2006) were able to correlate increased hippocampal neurogenesis with the number of activated microglia in the dentate gyrus. Microglia have also been reported to play a crucial role in a unique form of inducible neurogenesis (IN) specific to mixed glial cultures derived from the subependymal zone. In this paradigm the withdrawal of serum and mitogens triggers massive de novo neuron production. The presence of microglia has been shown to be require for IN, and their loss -due either to serial passaging or selected immuno-ablation- results

in a concomitant decline in the capacity for IN (Walton et al., 2006).

Microglia have also traditionally been viewed as functionally homogeneous throughout the neuraxis. Antecedent *in vitro* experiments typically have not involved the comparison of function by microglia from finely dissected anatomical regions, but rather use gross CNS areas (e.g., "forebrain") to generate microglial cultures (e.g., Giulian and Baker, 1985). Recent data, however, are beginning to reveal additional functional roles for microglia (Walton et al., 2006), and to uncover substantial spatial heterogeneity in microglia function. For example, Goings et al. (2006) demonstrated that microglia in the adult SEZ are constitutively activated (as evidenced by their semi-amoeboid morphology and high density of CD45 and CD11b), in comparison to microglia in other regions. In addition, they showed that the SEZ contains more proliferating microglia than other areas, and that SEZ microglia respond uniquely to a variety of brain insults, theorized to be due in part to their being in a "primed" state of semi-activation. It has been reported that microglia are capable of performing both pro-neurogenic and anti-neurogenic roles in the injured brain, and that these dichotomous functions are temporally regulated. For example, endogenous microglia in the immediate vicinity of neurological insult become activated and begin to phagocytose dead cells while also secreting a variety of inflammatory chemokines such as tumor necrosis factor- α (TNF-α), interferon-γ (IFN-γ) and interleukin-1β, all of which are believed to play a role in the suppression of neurogenesis (reviewed in Ekdahl et al., 2009). However, in the weeks following injury, elevated numbers of insulin-like growth factor-1 (IGF-1) secreting microglia proliferate and accumulate in the SEZ, potentially playing a neuro-supportive role by promoting neuroblast migration to the site of injury. Finally, the secretion of TNF-α and IFN-γ by the insult-adjacent microglia plays a critical but indirect role in the induction of neighboring astrocytes to secrete ciliary neurotrophic factor, which supports re-myelination of neurons as well as neuronal survival (Simard and Rivest, 2004). Finally, we have previously reported that microglia from the SEZ are capable of massively greater population expansion than microglia from non-neurogenic brain regions (Marshall et al., 2008), suggesting a link between microglia proliferation and neurogenesis, and suggesting possible environmental factors within the neurogenic niche that promote or preserve proliferative capacity.

In the present study we ask two major questions. First, is the capacity for massive *in vitro* population expansion an intrinsic property of neurogenic zone microglia, or is it conferred by interactions within the neurogenic niche? Second, can *in vitro* neurogenesis be enhanced by manipulating the number of neurogenic zone microglia?

MATERIALS AND METHODS

ANIMALS

All cultures were derived from neonatal (2–3 days post-birth) C57BL/6 wild type (WT) mice or C57BL/6 mice homozygous for the green fluorescent protein (GFP) reporter gene (Hadjantonakis et al., 1998). Animals were housed at the University of Florida's Department of Animal Care Services in compliance with IACUC regulations.

GENERATION OF REGION-SPECIFIC CULTURES

Primary neural tissue was obtained from the brains of either WT or GFP+ neonatal mice, as schematized in **Figure 1**. Brains were dissected into discrete regions by first removing the olfactory bulbs and cerebellum with a sterile razor blade (blue lines in **Figure 1A**). The brain was then blocked by a coronal cut just anterior to the hippocampal formation (red line in **Figure 1A**) to generate fractions containing the SEZ and cerebral cortex (CTX, Numeral 1, **Figure 1A**), or hippocampus (Numeral 2, **Figure 1A**). The CTX was then separated from the SEZ by removing the tissue dorsal to the corpus callosum and lateral to the lateral ventricles (**Figure 1B**). The SEZ was further isolated by removing and discarding the white matter superior to the lateral ventricles. Finally, the hippocampus was isolated by removing the tissue surrounding the dentate gyri (dashed lines in **Figure 1C**). All tissue blocks were then separately minced with a sterile scalpel, and incubated for 15 min in ice-cold Dulbecco's Modified Eagles Medium (DMEM)/F12 media w/ HEPES and L-Glutamine (Gibco BRL, Carlsbad, CA; 11330-032) supplemented with antibiotic and anti-mycotic agents (Penicillin-Streptomycin, Gibco, 15140-122, and Fungizone Antimycotic, Gibco, 15295-017). The tissue was then pelleted by centrifugation at 400×g for 5 min, and incubated in 0.25% Trypsin/EDTA solution (Atlanta Biologicals, Atlanta, GA; B81310) at 37°C for 5 min. Trypsin activity was quenched by the addition of 1/5 volume fetal bovine serum (FBS: Atlanta Biological), after which a single-cell slurry was prepared by repeated trituration through a series of descending diameter fire-polished glass Pasteur pipettes. The slurry was washed in 5X volume DMEM/F12 and pelleted as above. Cells were re-suspended in neural growth medium (NGM) consisting of DMEM/F12 containing 5% FBS, N2 supplement (Gibco BRL, 17502-048), 20 ng/mL recombinant human epidermal growth factor (EGF; rhEGF, Sigma-Aldrich, St. Louis,

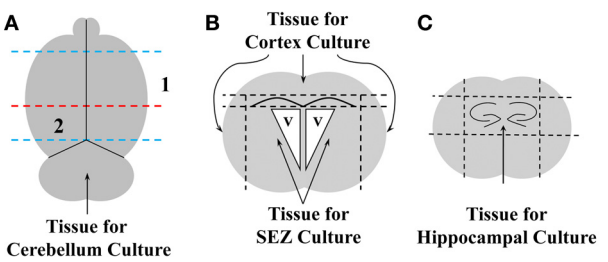

FIGURE 1 | Schematic representation of brain dissections. The dorsal surface of whole brain was viewed **(A)**, and a razor blade was used to remove the olfactory bulbs and the hindbrain (blue dashed lines). The remaining tissue was bisected through the frontal plane (dashed red line), and areas 1 and 2 were further dissected to obtain cerebral cortex (CTX), subependymal zone (SEZ), and dorsal hippocampus (HC). For dissection of CTX and SEZ, area 1 was viewed in the coronal **(B)**, and dorsal and lateral cortical tissue superficial to the subcortical white matter was removed with razor cuts (upper horizontal dashed line & vertical dashed lines). SEZ was then obtained by first removing the subcortical white matter (lower dashed horizontal line in panel B), then finely dissecting the tissue immediately subjacent to the lateral ventricles (v). Finally, HC was obtained by viewing area 2 in the coronal plane **(C)** and blocking the tissue around the two dentate gyri by making razor cuts indicated by the four dashed lines.

MO; E9644), and 10 ng/mL basic fibroblast growth factor (FGF; bFGF, Sigma-Aldrich, F0291), plated onto tissue culture T-25 flasks and incubated for 3 days at 37°C in 5% CO2. Confluent cultures were trypsinized and re-plated at a 1:3 density into T-75 tissue culture flasks in NGM (passage 1). Passage 1 cultures were supplemented every 2–3 days with EGF/FGF until confluency was reached. The passaging procedure was then repeated as described above. At each passage a small aliquot of cells was placed onto poly-ornithine (P-Orn) coated glass coverslips and fixed for immunophenotyping (see below).

FLOW CYTOMETRIC ANALYSIS OF CELL CYCLE AND Mcm2

Confluent passage-5 monolayer cultures were incubated in the presence of the thymidine analog EdU (Click-iT® EdU Alexa Fluor® 488 Flow Cytometry Assay Kit, Invitrogen, Carlsbad, California; Cat. No. C35002) for 18 h. Cells were then trypsinized, collected, fixed and labeled for EdU according to manufacturer's recommendation. Cells were subsequently immunolabeled with anti-CD11b primary antibody directly conjugated to phycoerythrin (BD Pharmingen, San Jose, California; Catalog number 553311), as well as with the kit-supplied cell cycle label (633-red). Controls were prepared by generating each of the following from each culture group: unstained negative control; EdU signal only; cell cycle only; and CD11b-conjugated to phycoerythrin (PE) only. Samples were processed using a BD LSRII flow cytometer (BD Biosciences, San Jose, California) utilizing FACSDiva software. Unstained and IgG stained control cells were used to determine compensation parameters sufficient for diminishing fluorochrome signal overlap and producing clearly defined labeled-populations. Data were analyzed by FlowJo software (Treestar, Ashland, Oregon).

Mini chromosome maintenance protein 2 (Mcm2) in microglia was determined by first trypsinizing, collecting and fixing confluent SEZ and CTX monolayers at passage 3, 4, and 5. Cells were then co-immunolabled with rabbit anti-Mcm2 primary antibody (D7G11, Cell Signaling Technologies, Danvers, MA) as well as the anti-CD11b primary antibody directly conjugated to phycoerythrin used above. Following washing to remove residual primary antibody, secondary staining to label the bound Mcm2 antibody was conducted using FITC-conjugated Goat anti-Rabbit secondary antibody (BD Pharmingen, Catalog Number 554020). Stained cells were washed to remove residual secondary antibody then resuspended in flow cytometry buffer consisting of phosphate-buffered saline (PBS) with 10% fetal bovine serum (FBS) and 1% Sodium Azide). Samples were processed using a BD LSRII flow cytometer (BD Biosciences, San Jose, California) utilizing FACSDiva software using unstained, primary Mcm2 antibody alone and IgG stained control cells to determine compensation parameters sufficient for diminishing fluorochrome signal overlap and producing clearly defined labeled-populations.

EXPANSION AND ISOLATION OF MICROGLIA FROM ADHERENT CULTURES

Microglia were expanded and isolated from adherent cultures as previously reported (Marshall et al., 2008). Briefly, NGM was replaced with microglial proliferation medium (MPM) consisting of DMEM/F12, 10% FBS, N2 supplement, and 20 ng/mL recombinant mouse granulocyte macrophage-colony stimulating factor (rGM-CSF, Stem Cell Technologies Inc; #02735). After 3 days in MPM, a microglia shake-off was performed by agitating the culture flasks for 10 minutes at room temperature (RT) on a rotary shaker set for 100 r.p.m. Detached cells was collected and quantified, while fresh MPM was added to the parent culture allowing for subsequent shake-offs at 3-day intervals. Isolated microglia were subsequently used as "donors" to supplement established mixed glial cultures (described below).

INDUCIBLE NEUROGENESIS IN ADHERENT SEZ CULTURES

Adherent WT SEZ cultures were established on P- Orn coated glass coverslips. Inducible neurogenesis (IN) was initiated upon confluence by serum starvation and mitogen withdrawal (Scheffler et al., 2005). The NGM was removed and replaced with DMEM/F12 media containing N2 supplement, but without serum or growth factors. Forty-eight hours after induction, small, phase-bright neuroblasts are abundant in the adherent cultures. Both induced and non-induced control cultures were fixed with 4% paraformaldehyde at 48 h. post- induction, and processed for immunolabeling as described below.

IMMUNOLABELING

All analyses were performed on confluent cultures, except for isolated microglia that were plated at low density and allowed to attach to coverslips for 24 h. Cells were fixed by immersion in PBS containing 4% paraformaldehyde (15 min @ RT), then washed with PBS for 5 min and blocked at RT for 1 h in PBS plus 0.01% Triton X-100 (PBSt) and 10% fetal bovine serum (FBS). Primary antibodies were applied to the cells overnight with gentle agitation at 4°C in PBSt containing 10% FBS. Primary antibodies: rat monoclonal anti-CD11b/Mac-1 (Becton Dickinson, Franklin Lakes, NJ: 550282, dilution 1:250) to label microglia; and rabbit polyclonal anti-β-III Tubulin (Covance; Princeton, NJ; PRB-435P, dilution 1:5000) to label neurons. Residual primary antibody was removed by 3 × 5 min washes with PBS. Secondary antibodies were applied at RT for 50 min in PBSt plus 10% FBS. Secondary antibodies were either goat anti-rat IgG conjugated to Alexa Fluor 568 (Molecular Probes, Eugene, OR: A11077), or goat anti-rabbit IgG conjugated to rhodamine (Molecular Probes, R-6394). Unbound secondary antibodies were removed by 3 × 5 min washes in PBS. Cells were coverslipped in Vectashield mounting medium containing the nuclear counterstain, DAPI (Burlingame, CA: H-1200).

MICROSCOPY AND QUANTITATIVE ANALYSIS

Cells were analyzed using a Leica DM2500 upright microscope (Leica Microsystems AG, Wetzlar, Germany) equipped with epifluorescence and aMagnafire digital camera (Optronics®, Goleta, California). Quantification was performed by capturing random fields of view at 20× magnification using DAPI filter cube to identify cell nuclei. The same field of view was photographed using first Leica I3 (GFP) filter followed by the TX2 filter (rhodamine). Merged images were then processed using ImageJ to determine the percent contribution of GFP and Rhodamine labeled cells to the total cell number. The values from 5 fields-of-view were combined and averaged to generate an estimate of the total

cellular distribution within each culture. Additonal images for photodocumentation were captured using the Olympus DSU-IX81 (Olympus, Center Valley, PA) spinning disc confocal microscope.

STATISTICAL ANALYSIS

Quantitative values obtained from ImageJ were entered into Excel spreadsheets for graphical presentation, and into Graphpad Prism 4 for statistical analysis using either one- or Two-Way ANOVA with a Bonferroni *post-hoc* test of significance (significance was defined as a p-value ≤ 0.05).

RESULTS

NEUROGENIC ZONE MICROGLIA HAVE MORE PROLIFERATIVE CELL CYCLE KINETICS THAN CTX MICROGLIA

We have previously shown that both SEZ and HC primary cultures of both neonatal and adult dissociates are capable of large-scale microglia while the CTX is capable only of relatively modest microglia production that is limited to neonatal tissue (see Marshall et al., 2008 and Figure S1). We therefore hypothesized that there are differential cell cycle kinetics between microglia from neurogenic zones and microglia from non-neurogenic zones. To test this hypothesis we compared the cell cycle of microglia in cultures derived from the SEZ, HC, and CTX of neonatal mice. Confluent cultures of these regions were incubated with the thymidine analog EdU for 18 h and then processed for flow cytometric cell cycle analysis. The number of CTX microglia in G1 phase is substantially greater than the SEZ- or HC-derived microglia, while the number of SEZ and HC microglia in S phase is greater than CTX-derived microglia, indicating that more microglia within the SEZ and HC cultures are actively undergoing cell division (**Figure 2**). Interestingly, there are no statistically significant differences in the cell cycle profiles between the non-microglia cells (CD11b−/EdU+) among these three brain regions, suggesting that the regional cell cycle heterogeneity of microglia is not due to global differences inherent to the tissue source, but rather is specific only to the microglia population (data not shown). In addition, we assessed immunolabeling differences in mini chromosome maintenance protein 2 (Mcm2), which is a critical component of the pre-replication Mcm complex crucial for the onset of DNA replication and cellular division (Lei and Tye, 2001). Mcm2 is frequently used as a marker for cells capable of -or "primed" for- mitosis (Shetty et al., 2005). We assessed Mcm2 in microglia by co-immunolabeling with antibodies against CD11b and Mcm2. Sequential FACS analysis of the microglial populations at passages 3–5 reveals that, while the percentage microglia co- expressing Mcm2 decreases with each passage, the SEZ contains more Mcm2+ microglia than the passage-matched CTX (Figure S2). This finding, together with the cell cycle results, suggests that microglia from neurogenic regions possess a substantially greater potential for cellular division and population expansion than microglia from non-neurogenic regions.

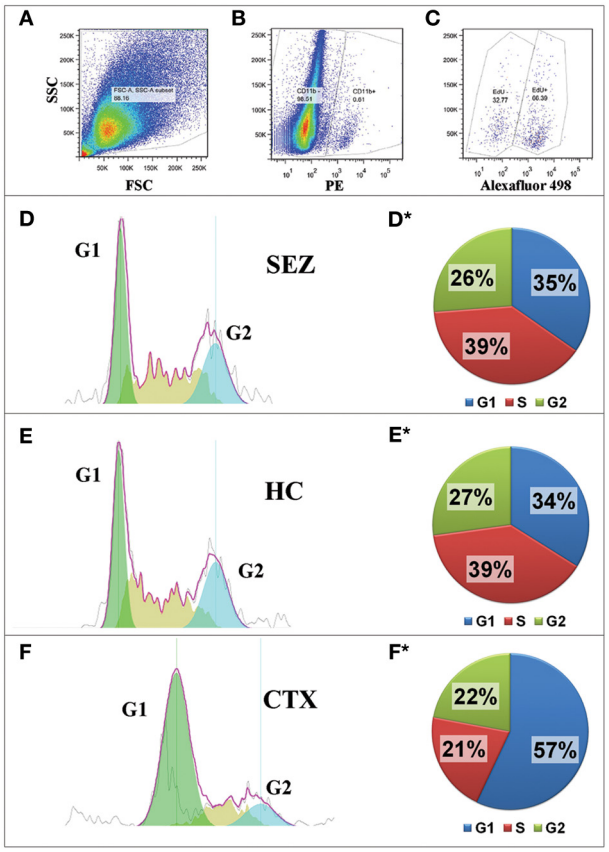

FIGURE 2 | Microglia from neurogenic regions are more proliferative than microglia from non-neurogenic regions. Cultures ($n = 1$) from the neurogenic SEZ and HC, and from the non-neurogenic CTX were exposed to the thymidine analog, EdU, for 18 h. Cells were then fixed and labeled for EdU and the microglial marker, CD11b. In addition, total DNA was labeled with propidium iodide. Analysis by flow cytometry was as follows: debris was excluded from the Forward/Side scatter plot **(A)** before the CD11b+ fraction (microglia) was gated from the CD11b- fraction **(B)**; mitotic microglia were identified (EdU+) from the total microglia population **(C)**; and cell cycle was assessed using FlowJo software to analyze DNA content **(D–F)**. Cell cycle analysis reveals that the SEZ **(D,D*)** and the HC **(E,E*)** have remarkably similar numbers of mitotic microglia (roughly 65% in S/G2), whereas only 43% of the CTX microglia **(F,F*)** are mitotic. Differential proliferation between neurogenic and non-neurogenic regions is not seen with CD11b-negative cells (not shown), suggesting that the elevated proliferation rate is unique to microglia of the brain's neurogenic zone.

SEZ MICROGLIA EXPANSION IS SUPPRESSED BY EXPOSURE TO CTX

The massive expansion capacity of neurogenic zone microglia compared to non-neurogenic zone microglia may be due either to cell-intrinsic properties or to extrinsic cues within the environmental milieu of the NSC niche. We therefore asked whether the expansion capacity of neurogenic zone microglia is altered by exposure to a non-neurogenic environment (i.e., CTX-derived cultures). The experimental paradigm we used in this study is schematized in Figure S3. We generated mixed, heterotypic cultures by combining age-matched dissociated primary slurries from WT SEZ and GFP+ CTX at a 1:1 ratio (Figure S3 and Methods: Generation and Analysis of Heterospatial Adherent Cultures). Microglia expansion within these heterotypic cultures was compared to the expansion within un-mixed sister cultures of SEZ alone and CTX alone over the course of 4 passages. At Passage

1 the overall number of microglia in the heterotypic cultures was calculated in order to determine the relative starting contribution of microglia from the SEZ (GFP−) and CTX (GFP+). Twenty-four hours after plating, microglia composed 4% of the total adherent cell population, with roughly half of these being derived from the SEZ and half from the CTX (Figure S4).

In order to determine the effect of SEZ-CTX interactions on region-specific microglia yield independent of the total yield in mixed cultures, we assessed the percentage of GFP+ and GFP- microglia after isolation (shake-off) to determine the relative contributions from CTX and SEZ. We and others have previously shown that CD11b is a reliable marker for labeling all microglia in culture (Sedgwick et al., 1991; Marshall et al., 2008), and we used anti-CD11b antibody to label and identify the isolated microglia (Figure S5). These assays were performed in order to analyze the number and source of microglial populations over time as compared to the microglia present in the initial cultures. As expected, we found that unmixed SEZ cultures produce significantly more microglia than unmixed CTX cultures (**Figure 3**, compare WT SEZ to GFP CTX at isolations 1 and 2, $p \leq 0.05$). In addition, at the first isolation SEZ microglia from mixed cultures (SEZ normalized) match the output of SEZ microglia from unmixed SEZ cultures (**Figure 3**, compare WT SEZ to SEZ normalized at isolation 1). However, with increased time *in vitro* mixed cultures produce significantly fewer SEZ microglia than the unmixed SEZ culture (**Figure 3**, compare WT SEZ to SEZ normalized at isolations 2 and 3, $p \leq 0.05$), suggesting that exposure to the CTX environment reduces the expansion rate or capacity of SEZ microglia. In contrast, the expansion of CTX microglia in mixed cultures (CTX normalized) did not substantially differ from the CTX microglia output in unmixed cultures (GFP CTX) at any of the three passages.

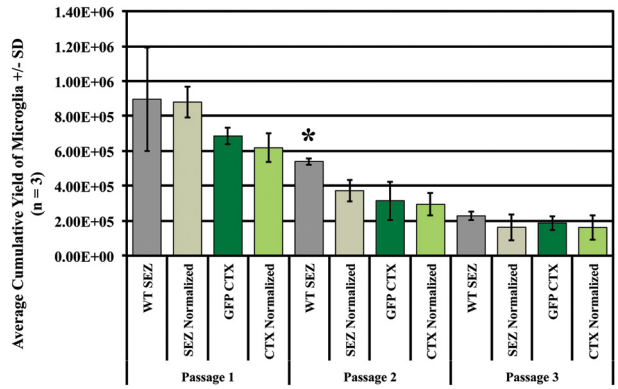

FIGURE 3 | Exposure to CTX cells *in vitro* suppresses SEZ microglia proliferation. Primary cultures were established from 2×10^6 SEZ cells (wild-type), 2×10^6 CTX cells (GFP+), or from a mixture of 1×10^6 SEZ + 1×10^6 CTX cells (see schema in Figure S3). In order to determine if exposure to the SEZ "environment" increases proliferation of CTX microglia, isolation via mitotic shake-off was performed three times. The number and source of microglia were determined, and SEZ and CTX microglia isolated from mixed cultures were normalized by doubling to allow direct comparison to their unmixed controls. The total yield of microglia decreases with each isolation regardless of tissue source, but SEZ yield (dark gray bars) is consistently greater than CTX yield (dark green bars) over time. Exposure to SEZ cells does not significantly alter the yield of CTX microglia at any isolation (compare dark green bars to light green bars). Values for "GFP CTX" and "CTX normalized" are not statistically different; if exposure to SEZ environment enhances CTX microglia yield, then "CTX normalized" would be higher than "GFP CTX." In contrast, exposure of SEZ microglia to the CTX environment seems to suppress SEZ yield (compare dark gray bars to light gray bars). In this case, "SEZ normalized" is lower than "WT SEZ" yield at passage 2 and 3, although only the difference at passage 2 reaches statistical significance. Yields were compared only within region and passage. One Way ANOVA. Asterisk indicates $p < 0.05$. $N = 3$ for all experiments.

DONOR SEZ MICROGLIA SUPPRESS HOST MICROGLIA EXPANSION IN HOMO- AND HETERO-SPATIAL CULTURES

The previous experiment with directly mixed primary dissociates indicates that the normal high rate of SEZ microglia expansion is attenuated by exposure to the CTX environment, while expansion of CTX microglia is unaffected by exposure to the SEZ environment. We hypothesized that the relative ratio of SEZ to CTX microglia may be an important variable for altering the proliferative behavior of CTX microglia, and therefore asked if the introduction of purified, expanded microglia to CTX cultures can improve CTX microglia expansion. By exploiting the *in vitro* expansion capacity of SEZ microglia this approach allows us to increase the ratio of donor-to-host microglia far beyond what can be achieved by mixing primary dissociates from different regions. As schematized in Figure S6 (also see Methods: Surface Supplementation of Established Adherent Cultures with Homo- and Hetero-typic Isolated Microglia) we seeded 1×10^6 passage 1 WT SEZ "donor" microglia onto confluent "host" GFP+ cultures derived from either the SEZ (homo-spatial) or CTX (hetero-spatial). Likewise, 1×10^6 passage-1 WT CTX donor microglia cells were seeded onto confluent GFP+ CTX (homo-spatial) and SEZ (hetero-spatial) host cultures. All cultures were maintained in standard NGM for 1 week, then transferred to MPM for 3 days. Microglia were then isolated, quantified and analyzed for the relative proportion of GFP+ microglia, as described above. We found that CTX cultures supplemented with either homo- or hetero-spatial microglia produce nearly identical total numbers of microglia (**Figure 4A**). However, within this total microglia population the proportion of donor-to-host derived microglia is significantly different. While homo-spatial CTX cultures produce nearly equivalent numbers of donor and host microglia, within heterotypic cultures 66% of the microglia are SEZ donor-derived (**Figure 4B**; $p < 0.05$). Likewise, SEZ cultures supplemented with either homo- or hetero-spatial microglia produce similar total numbers of microglia (**Figure 4C**). But here again, a significant difference is observed when the donor source is considered. Within heterotypic SEZ cultures the microglia population consists of nearly equal proportions of donor and host microglia. In contrast, 63% of the microglia in the homotypic cultures are derived from the donor SEZ microglia (**Figure 4D**; $p < 0.05$). To summarize, the presence of SEZ microglia does not enhance the capacity for CTX microglial expansion, indicating that the limited expansive potential of CTX-derived microglia cannot be enhanced by the proximity of SEZ-derived microglia or the factors they may secrete. In fact, SEZ-derived donor microglia inhibit the expansion of CTX and SEZ endogenous microglia populations.

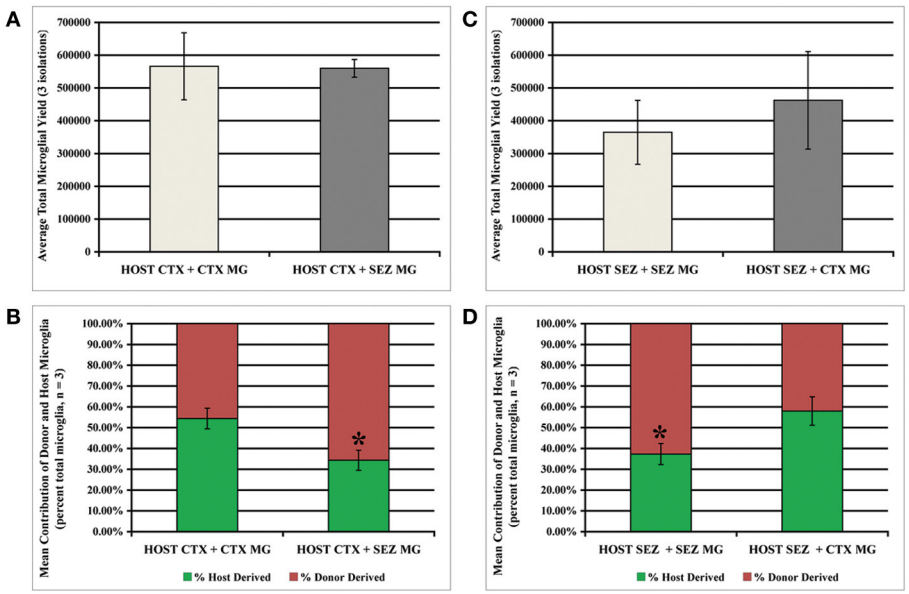

FIGURE 4 | Donor SEZ microglia suppress expansion of CTX and SEZ host microglia. Established CTX or SEZ cultures (4×10^6 GFP+ cells) were supplemented with 4×10^6 wild-type "donor" microglia isolated from SEZ or CTX cultures (see schema in Figure S6) in order to assess the effect of large numbers of homo- and hetero-typic microglia on the expansion of the host microglia population. Beginning 1 week after supplementation three microglia isolations were performed at 3-day intervals. The tissue source of donor microglia did not significantly affect total microglia yield from either CTX host **(A)** or SEZ host **(C)**. The relative contribution of microglia from donor and host was not affected by supplementation with CTX microglia, with statistically equal numbers of isolated wild-type and GFP+ microglia (**B**, left bar & **D**, right bar). However, supplementation with SEZ microglia reduced the donor microglia output from both CTX (asterisk in **B**) and SEZ (asterisk in **D**). $p < 0.05$ for both **(B)** and **(D)**, One Way ANOVA with Bonferroni's Multiple Comparison Test, ($n = 3$).

REPEATED SEZ MICROGLIA SUPPLEMENTATION PROMOTES NEUROBLAST SURVIVAL

Primary SEZ dissociates from heterogeneous adherent cell mixtures *in vitro* consist predominantly of astrocytes and microglia, but also contain a small population of neuroblasts (small β-III tubulin+ cells with rounded somas and short processes) that disappear upon repeated passaging (Scheffler et al., 2005). This loss of neurons is concomitant with declining numbers of microglia (unpublished observation), suggesting a role for microglia in maintaining neuronal survival in culture. Since these neuroblasts are unique to the SEZ we hypothesized that supplementing long-term, multi-passage SEZ cultures with microglia isolated from early-passage SEZ cultures would ameliorate the passage-related loss of neuroblasts, and that this protection would not be conferred by supplementation with early-passage CTX microglia. The experimental paradigm is schematized in Figure S7 (also see Methods: Microglia Supplementation of SEZ Cultures). SEZ cultures were generated from WT neonatal mice and grown to confluence. At the first passage, microglia isolated from either the SEZ or CTX of age-matched GFP mice were introduced to the suspended SEZ culture at 10% of the total cells prior to plating. This percentage was chosen since we have determined that the average percentage of microglia present in primary SEZ dissociates is ∼7–10% (data not shown). Cultures were supplemented with microglia and neurons were quantified at each of the first three passages. Additionally, test cultures were assessed at each passage for overall microglia yield 24 h after plating.

As expected, supplementation with either SEZ-derived or CTX-derived microglia results in significantly more total microglia at each of three passages, although no significant differences are observed between supplemented groups (**Figure 5A**). Repeated introduction of expanded SEZ-derived microglia into sequentially passaged SEZ cultures preserves neuroblast levels; by passage 3 there are significantly more neuroblasts as compared to both unsupplemented controls and to CTX- supplemented SEZ cultures, suggesting that SEZ microglia are uniquely capable of providing neurotrophic support to immature neurons (**Figure 5B**).

REPEATED SEZ MICROGLIA SUPPLEMENTATION PRESERVES INDUCIBLE NEUROGENESIS

Inducible neurogenesis (IN) from adherent SEZ cultures is a unique phenomenon of neurogenic astrocytes, whereby the withdrawal of mitogens and serum results in the de novo production of neuroblasts (Scheffler et al., 2005, and Figure S8). The capacity for IN may be linked to the presence of microglia, since the robustness of IN decreases concomitantly with diminishing microglia numbers over multiple passages and is eventually lost in high passage cultures that invariably contain fewer microglia. Additionally, there is evidence that IN can be restored by conditioned medium derived from microglia-rich, early-passage SEZ cultures (Walton et al., 2006). Here we asked if repeated supplementation of SEZ cultures with homotypic SEZ microglia can reduce or prevent the normal loss of IN that occurs during

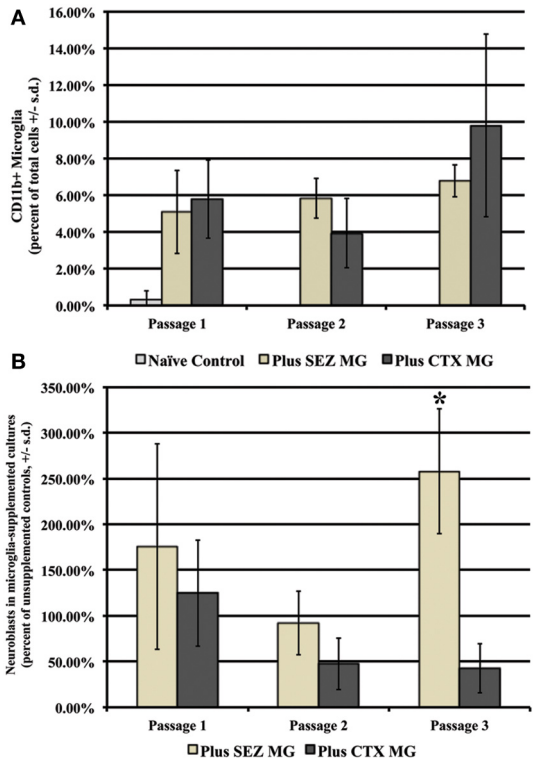

FIGURE 5 | Supplementation with SEZ but not CTX microglia increases neuroblast yield within SEZ cultures. First passage microglia isolated from either SEZ or CTX were added to established SEZ cultures at passages 1, 2, & 3. Both groups contained significantly more total microglia than unsupplemented control cultures at each passage, but contained similar levels of microglia as compared to each other **(A)**. While no significant difference in the number of neurons was observed in supplemented cultures at early passages, by passage 3 cultures supplemented with SEZ derived microglia contained significantly more neuroblasts than both unsupplemented cultures and cultures supplemented with CTX-derived microglia **(B)** (One Way ANOVA, $*p \leq 0.05$, $n = 3$).

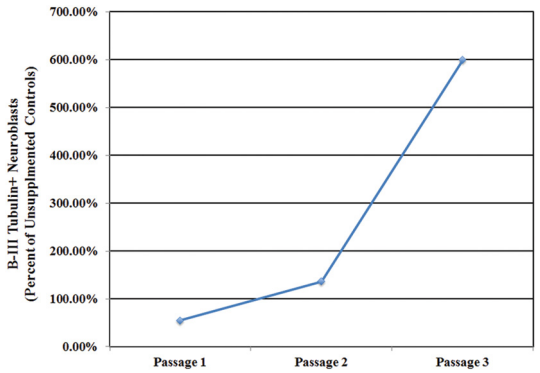

FIGURE 6 | Microglia supplementation preserves inducible neurogenesis. The repeated introduction of GFP+ SEZ-derived microglia into WT SEZ cultures prevents the passage-related decline in IN evident at passage 3 in control cultures, with neuroblast levels noticeably higher in supplemented cultures ($n = 1$ for control and treated cultures at each of three passages).

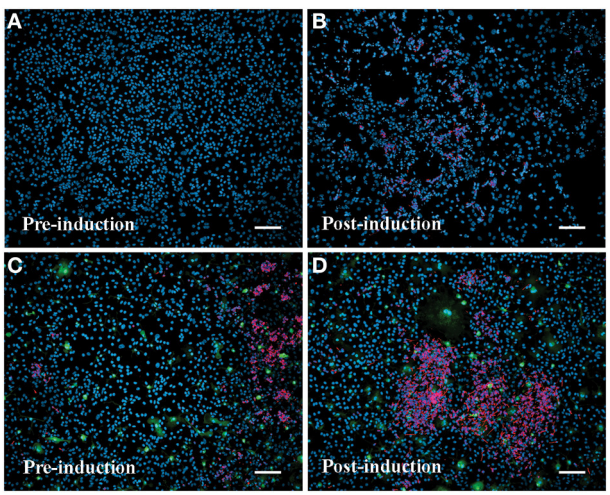

FIGURE 7 | Supplementation with SEZ microglia enhances inducible neurogenesis in SEZ cultures. Passage 3 control SEZ cultures in standard medium contain few or no neurons **(A)**. Following induction (withdrawal of serum and mitogens) control SEZ cultures show a small increase in neurogenesis **(B)**. In contrast, passage 3 SEZ cultures supplemented with SEZ microglia contain numerous neurons prior to induction **(C)**, and this level is dramatically increased after the induction protocol **(D)**. Scale bars = 50 μm; blue = DAPI, red = β-III tubulin+ neurons, green = GFP+ microglia.

sequential passaging of SEZ cultures. The experimental paradigm is schematized in Figure S9 (also see Methods: Supplementation of Neurogenic Cultures with Homotypic Microglia). Host cultures were derived from WT SEZ, as described above. At each passage purified microglia, isolated from age- and passage-matched GFP+ SEZ cultures, were again added at 10% of the total cell population. At each passage supplemented cultures were assessed for IN potential. Unsupplemented, passage-matched sister cultures served as controls. Sequential supplementation of SEZ cultures with isolated SEZ microglia leads to progressively increasing levels of IN as compared to unsupplemented sister cultures. Graphical representation of neuroblast production (**Figure 6**) shows that IN at passage 1 is lower than, but not statistically different from, the unsupplemented control. By passage 2 the supplemented group shows slightly higher IN than control, but again this difference is not statistically different. By passage 3, however, IN within the supplemented group is substantially and statistically higher than control. Photodocumentation of passage 3 IN clearly shows the robust difference in neuroblast production between unsupplemented and supplemented cultures (**Figure 7**; compare **A,B** to **C,D**). Interestingly, pre-induction supplemented cultures contain substantially more neuroblasts than pre- induction controls (compare **Figure 7C** to **Figure 7A**), presumably due to the increased neurotrophic effect of SEZ microglia as described in the previous experiment.

We also assessed the relative numbers of microglia present in both supplemented and unsupplemented controls at each passage. At Passage 1, microglia represent no more than 3% of all cells in the unsupplemented culture, but nearly 25% of all cells in the supplemented cultures (**Figure 8**, gray bars and left-side ordinate). Of this 25%, roughly 90% of are donor-derived (green

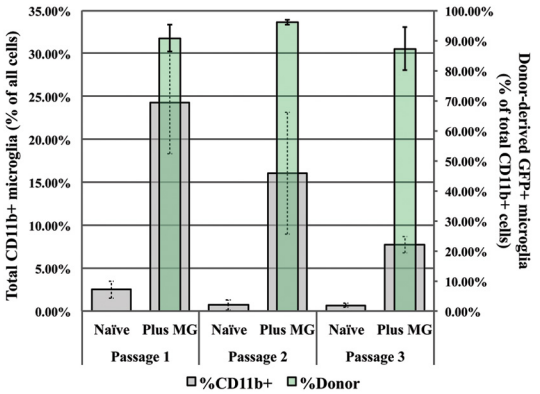

FIGURE 8 | Analysis of microglia populations in supplemented and naïve control cultures. Naïve cultures contain no more than 3% microglia, while nearly 25% of the cells in passage 1 supplemented cultures are microglia (gray bars, left y-axis). By passage 3, at the same time in which inducible neurogenesis is significantly restored, supplemented cultures are comprised of only 7% microglia. Yet at all passages, donor microglia in supplemented cultures comprised ∼90% of all microglia present (green bars, right y-axis, $n = 3$).

bars and right-side ordinate), indicating substantial proliferation by the donor microglia. At each subsequent passage the total microglia population is diminished in both supplemented and unsupplemented cultures, while the relative contribution by the donor microglia remains constant (∼90%). At passage 3 the total number of microglia present in the supplemented cultures is 8% compared to less than 1% in naïve cultures (**Figure 8**), correlating with the restored neurogenic potential seen in **Figure 7**. This finding supports the hypothesis that SEZ-derived microglia can preserve IN, and further suggests that the ratio of microglia to total cells may be important for determining the neurogenic potential of SEZ cultures.

DISCUSSION

Microglia have traditionally been thought of as functionally homogenous across brain regions. That is, while there may be some functional differences between infiltrating perivascular microglia and endogenous parenchymal microglia (Hickey and Kimura, 1988), the functions of microglia do not change substantially as one considers the different anatomical structures of the neuraxis. However, recent reports have begun to challenge this view, particularly with respect to unique functions of microglia in the neurogenic zones of the mammalian brain. Goings et al. (2006) showed that SEZ microglia and non-neurogenic zone microglia *in vivo* differ with respect to basal levels of proliferation as well as constitutive and injury-induced markers of activation. Additionally, our group has reported that SEZ microglia are capable of up to 20-fold greater *in vitro* expansion than non-neurogenic zone microglia (Marshall et al., 2008). In the present study we further examined the regionally specified functional variation between microglia from neurogenic and non-neurogenic regions of the mouse brain. Specifically, we assessed differential cell cycle kinetics and support of neurogenesis by these two broad categories of microglia.

MICROGLIA DISPLAY REGIONAL VARIATION IN CELL CYCLE KINETICS

Previous studies have suggested that microglia within the SEZ are more proliferative than microglia in other brain regions based upon the uptake of BrdU *in vivo* (Goings et al., 2006) as well as their capacity for large scale expansion *in vitro* (Marshall et al., 2008). However, the cell cycle kinetics of regionally discrete populations of microglia has not been reported. Our flow cytometry results are consistent with these antecedent studies in showing that a substantially higher percentage of microglia from the neurogenic SEZ and hippocampus are actively dividing compared to CTX microglia. We additionally assessed the protein density of the Mcm2 in SEZ vs. CTX microglia. The minichromosome maintenance protein family regulates the initiation of chromosomal replication in eukaryotic cells, and they can be expected to be expressed in cells that are competent to divide (Todorov et al., 1991; Kelly and Brown, 2000). As expected from the flow cytometric data, SEZ microglia express higher levels of Mcm2 than CTX microglia over multiple passages, confirming fundamental, spatially restricted differences in proliferative activity between neurogenic region and non-neurogenic region microglia. Because of morphological and immunophenotypic overlap, our studies do not distinguish between microglia and circulating macrophages. However, our results are consistent with antecedent *in vivo* work with parenchymal microglia. In addition, regional differences functions still apply, and still indicate functional differences between neurogenic and non-neurogenic brain regions. Further, increased neurogenic region yield cannot be accounted for by the potential presence of greater numbers of macrophages in neurogenic regions, since all of our quantification was normalized when we calculated fold expansion.

PROLIFERATIVE CHARACTERISTICS OF MICROGLIA ARE INFLUENCED BY ENVIRONMENTAL CUES

The finding that neurogenic zone and non-neurogenic zone microglia differ with respect to proliferative activity raises the question of whether this difference is intrinsic to the separate populations of microglia, or whether the difference is due to the specific microenvironment that the microglia are exposed to. Specifically, is there something about the neurogenic niche of the brain that promotes microglial proliferation, or maintains microglia in a competent state to divide? The idea that neurogenic regions of the brain might be uniquely supportive of proliferation was suggested by experiments showing the existence of a unique vascular niche within the hippocampus that seems to be the proliferative focus of neurons, glia, and endothelial cells (Palmer et al., 2000). Conversely, it may be that the microenvironment of non-neurogenic regions suppresses or fails to support the intrinsic proliferative capacity of microglia residing there. Thus, we performed two types of co-culture experiments to determine whether exposure to the SEZ culture microenvironment improves the proliferation dynamics of CTX microglia and—vice versa—whether exposure of SEZ microglia to the CTX environment reduces proliferation dynamics. Obviously, the *in vitro* microenvironment is not an exact replica of the complex *in vivo* environment; nevertheless, we hypothesized that these two approaches would provide a reasonable simulacrum sufficient to reveal gross effects on proliferation. Indeed, both studies show

clear and consistent environmental effects, arguing for the validity of this approach. Specifically, in both experiments SEZ microglia proliferation is suppressed by exposure to the CTX environment, either by direct mixing of primary dissociates (**Figure 3**) or by supplementation of SEZ cultures with purified CTX microglia (**Figure 4**). Conversely, in neither instance is the proliferation of CTX microglia altered by exposure to the SEZ environment. These data can be interpreted to indicate that SEZ and CTX microglia do possess intrinsically different programs of proliferation given a permissive environment, and that some aspect of the CTX environment is non-permissive for the SEZ program. Alternatively, it may be that differential proliferation between SEZ and CTX microglia results from regional environmental cues, and that some aspect of our culture paradigm allowed the CTX environment to alter SEZ microglia but not for the SEZ environment to alter CTX microglia. This may be due to a greater sensitivity of SEZ microglia to CTX cues, or perhaps to the necessity for a longer exposure time for CTX microglia to respond to SEZ cues.

From these data we conclude that our co-culture paradigms preserve at least some relevant *in vivo* environmental cues, and that these cues are functionally sufficient to alter microglia proliferation dynamics. Surprisingly, the microglia supplementation approach (**Figure 4**) showed that the proliferation rate of SEZ "host" microglia are suppressed by addition of both CTX and SEZ "donor" microglia. This results seems counterintuitive both because it is not expected that SEZ should inhibit SEZ, and because CTX "host" microglia are not suppressed by the addition of "donor" SEZ microglia. It may be, however, that SEZ microglia are uniquely sensitive to the relative ratio of microglia within a region. This interpretation is supported by our data showing that *in vitro* microglia-mediated support of neuroblasts is maximal within a narrow proportion of microglia to total cells (discussed below). A similar negative regulation may operate in the supplementation study, whereby "host" SEZ microglia reduce proliferation in an attempt to maintain a particular ratio within the culture environment.

SEZ MICROGLIA BUT NOT CTX MICROGLIA SUPPORT *IN VITRO* NEUROGENESIS

There is compelling evidence that microglia and associated immune factors influence neurogenesis both *in vivo* and *in vitro*. Beck et al. (2005) reported that hippocampal neurogenesis in transgenic mice lacking the cytokine IL-2 was significantly increased in comparison to littermate controls (although this effect was seen only in males). The authors proposed that this increase resulted from dysregulation of the mouse's neuroimmunological status acting on neuronal progenitor cells of the dentate gyrus. Another study, using an environmental enrichment paradigm known to augment adult hippocampal neurogenesis, showed that microglia activation was associated with increased neuronal production (Ziv et al., 2006). These authors also reported that immune-deficient SCID mice display impaired levels of neurogenesis under normal conditions, and that these levels are not responsive to environmental enrichment. Using an adrenalectomy model, Battista et al. (2006) were able to correlate increased hippocampal neurogenesis with the number of activated microglia in the dentate gyrus. Interestingly, they also found that this increased neurogenesis was likely due, at least in part, to the effects of TGFβ—an anti-inflammatory cytokine secreted by microglia. It has been reported that neurogenesis can either be blocked or enhanced by the action of microglia depending upon how they are activated (Butovsky et al., 2006). Although initial studies in rodents suggested that neuroinflammation (microglial activation) was detrimental to neurogenesis (Ekdahl et al., 2003; Kempermann and Neumann, 2003), more recently the tide of opinion has shifted and the prevailing notion now appears to be that the role of neuroinflammation is "much more complex" (Ekdahl et al., 2009). A number of publications suggest a beneficial role for microglia in the regulation of the migration, proliferation, and differentiation of neural stem/progenitor cells (Battista et al., 2006; Butovsky et al., 2006; Walton et al., 2006). More recently, Vukovic et al. (2012) have reported that exercise induced neural precursor activation in the hippocampus is mediated through the CX3CL1-CX3CR1 pathway. This signaling axis is reported to be critical for modifying the phenotype of microglia residing in the hippocampus toward a phenotype that increases neural progenitor cell activity. As CXCL1 is detectable on neurons of the dentate gyrus and exercise elevates protein density, the authors speculate that this may be the means by which regions of neurogenesis can direct neighboring microglia (which express CX3CR1 on their cell surface) to support neurogenesis, and may help explain why microglia from different regions of the brain perform different roles. One reason for the initially negative view on the neuroinflammation/neurogenesis nexus is likely to be found in the use of intraparenchymal or systemic administration of bacterial lipopolysaccharide (LPS) to induce widespread and non-specific inflammation, ignoring potentially direct toxic effects of LPS on neural cells (Monje et al., 2003; Lee et al., 2005). The sudden administration of LPS to experimental animals induces a condition similar to acute septic shock and it therefore does not mimic physiologically relevant neuroinflammation (Cohen, 2002).

In our present study we examined microglia support for neuroblast production in two *in vitro* models. Because both neuroblasts and microglia disappear from primary SEZ cultures upon repeated passaging, we first tested the effect of repeated microglia supplementation on the survival of SEZ neuroblast. We hypothesized that by adding microglia at each passage (at 7–10% of the total cell population as is seen in primary dissociations) we would enable neuroblasts to persist in culture longer and at greater numbers. Our data, represented in **Figure 5**, show that supplementation with SEZ microglia does result in increased numbers of neuroblasts over three sequential passages. In contrast, supplementation with equal numbers of CTX microglia results in fewer neuroblasts as compared to control, though these differences are not statistically significant.

We next performed a second assay designed to assess the effect of repeated SEZ microglia supplementation on inducible neurogenesis from SEZ cultures. Here, too, SEZ microglia supplementation leads gradually to an increase in inducible neurogenesis such that, by the third passage, there is a 6-fold increase in induced neuroblast formation. It is important to note that purity analysis shows that we are not introducing additional neuroblasts when we supplement with isolated microglia (Marshall et al., 2008

and Figure S3). Interestingly, this study also revealed that the relative proportion of microglia within the culture is important for determining functional effects, with inducible neurogenesis maximized when microglia represent between 5 and 10% of the total cell population. This ratio—remarkably similar to that seen in primary dissociates—may represent an optimal range for microglial regulation of neurogenesis.

Our analyses cannot discern the role that microgla proliferation, *per se*, plays in neuroblast support. That is, is proliferation itself required for this support, or is it high proliferation rate correlated with other aspects of microglia biology that support neuroblasts? Because we are studying co-cultures, we are currently unable to selectively block microglial proliferation without also perturbing neuroblast precursors contained within the culture.

CONCLUSION

From our results we conclude that microglia from neurogenic regions have a vastly greater proliferative cell cycle profile than microglia from non-neurogenic regions; furthermore, the higher proliferative capacity of neurogenic microglia can be modulated by the surrounding cellular environment, suggesting that it results at least partially from cell-extrinsic factors. We also conclude that neurogenic zone microglia are uniquely capable of supporting neuroblasts *in vitro*, although this support is not linear but rather depends upon the relative density of microglia within the cellular milieu.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fncel.2014.00180/abstract

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GENERAL COMMENTARY
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Activation of microglia bolsters synapse formation

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A commentary on

Brain inflammation induces post-synaptic changes during early synapse formation in adult-born hippocampal neurons
by Chugh, D., Nilsson, P., Afjei, S. A., Bakochi, A., and Ekdahl, C. T. (2013). Exp. Neurol. 250, 176–188. doi: 10.1016/j.expneurol.2013.09.005

Microglial cells in the central nervous system (CNS) are major players of innate immunity, the first line of defense in the presence of danger signals, from bacterial infections to mediators released by neurons upon cytotoxic insult (Kettenmann et al., 2011). Particular attention has been paid to the involvement of microglia in pathological conditions and their eventual dual role, as disease amplifiers and/or executors of brain repair, is still a matter of controversy (Benarroch, 2013); the fact that inflammation is itself a strategy to contain biological threats, which inevitably leads to variable degrees of damage, may explain the apparent duality of roles attributed to microglia. These microglial functions are mainly dependent on the release of a plethora of mediators (from trophic factors to anti- and pro-inflammatory cytokines or chemokines) and are accompanied by characteristic changes of morphology, ranging from highly ramified to amoeboid shapes (Kettenmann et al., 2011).

Although the role of microglia has been mostly approached from the angle of pathology, microglial cells are also active players in healthy conditions by constantly monitoring the brain parenchyma (Davalos et al., 2005; Nimmerjahn et al., 2005) and correcting deviations from homeostasis; accordingly, several studies reported that the genetic alteration of molecules selectively blunting specific microglial functions impacted on synaptic transmission and synaptic plasticity (Pascual et al., 2012; Ji et al., 2013; Zhang et al., 2014), leading to deficits of behavioral response, such as social interaction, motor learning, or short-term memory (Rogers et al., 2011; Parkhurst et al., 2013; Zhan et al., 2014). These functional effects of microglia on synaptic plasticity and the direct observation of physical apposition of microglia processes with synapses led to the emergence of the concept that microglia could directly interact with synapses (Wake et al., 2009; Tremblay et al., 2010), forming the so called quad-partite synapses (Schafer et al., 2013). This was re-enforced by the observed ability of microglia to engulf and remove synapses, a process named synaptic pruning, which is crucial both for adequate synaptic wiring during neurodevelopment, as well as during synaptic re-wiring following brain injury (Wake et al., 2009; Schafer et al., 2012; Bialas and Stevens, 2013; reviewed in Kettenmann et al., 2013).

The recent report of Chugh et al. (2013) provides evidence supporting a more complex role for microglia-associated neuroinflammation in the process of synaptic wiring. In fact, this report shows that the creation of an inflammatory environment in the hippocampus of adult mice triggers a region-selective increase in the number of thin dentritic spines endowed with PSD-95, indicative of enhanced synaptic connectivity of newborn neurons, without overt changes in neuronal or astrocytic morphology. This is in striking agreement with other reports showing that the functional impairment of specific microglia functions or the ablation of microglia triggered an increase of excitatory synaptic transmission (Pascual et al., 2012; Ji et al., 2013).

However, it remained to be shown if this ability of microglia to bolster synapse formation resulted from a direct signaling of microglia onto maturating synapses and there is still no information about the putative ability of microglia to control the axonal sprouting. This led us to design an experimental protocol to study the direct interaction between microglia and immature axon/pre-synaptic terminals. This was achieved using microfluidic chambers where hippocampal neurons are plated in one compartment and, as the neurons develop, the axons grow through the microgrooves to reach a second physically isolated compartment (Taylor et al., 2005; Neto et al., 2014). The growing axons, but not the cell bodies, were exposed to the N9 microglia cell line, which was previously *primed* with lipopolysaccharide (LPS), a "classic" microglia activator. When we measured the density of nerve terminals by immunofluorescence against

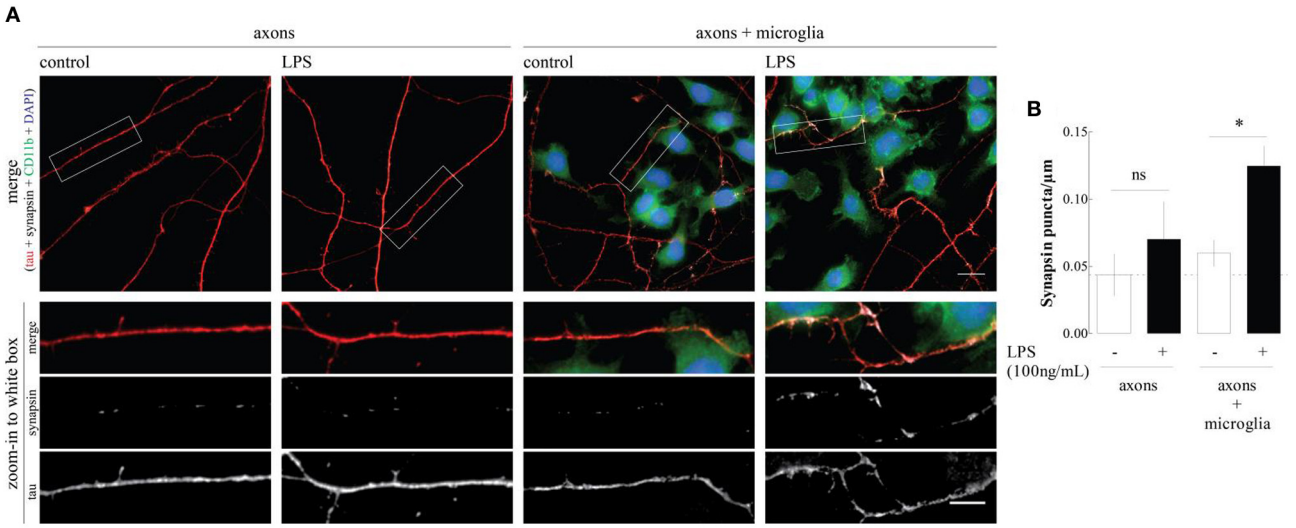

FIGURE 1 | LPS-*primed* microglial cells increase synapsin puncta along axons. (A) Representative images of the axonal/microglia compartment of microfluidic chambers in which rat embryo hippocampal neurons were allowed to grow for 7 days. When indicated, before axons arrival to the axonal compartment a microglia cell line was cultured (DIV3) and activated with LPS for 6 h (DIV4). Cultures were stained for CD11b (microglia marker, green), DAPI (nuclei, blue), tau (axonal marker, red), and synapsin I (synaptic vesicles marker, white). Scale bars are 10 and 5 μm for upper and lower zoom-in images, respectively. **(B)** Quantification of the number of synapsin puncta per axonal length. Results, obtained analyzing 20 images per condition, are expressed as mean ± s.e.m. of three independent experiments, (*$p < 0.5$, by Two-Way ANOVA followed by Bonferroni *post-hoc* test).

the pre-synaptic marker synapsin I, we found that activated microglia caused a clear-cut increase in the axonal density of the protein, which was not observed in the presence on non-*primed* microglia or in isolated axons (**Figure 1**). Recently, Parkhurst et al. (2013) demonstrated that microglia regulates synapse formation through brain-derived neurotrophic factor (BDNF), which is in line with our previous work (Gomes et al., 2013), showing the ability of LPS to increase BDNF release by N9 microglial cells. The increase in the number of synapsin clusters is, however, more striking in axons which are in contact with microglial cells, suggesting that a cell adhesion molecule might also mediate this effect (inset, **Figure 1**). Further studies using conditioned medium from LPS-*primed* microglial cells may help clarify the relative contribution of diffusible mediators (such as BDNF) and axon-microglia physical contact in the ability of *primed* microglia to bolster synapse formation.

Overall, these finding show that microglia are more than synaptic strippers, but are actually important controllers of synapse formation. This novel role of microglia on pre-synaptic differentiation may be of particular importance to better understand neurodevelopment disorders characterized by aberrant synapse formation associated with maternal infections during pregnancy.

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