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Targeting innate immunity for neurodegenerative disorders of the central nervous system

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Conflict of interest disclosures

The authors have no conflict of interests to declare. All experiments were conducted with the ARRIVE guidelines.

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Abstract

Neuroinflammation is critically involved in numerous neurodegenerative diseases, and key signaling steps of innate immune activation hence represent promising therapeutic targets. This mini review series originated from the 4th Venusberg Meeting on Neuroinflammation held in Bonn, Germany, 7-9th May 2015, presenting updates on innate immunity in acute brain injury and chronic neurodegenerative disorders, such as traumatic brain injury and Alzheimer's disease, on the role of astrocytes and microglia, as well as technical developments that may help elucidate neuroinflammatory mechanisms and establish clinical relevance. In this meeting report, a brief overview of physiological and pathological microglia morphology is followed by a synopsis on PGE2 receptors, insights into the role of arginine metabolism and further relevant aspects of neuroinflammation in various clinical settings, concluded by a presentation of technical challenges and solutions when working with microglia and astrocyte cultures. Microglial ontogeny and induced pluripotent stem cell derived microglia, advances of TREM2 signaling, and the cytokine paradox in Alzheimer's disease are further contributions to this series.

Graphical abstract

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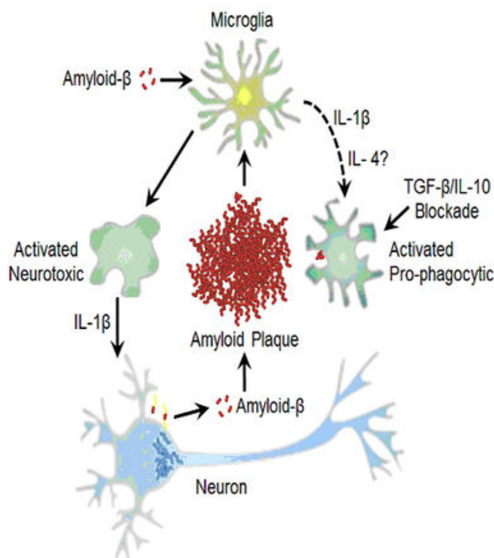

Keywords

Alzheimer's disease; blood-brain barrier (BBB); cytokines; microglia; innate immunity; Long-term potentiation (LTP); macrophage colony stimulating factor receptor (CSF-1R); non-steroidal anti-inflammatory drugs (NSAIDs); Venusberg Neuroinflammation Meeting Bonn 2015

Morphological aspects of microglia in development, aging and disease

Niva Russek-Blum & Alon Monsonego

Introduction

Microglia, the immune resident cells of the brain, were first described many decades ago as migratory phagocytic cells of the central nervous system (CNS) (Del Rio-Hortega 1932; Rezaie and Male 2002). In his seminal paper, del Rio-Hortega suggested that microglia exhibit an amoeboid morphology when they enter the brain during early development, then transform into a branched, ramified morphological phenotype. He observed that microglia are found almost evenly dispersed throughout the adult brain, and that, following injury, they undergo a transformation into an amoeboid morphology and have the capacity to migrate, proliferate and phagocytose similar to their characteristics during early development. Although these early morphological observations did not change dramatically since they were first described, they did undergo some refinements. Recently, the combination of advanced imaging techniques, such as confocal and two-photon microscopy, with the use of genetically based cell-specific markers and thinned-skull preparations, has allowed high resolution studies of microglia also in the living animal. This review summarizes our current understanding of structural-functional aspects of microglia, from development to the adult functioning brain, and to aging and age-related diseases.

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Microglia morphology in the developing CNS

Developmental studies show that microglia cells originate from yolk sac-derived precursors in a Myb-independent manner via PU.1 and interferon regulatory factor (IRF8)-dependent pathways (Figure 1). This lineage is primarily regulated by macrophage colony stimulating factor receptor (CSF-1R), its ligand IL-34, and transforming growth factor-β (TGF-β). Microglia population is established during prenatal development, but how these cells are maintained throughout the organism's lifespan remains an ongoing debate (Eglitis and Mezey 1997; Herbomel, Thisse et al. 2001; Ginhoux, Greter et al. 2010; Schulz, Gomez Perdiguero et al. 2012; Varvel, Grathwohl et al. 2012; Kierdorf, Erny et al. 2013; Shiau, Monk et al. 2013; Elmore, Najafi et al. 2014 Bruttger, Karram et al. 2015). After invading the brain's parenchyma, these precursor cells acquire a ramified and quiescent phenotype, unique to the CNS milieu. The mechanisms underlying the differentiation of yolk sac precursors to "resting", ramified microglia phenotype are mostly unknown. Cell-culture studies have suggested a few candidates that affect the ramification process such as cytokines, namely, TGF-β, macrophage colony-stimulating factor (M-CSF), and granulocyte/macrophage colony stimulating factor (GM-CSF) (Schilling, Nitsch et al. 2001; Abutbul, Shapiro et al. 2012). TGF-β, M-CSF and GM-CSF were shown to induce ramification and up-regulation of delayed K^+ channels in newborn mice-derived primary microglia. Transformation from amoeboid into ramified morphology induced in microglia by exposure to astrocyte-conditioned medium (ACM) was inhibited by neutralizing antibodies against TGF-β, M-CSF or GM-CSF, while ACM-induced delayed rectifier (DR) K^+ channel expression was exclusively inhibited by antibodies against TGF-β (Schilling, Nitsch et al. 2001). The role of TGF-β in acquiring a microglia-like quiescent phenotype along with ramified morphology was studied in our laboratory by using co-cultures of hematopoietic progenitors or bone marrow derived dendritic cells (BMDCs) with organotypic hippocampal slices or primary glia. TGF-β signaling via SMAD2/3 phosphorylation was found to be essential for myeloid-derived cells to upregulate CX3CR1 and to acquire a microglia-like ramified morphology and a quiescent phenotype (Abutbul, Shapiro et al. 2012). These findings were further supported by the identification of a unique microglial molecular signature, which depends on TGF-β signaling (Butovsky, Jedrychowski et al. 2014). Furthermore, study of transcriptomes and enhancer landscapes of microglia indicated specificity of the SMAD motif, which collaborates with PU.1 to establish microglia-specific enhancers (Gosselin, Link et al. 2014). The cellular and molecular mechanisms that mediate the specification, migration, and differentiation of developing microglia remain mostly unknown and raise fundamental questions regarding the cellular source and signaling pathways of TGF-β in differentiating microglia, and how it is orchestrated with other signaling cues in the developing brain.

Structural-functional aspects of microglia

In the mature brain, microglia show remarkable diversity with respect to brain anatomy (Olah, Biber et al. 2011; Grabert, Michoel et al. 2016). Under physiological conditions, their cellular density can vary considerably between different brain regions by as much as five-fold in mice (Lawson, Perry et al. 1990) and ten-fold in humans (Mittelbronn, Dietz et al. 2001). In both mice and humans, microglia are most numerous in the telencephalon,

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followed by the diencephalon, mesencephalon, and rhombencephalon (Lawson, Perry et al. 1990; Savchenko, McKanna et al. 2000). Myelinated regions are known to contain higher density of microglia than non-myelinated tissues of the same anatomical region (Lawson, Perry et al. 1990; Mittelbronn, Dietz et al. 2001). While white-matter microglia show elongated somata and processes that are preferentially oriented along fiber tracts, microglia in the circumventricular organs- regions characterized by a leaky blood-brain barrier, exhibit a compact morphology with a few short processes, which may resemble a primed morphology. In contrast, gray-matter microglia exhibit many elaborated, radially oriented arbors (Lawson, Perry et al. 1990).

The basic morphology of quiescent cortical microglia, as described in mice, is generally represented in a complexed branch order as follows: (i) the 1st branch order, usually between 7–9 main processes, which extend directly from the cell soma; (ii) the 2nd branch order are medium processes, which branch from the main processes to several shorter and thinner processes; and (iii) the 3rd branch order-fine processes, which are mostly devoid of Iba-1 expression (Baron, Babcock et al. 2014). Similar to the typical morphological properties of resting microglia in the mouse cortex, resting microglia in zebrafish are also highly branched with dynamic processes, which end with stick-like or bulbous tips (Peri and Nusslein-Volhard 2008; Sieger and Peri 2013).

The bulbous tip, a unique morphological feature of microglia, is formed rapidly through expansion from a stick-like ending, and stalls on the contacted neuronal soma for several minutes before gradually shrinking back (Li, Du et al. 2012). Functionally, the bulbous ending mediates the preferential contact of the microglia with neurons that show high levels of activity to ultimately regulate the spontaneous and evoked activity of the contacted neurons (Li, Du et al. 2012). Another unique morphological specialization of microglia, described based on electron microscopy studies, is finger-like protrusions that wrap around dendritic spines, followed by phagocytic inclusions of synaptic elements into the microglia (Tremblay, Lowery et al. 2010). These elements are found in the postnatal developing brain, wherein microglia play a fundamental role in “synaptic pruning”, namely, in eliminating excess synaptic connections and mediating proper maturation of excitatory synaptic transmission (Paolicelli, Bolasco et al. 2011; Zhan, Paolicelli et al. 2014). The process of synaptic pruning is dependent on neuronal activity and is achieved by microglial engulfment of both pre- and postsynaptic elements (Tremblay, Lowery et al. 2010; Paolicelli, Bolasco et al. 2011; Kettenmann, Kirchhoff et al. 2013; Schafer, Lehrman et al. 2013). Most microglial processes can contact more than one synaptic element simultaneously (Wake, Moorhouse et al. 2009; Tremblay, Lowery et al. 2010). As microglia play a role in regulating activity-triggered synaptic plasticity (Rogers, Morganti et al. 2011; Koeglsperger, Li et al. 2013; Schafer, Lehrman et al. 2013), engulfing cells undergoing apoptosis (Peri and Nusslein-Volhard 2008; Sierra, Abiega et al. 2013) and remodeling neural circuits (Schafer, Lehrman et al. 2012; Squarzoni, Thion et al. 2015), it is plausible and further shown that they play an important role in learning and memory (Rogers, Morganti et al. 2011; Tremblay and Majewska 2011; Parkhurst, Yang et al. 2013).

Although microglia in the healthy brain are typically considered to be resting and quiescent, they possess a ramified morphology with highly dynamic fine processes, continuously

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undergoing cycles of *de novo* formation and withdrawal on a time scale of minutes (Davalos, Grutzendler et al. 2005; Nimmerjahn, Kirchhoff et al. 2005; Wake, Moorhouse et al. 2009; Tremblay, Lowery et al. 2010). The dynamics of microglial process motility in the healthy brain is not a seemingly random process; rather, it was shown that the motility of the processes of resting microglia is affected by glutamatergic and GABAergic neurotransmission (Fontainhas, Wang et al. 2011), and that the frequency and duration of microglia–neuronal interactions can be influenced by local neuronal activity (Wake, Moorhouse et al. 2009; Tremblay, Lowery et al. 2010; Li, Du et al. 2012). Upon traumatic brain injury, ATP is released from damaged neural tissue and surrounding astrocytes inducing rapid microglial response towards the injury site, including chemotaxis of microglial cell bodies and processes (Davalos, Grutzendler et al. 2005; Nimmerjahn, Kirchhoff et al. 2005). ATP together with glutamate mediates rapidly propagating Ca^{2+} waves that provide positional information via ATP to P2YR12-expressing microglia. These Ca^{2+} waves define which microglia are targeted to neuronal injuries (Sieger, Moritz et al. 2012). Microglia activated simply by nerve injury involving release of ATP may then be involved in regenerative responses and phagocytotic clearance of cell debris (Streit 2002; Streit 2005).

Equipped with a rich repertoire of sensing receptors (Kettenmann, Hanisch et al. 2011), ramified surveilling microglia respond to a variety of triggers, ultimately facilitating neuronal remodeling and homeostasis. It can be hypothesized that the local environment shapes the molecular signature of these microglia, which is then translated into spatial and temporal mechanisms of surveillance morphology, contact with the target and function.

From the adult functioning brain to aging and disease

Although they maintain a quiescent phenotype in the intact brain, microglia can be activated by pattern recognition receptors (PRRs) that can sense and respond to damage-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs). Such PRRs include CD36, CD14, Toll like receptors (TLRs), scavenger receptors (SRs), purinergic receptors, triggering receptor expressed by myeloid cells 2 (TREM2) and CD33 (Ransohoff and Perry 2009). The activation process is characterized by morphological changes and upregulation of a spectrum of proinflammatory and anti-inflammatory cytokines (Heneka and O'Banion 2007; Ransohoff and Perry 2009; Perry and Holmes 2014; Gomez-Nicola and Perry 2015). Inflammatory reactions result in thicker, less branched and motile microglial processes, which, overall, exhibit a reduced coverage area (Cunningham 2013). While aging and/or neurodegeneration may cause microglia priming characterized by morphological changes and a very mild inflammatory response, peripheral inflammation can shift this priming into a more robust microglial inflammatory response with neurotoxic characteristics (Cunningham 2013; Perry and Holmes 2014; Gomez-Nicola and Perry 2015).

Numerous studies in mice and humans have shown that, during aging, microglia acquire an activated or primed phenotype characterized by process shortening and thickening (DiPatre and Gelman 1997; Sierra, Gottfried-Blackmore et al. 2007). These aging-related morphological changes, from a typical resting state to an activated phenotype, are associated

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with increased production of proinflammatory mediators, such as TNF-α, IL-1β, and IL-6 (Perry, Newman et al. 2003; Lucin and Wyss-Coray 2009; Baron, Babcock et al. 2014).

Microglia in the aging CNS have been described also as dystrophic with aberrant morphological features, including de-ramification, shortening, and process twisting and fragmentation (Streit 2004; Streit, Miller et al. 2008; Baron, Babcock et al. 2014; Streit, Xue et al. 2014). It should be noted, however, that microglial senescence (dystrophy) as it appears in aging human brain differs from the aged rodent brain and is much more robust (Smith and Dragunow 2014; Streit, Xue et al. 2014). It was suggested that in humans who live much longer than rodents and are exposed to diverse environmental effects, the rates at which microglial dystrophy occurs are much higher, causing a decline in microglial neuroprotection and cognitive function (Streit, Xue et al. 2014).

Morphometric analyses of individual microglia, based on three-dimensional reconstructions of the cells or on live-imaging analyses, quantitatively defined the alterations in the fine structure of microglia processes during aging (Damani, Zhao et al. 2011; Baron, Babcock et al. 2014; Hefendehl, Neher et al. 2014). Quantitative analysis of microglial processes revealed fewer bifurcations and branches, and reduced total branch length, in old mice as compared with younger ones. Moreover, microglial coverage volume was found to be reduced by more than 50% in old mice, as compared with young mice (Baron, Babcock et al. 2014).

Similar to the changes they undergo during aging, the complexity and motility of microglia processes also appear to be compromised under neurodegenerative conditions, including Alzheimer's disease (AD) and amyotrophic lateral sclerosis (ALS), presumably resulting in reduced surveillance capacity (Damani, Zhao et al. 2011; Dibaj, Steffens et al. 2011; Krabbe, Halle et al. 2013; Baron, Babcock et al. 2014; Hefendehl, Neher et al. 2014).

In AD mouse models, microglia have been shown to be more abundant at sites of Aβ plaques, with an altered morphology reminiscent of cell activation (Mucke, Masliah et al. 2000; Hickman, Allison et al. 2008; Eisenberg and Jucker 2012). In such models, microglia are clustered around Aβ plaques, leaving the surrounding tissue covered by fewer processes than usually occurs in age-matched wild-type (WT) mice (Baron, Babcock et al. 2014). Detailed analyses showed that microglia from adult amyloid precursor protein (APP)-transgenic (Tg) mice exhibit curved and twisted processes and a significant reduction in the number of bifurcations and branches in the total microglial process length. Furthermore, coverage volume by individual microglia is significantly reduced in adult APP-Tg mice by almost 50%, as compared with age-matched WT mice (Baron, Babcock et al. 2014). Co-expression of activation markers and CD39 (also known as ectonucleoside triphosphate diphosphohydrolase 1), which plays a key role in microglial migration, process formation toward ATP and phagocytosis (Davalos, Grutzendler et al. 2005; Farber, Markworth et al. 2008; Sieger, Moritz et al. 2012; Bulavina, Szulzewsky et al. 2013) was evaluated in APP/PS1 Tg mice. Overall, three layers of cells surround the plaque: first, a layer of Iba-1+/ CD39^{high} amoeboid cells, which are found in close proximity to the plaque and may resemble the activated form of microglia and/or some infiltrating monocytes; second, a layer of Iba-1+/CD39^{high} cells that have an enlarged cytoplasm and a decreased morphological

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complexity (as compared with cells found in plaque-free areas), which may resemble primed cells; and third, a layer of Iba-1+/CD39+ cells, with a marked population of cells expressing low levels of CD39 and exhibiting decreased morphological complexity, as compared with cells found in plaque-free areas (Baron, Babcock et al. 2014). Microglia stratified in all three layers around Aβ plaques have a morphology and process characteristics that are distinct from those of ramified resting microglia in the healthy CNS. Interestingly, in humans, microglia appear as either ramified cells in diffuse (early) plaques or as dystrophic cells in neuritic (late) plaques, suggesting that aggregated Aβ in human amyloid plaques may promote microglial degeneration (Streit, Xue et al. 2014).

Overall, accumulating data show that, whereas microglia are tightly packed and cover the tissue uniformly in younger mice, their coverage is impaired in older mice and, even more severely so, in APP-Tg mice, in which tissue space is left devoid of microglia processes. Considering the dynamic nature of the microglial processes, such a robust loss of branches during aging and disease may significantly impair the overall sensing capacity of microglia.

It is well known that the levels of proinflammatory cytokines of the innate immune system increase in the CNS during aging (Perry, Newman et al. 2003; Lucin and Wyss-Coray 2009; Baron, Babcock et al. 2014). This suggests that, during the process of aging, microglia exhibit a proinflammatory profile that, plausibly, underlies their reduced morphological complexity.

It has been shown that the number of fine microglia processes is significantly reduced in older mice, and that overexpression of the human mutated APP and the deposition of plaques in the brain significantly accelerate this reduction (Baron, Babcock et al. 2014). In addition, in an AD mouse model, a significant reduction in the number of microglial processes surrounding Aβ plaques was observed (Baron, Babcock et al. 2014). Microglial accumulation near Aβ plaques may thus not only shift the molecular and cellular milieu to one that can enhance neurotoxicity (Varvel, Grathwohl et al. 2012; Heneka, Kummer et al. 2013), but it also causes a progressive decrease in microglial process complexity, which may impair the clearance of Aβ oligomers and modulate the synaptic network or neuronal repair processes.

A key emerging question is whether the primed or dystrophic microglia play a role in facilitating aging or age-related neurodegenerative diseases. In aging, it has been suggested that numerous microglia processes protrude into the synaptic cleft, inducing synaptic stripping (Tremblay, Zettel et al. 2012). In addition, thickening of the microglia cell body, increased granulation, impairment of remodeling, and retraction of microglia processes have been described in various brain areas (Sierra, Gottfried-Blackmore et al. 2007; Damani, Zhao et al. 2011; Tremblay, Zettel et al. 2012; Kettenmann, Kirchhoff et al. 2013). Do such impaired morphologies and functions of microglia compromise their reaction to neuronal abnormalities? Does the impairment in synaptic plasticity, caused by microglia, accelerate the cognitive decline associated with aging? Further high-resolution morphological analyses of microglia in the adult brain, and of the changes that they undergo during aging and disease, may reveal key aspects of neurodegeneration and cognitive impairment.

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Conclusion

What can we learn from microglia morphology? Activation of human macrophages by various signals had led to the acquisition of 299 macrophage transcriptomes. Analysis of this dataset revealed a spectrum of macrophage activation states, which extends the current M1 versus M2-polarization model. Network analyses have identified central transcriptional regulators associated with many types of macrophage activation, complemented by regulators related to stimulus-specific programs (Xue, Schmidt et al. 2014). Microglia differ decisively from peripheral macrophages, and their activation likely does not follow these precise pathways; nevertheless, it is instructive to consider how the underlying concept of macrophage heterogeneity might apply to microglial responses. Stimulated microglia, like macrophages in other tissues, are not simply beneficial or deleterious; rather, they demonstrate a highly plastic response, modified by the nature of the stimulus and by the molecular repertoire that it affects (Gordon 2003; Ransohoff and Perry 2009). The combination of morphological criteria and molecular profiles can be useful to describe the activity of microglia in response to diverse conditions and stimuli. The functional implications of the different morphological states and dynamic changes are still not fully understood; our challenge will be to utilize state-of-the-art morphological analysis tools, in both zebrafish and mouse models, to define phenotypically heterogeneous microglia responding to distinct challenges. Such investigations may lead to further insights into neurodegeneration, aging, and cognitive decline.

Microglial ontogeny and induced pluripotent stem cell-derived microglia

Donovan Low, Kazuyuki Takata and Florent Ginhoux

Introduction

Microglia, the resident macrophages of the central nervous system (CNS), play important roles in both CNS homeostasis and inflammation. Dysregulated microglial activity is involved in the pathogenesis of neurodegenerative disorders and neuroinflammatory diseases, rendering modulation of microglial responses an attractive therapeutic target. However, rational design of novel interventions focusing on the microglial compartment must be underpinned by a thorough understanding of the origin and developmental pathways of this unique and intriguing cell type, which until now has been hindered by the difficulties in isolating microglia or generating them *in vitro* for study. Here, we describe the current knowledge of microglial ontogeny and its implication for the generation of microglia *in vitro*, with a focus on the potential advances of utilizing induced pluripotent stem cells.

The term "microglia" was first introduced by del Rio-Hortega based on a set of morphological and functional differences (such as "migratory activity" and "phagocytosis") which delineated a distinct population of non-neuronal, non-astrocytic cells of mesodermal origin within the central nervous system (CNS). While the existence of the microglial population was undeniable, del Rio-Hortega's claim of their mesodermal origin was highly debated as it had long been believed that microglia shared the neuro-ectodermal origin of neurons and other glial cells (Ginhoux, Lim et al. 2013; Ginhoux and Prinz 2015). The subsequent discovery that microglia express monocyte/macrophage antigens led to

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widespread acceptance of their myeloid nature and mesodermal origin, but for many years accurate definition of their precise ontogeny remained elusive. Initial studies proposed an embryonic origin of microglia, suggesting they were derived from yolk sac (YS) progenitors that seeded the brain rudiment during early fetal development. However, these reports could not exclude the possibility that other progenitors might supersede the YS contribution with time or under certain physiological conditions, leaving the questions on the composition and maintenance of the adult microglial compartment incompletely answered.

In fact, data continued to emerge that suggested a requirement for the contribution of blood-borne cells, such as monocytes, to both generate the post-natal microglial compartment, and to maintain it into adulthood (Ginhoux, Lim et al. 2013; Ginhoux and Prinz 2015). Microglia are essential for the immune protection of the brain against foreign pathogens (Perry and Gordon 1988) as well as for brain development and homeostasis. Microglia are involved in the proper development of the brain such as synaptic pruning (Paolicelli, Bolasco et al. 2011), interneuron positioning (Squarzoni, Oller et al. 2014), and in the regulation of neuronal activity (Li, Du et al. 2012). By clearing debris from dying neurons in steady-state and upon brain injury, microglia may also be essential to avoid plaque formation and likely play a critical role in Alzheimer's disease (McGeer, Schulzer et al. 1996) and Parkinson's disease (Doorn, Lucassen et al. 2012). Altogether, it is now clear that microglia serve essential functions required for tissue homeostasis far beyond their sole role of immune sentinels. Given the central role of microglia in maintaining optimal CNS function and in mediating the pathology of neurodegeneration and neuroinflammation, recent years have seen intense interest in how the microglial population might be targeted therapeutically. With advances in stem cell research, for the first time it has become feasible to consider how we might use our knowledge of microglial ontogeny and function to generate microglia-like cells *in vitro* for therapeutic use and study (Figure 2). Here we review the latest advances in our knowledge of the origins of the microglial population and how this might be harnessed for clinical use.

Ontogeny of microglia: A primitive ancestry

Initial studies broadly described the emergence of microglia early in the developing brain at a point preceding the initiation of fetal liver and bone marrow (BM) hematopoiesis, and concomitant with the appearance of YS primitive hematopoiesis (Takahashi and Naito 1993). Primitive hematopoiesis occurs in the blood islands of the extra-embryonic YS at embryonic day 7 (E7) giving rise to 'early' erythro-myeloid precursors (Parkhurst, Yang et al. 2013) mainly forming primitive nucleated erythrocytes and primitive macrophages (Orkin and Zon 2008; Hoeffel, Chen et al. 2015). However the truly unique ontogeny of microglia has only recently been established following the development of new fate-mapping mouse models that allow accurate tracing of YS progenitors (Ginhoux, Greter et al. 2010; Schulz, Gomez Perdiguero et al. 2012; Sheng, Ruedl et al. 2015). Ginhoux et al. used a lineage-tracing model to label YS progenitors, including YS macrophages, based on their expression of runt-related transcription factor 1 (Runx1), which is critical for the emergence of hematopoietic stem cells. During early embryogenesis, Runx1 expression is restricted to cells of the extra-embryonic YS, allowing tracing of YS progenitors after activation of a reporter gene by tamoxifen treatment of the pregnant female within a specific timeframe

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(Samokhvalov, Samokhvalova et al. 2007). Using this model, the authors showed that the majority of both embryonic and adult microglia arose from YS Runx1$^+$ progenitors (Ginhoux, Greter et al. 2010); furthermore there was minimal contribution from either fetal or adult monocytes to microglia in the brain (Ginhoux, Greter et al. 2010; Hoeffel, Chen et al. 2015), while other tissue macrophages did arise from fetal monocytes (Ginhoux, Greter et al. 2010; Hoeffel, Wang et al. 2012; Hoeffel, Chen et al. 2015; Sheng, Ruedl et al. 2015). The absence of fetal monocyte contribution to the microglial pool could result from a lack of intrinsic potential or a lack of access to the developing brain (Hoeffel, Chen et al. 2015) due to the nascent blood brain barrier as early as E13.5 (Daneman, Zhou et al. 2010).

The YS progenitor population has since been dissected further, with Kierdorf et al. first identifying the precursors of the primitive YS macrophages/microglia: at E8, c-kit$^+$ EMPs in the YS differentiate into CD45$^+$c-kit^{lo}CX3CR1$^-$ early precursors that mature and migrate into the developing brain as CD45$^+$c-kit$^-$CX3CR1$^+$ cells (Kierdorf, Erny et al. 2013). More recently, Hoeffel et al. extended the characterization of YS EMPs using an *in utero* macrophage depletion system combined with genetic tagging of YS and fetal liver hematopoietic cells. Using the same Runx1 fate mapping model, the authors compared the cell populations that arose from YS progenitors labeled by tamoxifen treatment at either E7.5 or E8.5, revealing that two waves of EMPs arise sequentially in the YS: an early wave at E7.5 which differentiates locally into the YS macrophages that are the primary source of microglia, and a late wave of E8.5 EMPs that can either differentiate locally into YS macrophages, or migrate to seed the fetal liver following the establishment of blood circulation from E9.0 (Hoeffel, Chen et al. 2015). However, this study clearly showed that most of microglia arise from YS macrophages deriving from the early wave of EMPs.

Further experiments in both newborn transplant models (where host CD45.2 mice were sub-lethally irradiated and subsequently reconstituted with hematopoietic cells from congenic CD45.1 mice) and parabiotic mice (where two mice are surgically attached to allow shared blood circulation while retaining separate organs) indicated that postnatal circulating hematopoietic progenitors contribute only negligibly to the adult microglial population (Ginhoux, Greter et al. 2010; Ajami, Bennett et al. 2011; Hoeffel, Chen et al. 2015). Furthermore, recent ablation experiments in adult mice showed that the cells replacing microglia after depletion are derived from CNS-intrinsic precursors (Waisman, Ginhoux et al. 2015). Of note, following depletion of microglia either in the embryo or in the adult, the microglial compartment is replenished rapidly, within 7 days (Squarzoni, Oller et al. 2014; Waisman, Ginhoux et al. 2015). The precursors of these new repopulating microglia are not yet fully characterized and it remains unknown whether they possess the same characteristics as their YS embryonic counterparts in terms of their roles in CNS development, homeostasis and neuropathologies. Altogether these data show that adult microglia unequivocally arise from YS macrophages that seed the brain at E9.5, where these cells establish the nascent microglial population, proliferate *in situ* and are maintained throughout adulthood. Thus the majority of the postnatal microglial compartment exists independent of circulating hematopoietic cells and can be self-renewed by local radio-resistant prenatally-seeded progenitors.

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The unique ontogeny of microglia in the steady state has been established, but the situation is not as clear during certain situations, such as inflammation, where hematopoietic cells from the BM can infiltrate the brain. Several studies have reported BM-derived cells migrating into the CNS following BM transplantation and differentiating into parenchymal microglia-like cells (Ginhoux and Jung 2014; Greter, Lelios et al. 2015). Circulating myeloid cells such as monocytes can only enter the CNS if the blood-brain barrier is damaged, such as when the CNS is inflamed or upon lethal irradiation (Ajami, Bennett et al. 2007; Mildner, Mack et al. 2009). Under such conditions these cells recapitulate several of the phenotypic and functional characteristics of the pre-existing adult microglial population, giving rise to 'microglia-like cells' (Ajami, Bennett et al. 2011; Takahashi, Kakuda et al. 2015), which may or may not fulfill the same role as their embryonic counterparts in terms of CNS homeostasis and neuroinflammation. Interestingly, these microglia-like cells did not persist once the inflammation was resolved in a model of experimental autoimmune encephalomyelitis (Ajami, Bennett et al. 2011). Furthermore, it was also shown that monocyte-derived microglia-like cells accumulate to a much lesser extend compared to resident microglia close to amyloid plaques in the brains of Alzheimer's disease mouse models (Prokop, Miller et al. 2015; Varvel, Grathwohl et al. 2015), suggesting that they play a different role.

Pluripotent stem cell-derived macrophages as an adequate source of microglia

Efforts to elucidate the biology of microglia have been limited by the lack of access to sufficient numbers of cells for comprehensive *in vitro* studies. Isolation of primary rodent microglia is generally achieved either by cell sorting or stepwise cell culture from newborn rodent brains (Ju, Zeng et al. 2015), both of which are time-consuming and often yield few cells. While of some limited use in rodents, this approach is entirely unfeasible for obtaining human microglia. Although there has been some success producing microglia-like cells from BM stem cells and circulating monocytes, as these cell populations do not share the embryonic origin of the vast majority of microglia in the homeostatic brain, they are likely to be poor models of adult microglia.

This inability to access or generate sufficient numbers of cells of true microglial origin has prevented comprehensive or large scale *in vitro* studies. However, a potential source of microglia might be embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs), which are reprogrammed terminally-differentiated somatic cells that resemble ESCs (Takahashi and Yamanaka 2006; Takahashi, Tanabe et al. 2007). ESCs and iPSCs have unlimited expansion potential and the ability to differentiate toward any cell type of the three germ layers (De Los Angeles, Ferrari et al. 2015). In addition, iPSCs do not suffer the limitation of having a fertilized egg as their source; instead they can be derived from a wide range of different cell types even from cancer cells and could be generated in a patient-specific manner for future therapies, thereby circumventing two of the major ethical and barrier/immune issues associated with the use of ESCs. For these reasons the use of iPSCs has opened-up new avenues for stem cell research, developmental biology, cancer research, various types of disease modeling, and the development of patient-specific disease

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interventions (Ohnuki and Takahashi 2015); ESCs and iPSCs are already used for research into embryonic development, as well as for cellular transplantation therapies for neurodegenerative diseases (Park, Arora et al. 2008; Kiskinis and Eggan 2010; Ben-David, Kopper et al. 2012). Moreover, the application of autologous iPSCs in the field of regenerative medicine is anticipated to provide much-needed improvements in our ability to treat/prevent organ rejection in recipient patients after engraftment, and furthermore the concept of haplo-banking with human leukocyte antigen homozygous of iPSCs has been established (Nakajima, Tokunaga et al. 2007; Nakatsuji, Nakajima et al. 2008; Zimmermann, Preynat-Seauve et al. 2012; de Rham and Villard 2014).

The differentiation pathways of hematopoietic lineages have also been investigated using iPSCs (Ackermann, Liebhaber et al. 2015). Recently, selective induction of primitive or definitive hematopoiesis has been shown to rely on Wnt signaling, providing simple selective differentiation strategies for the generation of primitive or definitive hematopoietic progenitors by Wnt-β-catenin manipulation (Kennedy, Awong et al. 2012; Sturgeon, Ditadi et al. 2014). In addition, large-scale differentiation of macrophages from human iPSCs has been reported (Lachmann, Ackermann et al. 2015). We have also developed our own "in house" xeno/serum-free differentiation protocol for the generation of immature macrophages, similar to those expected to arise from primitive hematopoiesis, as a microglial source. In this protocol, mouse iPSCs start to differentiate into primitive macrophages, which express markers including F4/80, CD11b, and CX3CR1, and exhibit phagocytic function after 8-12 days of culture. Crucially, as these cells share the same embryonic origin as microglia, they may well represent the first adequate and appropriate *in vitro* source of microglia for study and therapeutic investigation. We have also been establishing methods for the co-culture of iPSC-derived primitive macrophages with neurons derived from the same mouse iPSC batch, which we hope will provide insights into the mechanism of primitive macrophage maturation into microglia, and the interactions of microglia and neurons in both physiological and pathological neuroinflammatory conditions (Figure 2). Furthermore, the co-culture model has the potential to serve as an *in vitro* drug screening system for the development of novel therapies against brain diseases. Taken together, iPSC-derived primitive macrophages could be a useful microglial source for biological, pathophysiological and therapeutic studies.

Conclusion

Our composite knowledge of microglial origin at this time supports the idea that immature macrophages arise from primitive hematopoiesis in the YS before birth. This knowledge may be further supplemented in the near future with the advent of iPSC-based techniques that might enable us to establish an *in vitro* system able to generate microglia of appropriate ontogeny and in sufficient quantities for meaningful study. This will allow us to gain a better understanding of microglial biology in health and disease, and will facilitate the refinement of accurate developmental and pathophysiological study models as well as drug screening systems for new microglia-targeted therapies. Extending this to the human setting, we may soon have the opportunity to generate patient specific iPSC-derived microglia and use them for disease modeling, drug screening and even cell therapy. More focused on brain macrophages, plenty of questions remain: for example, how do resident microglia and

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meningeal and perivascular macrophages in the brain differ in function and cooperate to maintain a healthy CNS? This gap in knowledge also extends to macrophages arising from primitive and definitive hematopoiesis. To address these issues we will need to employ an integrated approach, incorporating *in vivo* studies of ontogeny as well as *in vitro* iPSC-based culture models; only then can the potential be realized for the development of novel drugs and/or cell replacement therapies capable of targeting microglia implicated in the pathology of brain diseases.

The Cytokine Paradox in Alzheimer's Disease Pathogenesis

Terrence Town and M. Kerry O'Banion

Introduction

The recognition of activated glia together with inflammatory cytokines and other mediators in the brains of individuals with Alzheimer's disease (AD) and evidence that non-steroidal anti-inflammatory drugs might protect against AD led to an early view that neuroinflammation contributed to AD pathogenesis (Akiyama, Barger et al. 2000). Over time this view has been modified to encompass a growing body of experimental evidence that neuroinflammation has both beneficial as well as detrimental roles in AD that vary with context. For example, experiments using pro-inflammatory stimuli in AD mouse models have demonstrated reduction of amyloid deposits, raising the notion that, at least under certain conditions, neuroinflammation can play a protective role (Shaftel, Griffin et al. 2008). Such stimuli include lipopolysaccharide (DiCarlo, Wilcock et al. 2001), interleukin (IL)-1β (Shaftel, Kyrkanides et al. 2007), interferon γ (Chakrabarty, Ceballos-Diaz et al. 2010), IL-6 (Chakrabarty, Jansen-West et al. 2010), and tumor necrosis factor α (Chakrabarty, Herring et al. 2011). In all cases, plaque reduction was associated with increased gliosis, suggesting the possibility that these pro-inflammatory stimuli provoked increased phagocytosis of Aβ. Indeed, in a distinct model of microglial activation caused by deletion of CX3CR1, plaque reduction was associated with increased Aβ phagocytosis and evidence of increased IL-1β expression (Lee, Varvel et al. 2010).

Interleukin-1

Using models of virally delivered IL-1β dependent reduction in local Aβ deposition, O'Banion and coworkers have investigated possible mechanisms underlying the apparent benefits of pro-inflammatory cytokine expression on AD pathology. In initial studies, overexpression of human IL-1β for one month using a viral cre-activated transgene in APP/PS-1 mice led to local reduction (e.g. one hippocampus vs. the contralateral side) in Aβ plaque load, an effect that did not appear to involve changes in APP production or Aβ processing (Shaftel, Kyrkanides et al. 2007). Further work demonstrated similar changes regardless of animal age or duration of IL-1β overexpression (1-3 months) in both the APP/PS1 and triple transgenic models (Matousek, Ghosh et al. 2012; Ghosh, Wu et al. 2013). In all cases, IL-1β overexpression was associated with increased density of Iba-1 positive cells surrounding amyloid plaques (Shaftel, Kyrkanides et al. 2007; Ghosh, Wu et al. 2013) as well as dramatic evidence of peripheral cell infiltration and a compromised blood-brain barrier (Shaftel, Carlson et al. 2007). To test the possibility that infiltrating

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peripheral macrophages might be responsible for the observed effects, Rivera-Escalera et al. carried out a series of studies in APP/PS-1 mice using AAV2-hIL-1β as an alternate means to drive sustained IL-1β expression (Rivera-Escalera, Matousek et al. 2014). These mice also showed reduced plaque deposition, and while bone marrow chimera experiments clearly demonstrated infiltrating Iba-1 positive cells associated with plaques, experiments with CCR2 deficient mice still showed IL-1-dependent plaque clearance despite almost complete inhibition of monocyte/macrophage infiltration (Rivera-Escalera, Matousek et al. 2014). More recently, Cherry et al. reported that sustained IL-1β overexpression induced a population of arginase-1 positive cells, derived from microglia, that appeared to have increased capacity for Aβ phagocytosis (Cherry, Olschowka et al. 2015). Sustained IL-1β overexpression also led to increased IL-4 levels in the brain, some of which appeared to derive from infiltrating CD3 positive T cells.

Indeed, when APP/PS-1 mice overexpressing IL-1β were treated with a neutralizing antibody to IL-4Rα, there was a reduction in arginase-1 positive cells that correlated with abrogation of the IL-1β dependent benefits on plaque clearance (Cherry, Olschowka et al. 2015). Moreover, IL-4 injection alone led to rapid induction of arginase-1 positive cells associated with plaque clearance in APP/PS-1 mice, an observation consistent with previous observations from some (Kiyota, Okuyama et al. 2010; Kawahara, Suenobu et al. 2012), but not all (Chakrabarty, Tianbai et al. 2012) investigators. These observations suggest that homeostatic mechanisms reacting to increased neuroinflammation in the setting of focal IL-1β overexpression result in alternative microglial activation, a process that might be harnessed for benefit in AD therapy. Interestingly, evidence of alternate microglial activation was also observed in a different model where deletion of the NLRP3 inflammasome, and subsequent failure to generate and release mature IL-1β, reduced amyloid plaque deposition in APP/PS1 mice (Heneka, Kummer et al. 2013). These findings highlight the paradoxical effects of cytokines on AD pathology: overexpression of IL-1β and absence of IL-1β both generate a similar phenotype, at least with regard to amyloid deposition. Whether the paradox can be explained by modulation of a single mechanism (e.g. microglial phenotype) or multiple mechanisms requires further investigation.

Understanding mechanisms that underlie the benefits of pro-inflammatory cytokine expression on Aβ deposition is an especially important undertaking in consideration of effects observed on tau pathology. For example, while IL-1β overexpression in triple-transgenic mice reduced Aβ plaque density, it exacerbated tau phosphorylation (Ghosh, Wu et al. 2013). Similar findings have been reported for lipopolysaccharide (Kitazawa, Oddo et al. 2005; Lee, Rizer et al. 2010) and for loss of CX3CR1 (Bhaskar, Konerth et al. 2010; Maphis, Xu et al. 2015), processes which both appear to depend on IL-1 signaling. In addition, bilateral overexpression of IL-1β in the murine hippocampus led to behavioral deficits in two hippocampal-dependent learning tasks (contextual fear conditioning and Morris water maze) (Moore, Wu et al. 2009; Hein, Stasko et al. 2010). Taken together, these results raise clear concerns about the possible application of enhancing inflammation to intervene with AD, an issue illustrated by the negative outcomes reported in initial trials of active Aβ immunization (Robinson, Bishop et al. 2004).

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Rebalancing neuroinflammation to homeostasis by blocking immunosuppressive cytokines

Transforming growth factor-β

While much attention has been directed toward understanding the role(s) of pro-inflammatory cytokines in AD pathogenesis, considerably less effort has been placed on factors that limit the extent and duration of inflammatory processes. As a key immunoregulatory cytokine, transforming growth factor-β (TGF-β) ensures that inflammatory responses are carried out in an acute, controlled fashion without becoming chronic and inducing excessive tissue damage (Li and Flavell 2008). Prolonged, low-level activation of brain inflammatory processes occurs in AD and is likely pathogenic (Wyss-Coray, Masliah et al. 1997; Selkoe 2001); raising the paradoxical question of whether *immunosuppressive* pathways are overly activated and contribute to AD evolution. The first supporting evidence came when a transgenic mouse line overexpressing TGF-β1 (Wyss-Coray, Feng et al. 1995) was crossed with the PDAPP mouse model of cerebral amyloidosis (Games, Adams et al. 1995). In this setting, vascular Aβ deposits were markedly accelerated; suggesting an amyloidogenic role for TGF-β1 *in vivo* (Wyss-Coray, Masliah et al. 1997). Despite increases in vascular Aβ deposits, these bigenic animals had less parenchymal Aβ burden–suggesting opposing effects of TGF-β1 on vascular vs. parenchymal Aβ deposition (Wyss-Coray, Lin et al. 2001). These results were supported by findings in AD patients' brains from the laboratories of Tony Wyss-Coray and Lennart Mücke. Those investigators found that TGF-β1 mRNA levels were ~3-fold increased in AD patient frontal cortex vs. non-demented controls. Further, positive correlation was noted between TGF-β1 mRNA levels and vascular Aβ deposits (Wyss-Coray, Masliah et al. 1997) while an inverse association was found between TGF-β1 mRNA and parenchymal Aβ deposits (Wyss-Coray, Lin et al. 2001). From an innate immune perspective, increased TGF-β1 abundance in AD frontal cortex may pathologically inhibit 'beneficial neuroinflammation' in the AD brain. Taken together, these findings raise the interesting and important question of the cellular mechanistic underpinnings of the TGF-β/AD pathology relationship.

TGF-β signaling often functions to suppress inflammation, and the initial report from the Town laboratory showed that inhibiting TGF-β signaling in peripheral macrophages led to brain infiltration of these cells and resolution of cerebral amyloidosis in concert with increased brain interleukin-10 (IL-10) levels (Town, Laouar et al. 2008). Specifically, TGF-β-Smad 2/3 signaling was genetically interrupted in peripheral macrophages (as opposed to brain-resident microglia) by engineering a CD11c promoter-driven dominant-negative TGF-β type II receptor transgene in C57BL/6 mice (CD11c-DNR mice) (Laouar, Town et al. 2008). CD11c-DNR mice were subsequently crossed with the Tg2576 AD mouse model; behavioral impairment and AD-like pathology were evaluated (Town, Laouar et al. 2008). Notably, bigenic animals exhibited partial amelioration of cognitive impairment and presented with reduced astrocytosis and concomitant ~90% attenuation of brain parenchymal Aβ and reduced cerebral amyloid angiopathy (Town, Laouar et al. 2008). These therapeutic effects were associated with increased accumulation of Aβ-containing peripheral mononuclear phagocytes in and around cerebral vessels and Aβ plaques. Further,

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Aβ could be localized within the cytoplasm of these cells, suggesting a productive Aβ phagocytosis/clearance response. Based on these findings, it appears that releasing TGF-β immune suppression on peripheral monocytes returns these cells to homeostasis; allowing them access into the brain while simultaneously maximizing their Aβ phagocytic potential. As ex vivo validation of the latter, there was ~3-fold increased Aβ phagocytosis by CD11c-DNR *vs.* wild-type macrophages (Town, Laouar et al. 2008). These results suggest that inhibition of TGF-β-Smad 2/3 signaling promotes peripheral mononuclear phagocyte recruitment to brains of cerebral amyloid-depositing transgenic mice and reduces Aβ burden *via* phagocytic clearance. At face value, our data and those of Wyss-Coray and Mücke might seem at odds with each other – blocking TGF-β signaling and overexpressing brain TGF-β1 both reduce parenchymal amyloid plaques. However, it is important to note that the act of genetically or pharmacologically inhibiting the TGF-β-Smad 2/3 pathway shifts downstream signaling to activate TGF-β-Smad 1/5/8 signaling in mononuclear phagocytes; thereby mimicking effects of TGF-β itself (Town, Laouar et al. 2008). Specifically, it seems that the act of blocking Smad2/3 signaling results in promotion of Smad1/5/8 signaling, which is associated with increased macrophage Aβ phagocytosis.

Interleukin-10

Often in concert with TGF-β, IL-10 suppresses overly exuberant inflammatory responses and inhibits effector function of myeloid cells by blocking pro-inflammatory cytokine pathways (Banchereau, Pascual et al. 2012). Several lines of evidence implicate aberrant IL-10 signaling in AD. Notably, elevated IL-10 signaling was observed in reactive glia neighboring β-amyloid plaques in aged Tg2576 mice (Apelt and Schliebs 2001). Additionally, a functional polymorphism within the IL10 gene (-1082 A>G) has been linked to increased risk for AD in certain populations (Lio, Licastro et al. 2003; Arosio, Trabattoni et al. 2004; Vural, Degirmencioglu et al. 2009), and two recent meta-analyses of pooled studies examining the IL10-AD risk relationship reported modest statistical significance; likely due to heterogeneity between studies (Zhang 2011; Di Bona, Rizzo et al. 2012). In the Di Bona and coworkers meta-analysis, it is interesting that the -1082 A risk relationship with AD was more evident in the oldest AD patients. While the IL10 -1082 A allele reportedly lowers IL-10 protein abundance in healthy control plasma (Ma 2005), due to unavailability of data from AD patient plasma it is not possible to draw a definitive conclusion. Nonetheless, these results could be interepreted as a ‘double edged sword,’ where blocking inflammation is beneficial early on and prior to conversion from cognitively healthy to AD-type dementia; whereas older individuals ‘on the cusp’ of dementia convert more quickly if immunity/inflammation is suppressed. This emerging concept has gained support from the first and only AD primary prevention trial for non-steroidal anti-inflammatory drugs (Breitner, Baker et al. 2011).

Interestingly, when CD11c-DNR mice were crossed with Tg2576 animals, we noted that brain IL-10 levels were elevated. There are several possible explanations for this result; cerebral IL-10 could function as 1) a compensatory response to abrogated peripheral innate immune TGF-β signaling, 2) an effector cytokine responsible for beneficial effects of TGF-β pathway blockade, or 3) simply a marker for altered innate immune phenotype.

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To further explore how anti-inflammatory IL-10 signaling affects Aβ pathology, we and others have investigated the effects of IL-10 modulation in brains of APP transgenic mouse models. After finding that the IL-10 signaling pathway was elevated in AD patient brains, we interrogated the role of this cytokine in the context of AD. Specifically, the APP/PS1 mouse model of cerebral amyloidosis was crossed with a mouse deficient for *Il10* (APP/PS1^{+}*Il10*$^{-/-}$) (Guillot-Sestier, Doty et al. 2015). Using a novel method to quantify activated Aβ phagocytic microglia by *in silico* 3D modeling technique (Guillot-Sestier, Doty et al. 2015), activated Aβ phagocytic microglia were observed to restrict cerebral amyloidosis in APP/PS1^{+}*Il10*$^{-/-}$ mice (Guillot-Sestier, Doty et al. 2015). Notably, genome-wide RNA sequencing of APP/PS1^{+} brains showed modulation of innate immune genes that are known to promote neuroinflammation. Moreover, *Il10* deficiency preserved synaptic integrity and mitigated cognitive disturbance in APP/PS1 mice, suggesting a detrimental role of IL-10 in Alzheimer pathophysiology. To probe the mechanism underlying these findings, *in vitro* knock-down of microglial *Il10-Stat3* signaling was performed–and led to augmented Aβ phagocytosis–while addition of exogenous IL-10 had the converse effect.

In a complementary approach, Todd Golde's group investigated the effects of adeno-associated virus (AAV2/1) delivered over-expression of IL-10 in brains of two APP transgenic mouse models. In this model, IL-10 expression resulted in increased Aβ accumulation and impaired learning and memory (Chakrabarty, Li et al. 2015). Interestingly, *Il10* deficient animals had reduced apolipoprotein E (ApoE) mRNA levels by RNA sequencing (RNAseq) and quantitative real-time PCR (qPCR) (Guillot-Sestier, Doty et al. 2015), while transcriptome analyses performed by Chakrabarty et al. demonstrated enhanced IL-10 signaling with concomitantly increased ApoE expression in IL-10 expressing APP mice (Chakrabarty, Li et al. 2015). Importantly, in the Chakrabarty and colleagues report, ApoE protein was selectively increased in the plaque-associated insoluble cellular fraction, which they linked to direct interaction of ApoE with aggregated Aβ in IL-10 expressing APP transgenic mice (Chakrabarty, Li et al. 2015). In both studies, ex vivo analyses showed that IL-10 and ApoE can separately impair microglial Aβ phagocytosis. Interestingly, Il10 deficiency also partially overcame isoform-specific inhibition of human ApoE on microglial Aβ uptake (E4 > E3 > E2) (Guillot-Sestier, Doty et al. 2015). Together, these results suggest two mechanisms by which *Il10* blockade enables cerebral Aβ clearance: 1) by decreasing microglial expression of ApoE, and 2) by opposing ApoE Aβ binding and therefore endorsing Aβ phagocytosis (Guillot-Sestier, Doty et al. 2015). It is important to note that our study showed that human ApoE isoforms inhibit microglial Aβ phagocytosis (E4 > E3 > E2); whereas others have recently noted beneficial effects of increasing mouse ApoE expression (Fitz, Tapias et al. 2015; Skerrett, Pellegrino et al. 2015). However, due to salient differences between human ApoE isoforms and mouse ApoE, it is not possible to compare results across species. Additionally, while both our study and that of Chakrabarty and coworkers specifically focus on ApoE in monocyte Aβ phagocytosis, this in no way precludes other function(s) of ApoE, such as its action as an Aβ molecular chaperone involved in plaque 'seeding' (Nilsson, Arendash et al. 2004). Collectively, these studies demonstrate a negative effect of IL-10 on Aβ proteostasis and cognition in APP mouse models, and suggest that 'rebalancing' innate immunity to homeostasis by blocking the IL-10 anti-inflammatory response may be therapeutically relevant for AD.

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Conclusion

In contrast to prevailing views about the negative consequences of neuroinflammation, a growing body of literature illustrates potential benefits of activating innate immunity to mitigate AD pathology (Figure 3). Although it needs to be more fully explored whether these benefits extend to synaptic and cognitive changes seen in AD, the independent and complementary results reported here, which utilized divergent approaches, demonstrate the complex interplay between innate immunity and proteostasis in AD. Rather than shutting neuroinflammation off completely, rebalancing it toward a beneficial, homeostatic innate immune response may allow us to harness innate immunity in the fight against AD.

Trem2 and the role of innate immunity in neurodegenerative disease

Bruce Lamb, Marco Colonna, and Gary Landreth

Introduction

Recent studies have linked genetic variants of the triggering receptor expressed on myeloid cells 2 (TREM2) with increased disease risk, most prominently of Alzheimer's disease. TREM2 is a critical regulator of microglia and macrophage phenotype. In the AD brain and in mouse models of AD, plaque-associated cells express high levels of TREM2. TREM2 expression increases with age and disease progression. Genetic inactivation of TREM2 results in the near absence of plaque-associated macrophages which is accompanied by alterations in plaque burden. A central question is whether these plaque-associated macrophages represent activated microglia or derive from blood borne monocytes. The expression of TREM2 and other phagocytic receptors can be induced by nuclear receptor agonists and stimulate phagocytosis. These studies have demonstrated a critical role for TREM2 in neurodegenerative disease pathogenesis.

TREM2 deficiency impacts amyloid pathology - Discrepant results on origin of myeloid cells surrounding amyloid plaques in AD models

Lamb's work emphasized how the cells of the innate immune system respond to disease or injury-related perturbations of neuronal function. There is a substantial body of evidence demonstrating that robust and continuous communication between neurons and microglia is required for normal brain homeostasis. The Lamb lab, and others, have shown that neuronal microglial communication is mediated by cell surface ligand-receptor pairs (Kierdorf and Prinz 2013). For example, fractalkine (CX3CL1) is expressed by neurons and directly interacts with its receptor, CX3CR1 on microglia (Wolf, Yona et al. 2013). This neuron-microglia interaction results in bi-directional communication between these two cell types.

When expression of CX3CR1 is abolished in amyloidogenic murine models of AD, a reduction in the number of activated microglia and dysregulated proinflammatory cytokine expression was observed (Lee, Varvel et al. 2010). This was accompanied by diminished plaque burden, likely owing to enhanced microglial phagocytosis. CX3CR1 loss in a mouse model of tau pathology resulted in enhanced phosphorylation and aggregation of tau in neurons which was associated with behavioral impairment (Bhaskar, Konerth et al. 2010).

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The tau pathology was postulated to arise from elevated levels of microglial-derived IL-1β, consistent with the view that neuroinflammation is mechanistically linked to tau-related neurodegeneration (Maphis, Xu et al. 2015). These data provided direct support for the idea that the neuron-microglia interactions in the normal brain act to suppress microglial 'activation' and promote neuronal homeostasis. The ability of microglial dysfunction to cause neurodegeneration by perturbation of signaling through a different microglial-neuronal interaction was demonstrated in individuals with Nasu-Hakola disease (Golde, Streit et al. 2013). Nasu-Hakola disease is a progressive neurodegenerative disorder that arises from loss of function mutations in the microglial cell surface receptor TREM2 or its intracellular signaling adapter, DAP12 (aka TYROBP). Importantly, genetic variants of TREM2 have recently been shown to confer a dramatically increased risk for Alzheimer's disease (Guerreiro, Wojtas et al. 2013; Johnson, Stewart et al. 2013; Jonsson, Stefansson et al. 2013) and other neurodegenerative diseases (reviewed in Painter, Atagi et al. 2015). The discovery of this linkage has focused attention on the critical roles of microglia / macrophages in neurodegenerative disease.

A recent study from the Colonna laboratory analyzed the impact of TREM2 deficiency and haplo-insufficiency on the microglial response to Aβ deposition in the 5XFAD mouse model of AD, which contains 5 mutations in human transgenes encoding the amyloid precursor protein (APP) and presenilin 1 (PS1) (Wang, Cella et al. 2015). Aβ deposition induced activation and accumulation of microglia around Aβ plaques in TREM2-sufficient mice; in contrast, microglia did not cluster around Aβ plaques in TREM2-deficient or haplo-insufficient mice, indicating a defective response to Aβ deposition. Moreover, transcriptional profiling of microglia demonstrated that TREM2 deficiency reduced the expression of genes associated with microglial activation, such as phagocytic receptors, costimulatory molecules, inflammatory cytokines and trophic factors. The lack of trophic factors correlated with apoptosis of TREM2 deficient microglia *in vitro*. These results indicate that TREM2 promotes a broad array of microglial functions in response to Aβ deposition. TREM2 deficiency resulted in increased Aβ accumulation in the hippocampus of 5XFAD mice at 8 months of age. TREM2 haploinsufficient 5XFAD mice also displayed a trend toward an increase in Aβ immunoreactivity and a significant increase in insoluble Aβ by ELISA compared to TREM2 sufficient 5XFAD mice. This finding contrasts with what was observed in 3-7 month old APP/PS1 mice hemizygous for TREM2 which exhibited no significant change in plaque burden (Ulrich, Finn et al. 2014). It will be important to determine whether these different results are due to the different AD models used or whether TREM2 might have different roles at different stages in pathology. Nonetheless, the data suggest that the TREM2-dependent activation of microglia is required to limit Aβ pathology.Wang et al. also investigated potential ligands of TREM2, which may be relevant to AD. We found that TREM2 binds anionic and zwitterionic phospholipids that may become exposed during Aβ accumulation due to neuronal and glial apoptosis, myelin degradation and formation of aggregates between Aβ and phospholipids (Wang, Cella et al. 2015). In contrast, microglia expressing the R47H variant of TREM2 associated with AD had a reduced capacity to bind phospholipids. The model emerging from this study is that TREM2 senses changes in the lipid microenvironment that result from Aβ accumulation and neuronal degeneration, which triggers signals that activate microglial capacity to limit further Aβ accumulation.

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Finally, the Colonna lab addressed the origin of myeloid cells around the amyloid plaques using parabiosis experiments using TREM2-sufficient and TREM2-deficient 5XFAD mice developing Aβ accumulation. Preliminary results suggest that, in these settings, myeloid cells clustered around Aβ plaques derive from brain resident microglia rather than peripheral blood monocytes.

Recent studies reported by Jay et al. (Jay, Miller et al. 2015) found that in two mouse models of AD (APPPS1 and 5XFAD) and in humans with AD, TREM2 was highly expressed by macrophages that were associated with amyloid plaques. TREM2 expression increased progressively with age and Aβ pathology in the mouse models. Closer examination using immunohistochemistry and flow cytometry revealed that the TREM2+ cells expressed markers, which identify a subset of blood-borne monocytes. Indeed, they found an age-dependent increase in the number of cells co-expressing TREM2 and high levels of the peripheral monocyte marker CD45. These cells also expressed the canonical monocyte marker Ly6C, consistent with their systemic origin. Immunohistochemical analysis revealed that the resident microglia, identified by expression of P2YR12, did not express detectable levels of TREM2 and were not associated with plaques, whereas the TREM2+, CD45 brightly staining cells were invariably plaque-associated. These data suggest that circulating monocytes infiltrate the AD brain and migrate to plaques in the above mentioned mouse models and at the respective ages. Remarkably, plaque-associated macrophages were virtually absent in the Trem2$^{-/-}$ mice. Moreover, mRNA levels for CD11b, CD68, F4/80 and CD45 were dramatically reduced in *Trem2*$^{-/-}$ mice, while the microglial marker P2YR12 was unchanged. These findings provide direct evidence that TREM2 expression is required for the infiltration and/or survival of these myeloid cells and their association with deposited Aβ.

The plaque-associated macrophages in the AD brain exhibit an ‘activated’, proinflammatory phenotype, secreting cytokines and other inflammatory molecules. The *Trem2*$^{-/-}$ mice had lower transcript levels of IL-6 and IL-1β, but had elevated expression of anti-inflammatory markers Ym1 and Fizz1, reflective of the critical influence of TREM2 expressing cells on the inflammatory status in the AD brain.

The effect of TREM2 deficiency on neuroinflammation and macrophage number and distribution led to the examination of its effect on plaque burden. APP/PS1-21 (herein termed APP/PS1) mice exhibit robust plaque deposition at 4 months of age in both the cortex and the hippocampus (Radde et al., 2006). In APP/PS1 mice lacking TREM2 expression, there was a 70% reduction in plaque burden, as evaluated by 6E10 or Thioflavin S staining in the hippocampus. Analysis of Aβ40/42 levels revealed a similar reduction in both soluble and insoluble Aβ peptide concentrations. This effect was restricted to the hippocampus, as there were no significant changes observed in the cortex. It should be noted that Ulrich et al. found no change in plaque levels in APP/PS1 mice heterozygous for TREM2 deficiency (Ulrich, Finn et al. 2014).

Finally, as presented by Dr. Richard Ransohoff, the Lamb lab tried to address the origin of myeloid cells around the amyloid plaques by bone marrow chimera experiments using head sparring radiation of APP/PS1 mice, whose bone marrow was replaced with that from

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Trem2$^{LacZ/+}$ mice. Preliminary results suggest that, following five months of reconstitution, myeloid cells clustered around Aβ plaques stained positive for β-galactosidase expression, indicating the origin of some the TREM2+ cells from peripheral blood monocytes rather than brain resident microglia.

Thus, these data suggest an unexpected role for TREM2 in AD pathology, enhancing inflammation through allowing the accumulation of peripherally derived myeloid cells around plaques and promoting amyloid accumulation. This is of particular interest since an unexplained facet of the AD is the inability of the macrophages to mount a phagocytic response and clear the deposited amyloid, despite the presence of cell surface receptors which can stimulate this response, such as TREM2, MerTK and Axl. Heppner and colleagues have demonstrated that there is an amyloid-dependent inactivation of phagocytosis, but the basis of this effect is unknown (Krabbe, Halle et al. 2013).

The phenotype of myeloid cells is regulated through the actions of ligand activated, heterodimeric type II nuclear receptors which act broadly to suppress inflammatory gene expression and promote the expression of genes associated with tissue repair and phagocytosis. In the brain, the principal type II nuclear receptors are PPARγ, PPARδ and LXR, that heterodimerize with retinoid X receptors (RXR) to form a functional transcription factor. The dimeric nuclear receptors bind to response elements positioned in the enhancers and promoters of their target genes, and directly regulate gene expression (Gosselin, Link et al. 2014). It has recently been appreciated that TREM2 expression is directly regulated by agonists of RXR (Daniel, Nagy et al. 2014; Lefterov, Schug et al. 2015). Importantly, Savage et al. (Savage, Jay et al. 2015) found that agonists of PPARγ, PPARδ and RXR stimulate the expression of the phagocytic receptors MerTK and Axl by microglia and macrophages in the brain of two different murine models of AD. Importantly, previous work documented that treatment with nuclear receptor agonists stimulated phagocytosis by these cells *in vitro* and in vivo, in the latter case this resulted in a reduction in plaque burden (Skerrett, Malm et al. 2014).

Immunohistochemical staining of the brains of AD mice revealed that MerTK and Axl expression was found almost exclusively on plaque-associated macrophages, and was not detected on parenchymal microglia. This finding led to experiments testing if these cells also expressed TREM2. We found that MerTK expressing macrophages were TREM2+ when evaluated by immunohistochemical staining or by flow cytometry. Moreover, the MerTK/Axl expressing cells were also CD45^{hi}, suggesting that these cells were derived from infiltrating peripheral monocytes. Treatment of mice with the RXR agonist bexarotene resulted in no change in the number of MerTK/Axl or TREM2 expressing cells, but elevated the levels of receptor expression. Thus, these data clearly demonstrate that plaque-associated macrophages express three different receptors that function to regulate phagocytosis and these cells exhibit markers consistent with their origin as blood-borne monocytes which infiltrate the AD brain.

Subsequent studies tested if nuclear receptor activation could restore phagocytic competence to plaque-associated macrophages in the AD brain. Using an ex vivo phagocytosis assay (Krabbe, Halle et al. 2013), Krabbe and colleagues reported that phagocytosis by plaque-

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associated macrophages was suppressed in the AD brain. These findings are consistent with the age-dependent accumulation of plaque burden in AD patients and mouse models. We replicated these findings using 12-month-old APP/PS1 mice. We found that treatment of AD mice with bexarotene for 5 days before preparing brain slices restored phagocytic competence to the plaque-associated macrophages, coincident with the induction of MerTK/Axl expression. Moreover, activation of MerTK/Axl in APP/PS1 brain sections with a specific ligand, Gas6, resulted in phagocytic activity that was similar to that of non-transgenic mice. Importantly, an antibody blocking MerTK function inhibited phagocytosis in both wild-type and mouse models of AD. These studies directly demonstrated that nuclear receptor-stimulated phagocytosis is reliant upon MerTK expression and function.

These studies support the hypothesis that the plaque-associated macrophages are derived from monocytes in mouse models of AD. These cells express high levels of TREM2 and the phagocytic receptors MerTK and Axl, and their expression is coordinately regulated by nuclear receptors. The presence of plaque-associated macrophages is reliant upon TREM2 expression. Significantly, the TREM2+ macrophages exhibit a robust inflammatory phenotype, a finding that contravenes the postulated anti-inflammatory actions of TREM2. The phagocytic activity of these cells can be stimulated by treatment of AD mice with nuclear receptor agonists.

Conclusions and controversies

One important and consistent conclusion arising from studies in the three laboratories is that TREM2 deficient mouse models of AD exhibit reduced inflammation and myeloid cell accumulation around plaques. However, there are also a number of inconsistencies across the three studies, including the plaque burden in TREM2 deficient mouse models of AD and the findings concerning the origin of the respective myeloid cell population. Reduced plaque burden was observed in the hippocampus of 4-month-old APP/PS1 mice, whereas significantly increased plaque levels were found in the hippocampus of 8-month-old 5XFAD mice. These findings raise the possibility of age or disease progression-dependent difference in the TREM2 deficient macrophages. These studies were performed in two different mouse models of AD and TREM2 KO mice which could also influence the experimental outcomes. Efforts are currently underway to reconcile these disparate findings.

Another point of controversy is whether the plaque-associated macrophages represent the activation and accumulation of resident microglia or alternatively that these cells derived from blood borne monocytes that infiltrate the AD brain. Parabiosis experiments conducted in the Colonna lab argue that these cells are microglia, whereas flow cytometry and bone marrow chimera experiments from the Lamb and Landreth labs suggest they arise from blood borne monocytes. Clearly, additional work using genetic models is needed to resolve this important issue.

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Inflammatory PGE_2 receptors in models of Alzheimer's disease

Katrin I. Andreasson

Introduction

Pathological changes in AD consist of amyloid ß accumulation, tau phosphorylation, and synaptic and neuronal loss, which develop steadily over years to decades in the context of a chronic and non-resolving pro-inflammatory response. Recent genome-wide association studies (GWAS) and systems biology approaches have confirmed a central role for the microglial inflammatory response in AD development (Lambert, Heath et al. 2009; Augustin, Lichtenthaler et al. 2011; Hollingworth, Harold et al. 2011; Naj, Jun et al. 2011; Cruchaga, Kauwe et al. 2013; Guerreiro, Wojtas et al. 2013; Lambert, Ibrahim-Verbaas et al. 2013; Zhang, Gaiteri et al. 2013; Jiang, Yu et al. 2014; Luo, Li et al. 2014; Gjoneska, Pfenning et al. 2015), and support the hypothesis that maladaptive inflammatory responses play a central role in the pre-clinical development of AD (Heneka, Golenbock et al. 2015; Malm, Jay et al. 2015; Mhatre, Tsai et al. 2015).

NSAIDs in Alzheimer disease

These recent findings complement conclusions from epidemiologic studies demonstrating reduced risk of developing AD in cognitively healthy aging populations taking non-steroidal anti-inflammatory drugs (NSAIDs) (McGeer, Schulzer et al. 1996; Stewart 1997; in t' Veld, Ruitenberg et al. 2001; Szekely, Thorne et al. 2004; Vlad, Miller et al. 2008; Breitner, Baker et al. 2011). A primary action of NSAIDs is the enzymatic inhibition of the inflammatory cyclooxygenases COX-1 and COX-2, cytosolic enzymes that generate PGH_2 from membrane stores of arachidonic acid, the precursor of the prostaglandins PGE_2, PGD_2, PGI_2, and PGF_{2a}, and the thromboxane TXA_2. Structurally distinct NSAIDs reduce the risk of developing AD in large epidemiologic studies (Stewart 1997; in t' Veld, Ruitenberg et al. 2001; Vlad, Miller et al. 2008) suggesting that inflammatory prostaglandin signaling may play an important role in pre-clinical development of AD. NSAIDs, however, cannot be effective for large scale AD prevention because both toxic as well as beneficial downstream prostaglandin signaling pathways are inhibited, leading to adverse renal and gastric toxicities and an increased risk for vascular and cerebrovascular disease (Funk and FitzGerald 2007). Recent genetic studies of prostaglandin receptors in mouse preclinical models have identified beneficial prostaglandin signaling pathways downstream of NSAID action, notably the vasodilatory prostacyclin (PGI_2) receptor and the neuroprotective, anti-inflammatory, and vasodilatory PGE_2 EP4 receptor (Egan, Lawson et al. 2004; Funk and FitzGerald 2007; Shi, Johansson et al. 2010; Liang 2011; Woodling, Wang et al. 2014). In addition, findings point to a beneficial function of PGJ_2, a cyclopentenone metabolite that does not act via classical prostanoid G-protein coupled receptors, but exerts anti-inflammatory effects via the NFkB and PPARg transcription factors (reviewed in Straus and Glass 2001).

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Prostacyclin signaling

Levels of prostacyclin (PGI_2), considered to be anti-inflammatory and vasodilatory, are decreased in CSF of probable AD subjects (Montine, Sidell et al. 1999). Conversely levels of PGE_2, widely considered an immune modulatory signaling molecule, are increased 5-fold in the cerebrospinal fluid (CSF) of patients with early probable AD (Montine, Sidell et al. 1999; Combrinck, Williams et al. 2006), suggesting that PGE_2 signaling may play a role in promoting pre-clinical development of AD. PGE_2 binds four G-protein coupled receptors (GPCRs) termed E-prostanoid receptors (EP1-4) that have distinct downstream signaling cascades and cellular distributions in the brain. Figure 4 depicts mechanisms of PGE_2 action. In vivo, all four EP receptors are expressed in neurons, with EP2, EP3, and EP4 receptors, but not the EP1 receptor, expressed in microglia (Slawik, Volk et al. 2004; Shi, Johansson et al. 2010; Johansson 2013; Woodling, Wang et al. 2014; Johansson, Woodling et al. 2015). Activation of EP receptors leads to changes in the production of cAMP and/or phosphoinositol turnover and Ca^{2+} mobilization. EP2 and EP4 receptors couple positively to G_s to increase cAMP formation whereas EP3 couples negatively to cAMP through G_i.

APP transgenic models of AD display loss of synapses associated with development of spatial memory deficits along with inflammation. Synaptic injury and behavioral deficits in murine AD models are associated with the presence of Aß oligomers, which are directly toxic to synapses (Mucke and Selkoe 2012), and neuroactive inflammatory mediators, for example IL1-β (Hein, Stasko et al. 2010) or TNF-α (Medeiros, Prediger et al. 2007).

Early studies examining the in vivo function of the PGE_2 EP2 receptor in models of innate immunity demonstrated a dramatic reduction in lipid peroxidation following intracerebroventricular (ICV) administration of lipopolysaccharide (LPS) in EP2 null mice (Montine, Milatovic et al. 2002). Additionally, *in vitro* microglial EP2 signaling elicited paracrine neurotoxicity in co-cultures of neurons and LPS-primed microglia, an effect dependent on increased inducible nitric oxide synthase (iNOS) and COX-2 activities (Shie, Montine et al. 2005). *In vivo*, APP/PS1, global deletion of EP2 led not only to significant decreases in lipid peroxidation and amyloid accumulation (Liang 2007), but also to reduced expression of inflammatory enzymes iNOS and NADPH (Liang, Wang et al. 2008).

A cell-specific role for microglial PGE_2 EP2 signaling in suppressing phagocytosis of Aβ fibrils was initially demonstrated in an ex vivo preparation using AD brain sections coated with EP2-/- microglia (Shie, Breyer et al. 2005). This observation was subsequently confirmed in vivo, where deletion of EP2 in the APP/PS1 model reduced amyloid plaque load, an outcome reflecting both a more benign inflammatory milieu and an enhanced clearance of Aβ peptides (Liang, Wang et al. 2005; Keene, Chang et al. 2010). Recent conditional knockout strategies of microglial EP2 receptor using the Cd11bCre recombinase line, which excises loxP-flanked sequences in myeloid cells (Johansson 2013), restored healthy microglial responses to Aβ peptides (Johansson, Woodling et al. 2015), increasing microglial clearance of Aβ peptides and suppressing toxic inflammatory gene expression. Transcriptomics of microglia isolated from adult mice lacking microglial EP2 receptor additionally revealed that ablation of microglial EP2 receptor increased expression of insulin-like growth factor-1 (IGF-1) in response to Aβ peptide stimulation (Johansson,

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Woodling et al. 2015) and enhanced both IGF-1 and PPAR signaling; in this screen expression of RXRγ was enhanced with ablation of microglial EP2, and RXRγ along with its binding partner PPARγ reduce proinflammatory gene expression (Heneka, Landreth et al. 2007) and enhance clearance of Aβ peptides (Yamanaka, Ishikawa et al. 2012). The upregulation of these genes in microglial EP2 knockout mice suggests that EP2-deficient microglia respond to $A\beta_{42}$ peptides *in vivo* by inducing anti-inflammatory and Aβ-clearing nuclear hormone receptor signaling genes. In support of a beneficial effect of reduced EP2 signaling, conditional deletion of microglial EP2 in the APP/PS1 model prevented synaptic injury and spatial memory deficits (Johansson, Woodling et al. 2015).

Toxic effects of microglial E-prostanoid receptors

The toxic effects of microglial EP2 contrast significantly with the beneficial anti-inflammatory effects of microglial EP4 *in vivo*. Initial *in vivo* studies of innate immune responses to LPS identified a pronounced anti-inflammatory effect of microglial EP4 signaling associated with reduced nuclear translocation of NF-κB subunits p65 and p50 (Shi, Johansson et al. 2010) in myeloid cells. Subsequent studies confirmed the anti-inflammatory function of microglial EP4 signaling, where in primary microglial cells, EP4 stimulation attenuated levels of $A\beta_{42}$-induced inflammatory factors and potentiated phagocytosis of $A\beta_{42}$ (Woodling, Wang et al. 2014). Transcriptomic studies demonstrated that microglial EP4 signaling broadly opposed $A\beta_{42}$-driven gene expression changes, with a significant suppression of IRF1, IRF7, and NF-κB transcription factor regulated genes. In vivo, APP/PS1 mice deficient for microglial EP4 demonstrated increased inflammatory gene expression, oxidative protein modification, and Aβ deposition in brain at early but not late stages of pathology, suggesting an anti-inflammatory function of microglial EP4 signaling early in the APP/PS1 model. Interestingly, both EP2 and EP4 receptors are positively coupled to cAMP, yet have opposing inflammatory functions in models of Aβ peptide mediated neuroinflammation. This may be explained in part by the carboxy terminal cytoplasmic tail of the EP4 receptor, which is significantly longer than that of the EP2 receptor and may recruit distinct signaling molecules (Takayama, Sukhova et al. 2006; Minami, Shimizu et al. 2008).

The EP3 receptor is a central component in the regulation of the febrile response (Ushikubi, Segi et al. 1998; Lazarus, Yoshida et al. 2007). In the brain, EP3 is mainly expressed in neurons, although it can be induced in striatal microglia following injection of the excitotoxin quinolinic acid (Slawik, Volk et al. 2004). The function of EP3 signaling has been examined in models of AD, including the intracerebroventricular (ICV) Aβ injection model, which elicits a potent and long lasting inflammatory response to Aβ peptides (Letiembre, Liu et al. 2007; Walter, Letiembre et al. 2007) and in the APP/PS1 model. In a recent study (Shi, Wang et al. 2012), the induction of pro-inflammatory gene expression, cytokine generation, and lipid peroxidation following ICV $A\beta_{42}$ was markedly reduced in EP3 null mice, suggesting that inflammatory EP3 signaling triggers a deleterious response to Aβ peptides. In the APP/PS1 model, deletion of EP3 reduced expression of proteins capable of increasing oxidative injury, including iNOS, components of the NADPH oxidase complex, and COX-2. Moreover, suggestive of a role in Aβ clearance, APP/PS1 mice lacking either one or both EP3 alleles exhibited lower amyloid accumulation. However, EP3

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deletion also affected the generation of Aβ peptide from APP, as prior studies have correlated increased oxidative stress and inflammation with increased expression and activity of β-secretase (BACE) (Tamagno, Bardini et al. 2002; Apelt, Bigl et al. 2004). Indeed, deletion of the EP3 receptor in the APP/PS1 background decreased BACE expression and activity. This would suggest that in the APP/PS1 model, EP3 signaling both suppresses Aβ clearance and enhances Aβ peptide generation through increased BACE expression and activity. Interestingly, loss of just one allele of EP3 in the APP/PS1 background had beneficial effects in lowering Aß peptide levels.

Conclusion

In conclusion, studies examining mechanisms underlying the preventive effects of NSAIDs in AD development have identified maladaptive inflammatory responses mediated by the EP2 and EP3 receptors, but at the same time beneficial immune modulatory responses mediated by the EP4 receptor. This dichotomy of beneficial vs toxic prostaglandin signaling argues that NSAIDs are not an optimal mechanism to halt preclinical development of AD. The opposing actions of the EP2/EP3 and EP4 receptors highlight the importance of targeting selected EP receptors downstream of COX-1/COX-2. This is a potential future indication, as we await the identification and validation of biomarkers that can reliably predict subjects at risk for AD.

Arginine Metabolism: Neuroinflammation and Neurodegenerative Disorders

Leslie A. Sandusky, Maj-Linda B. Selenica, Daniel C. Lee

Introduction

Arginine metabolism represents a critical branch-point that impacts several systems, notably the production of nitric oxide (NO) signaling and polyamines. Arginases (Arg) (including arginase 1 and 2; Arg1 and Arg2, respectively) or nitric oxide synthases (NOSes), comprised of inducible, neuronal, and epithelial nitric oxide synthase, metabolize arginine to generate either ornithine and subsequent polyamines (putrescine, spermidine, and spermine) or nitric oxide, respectively (Morris 2004) (Figure 5). Polyamines remain critical for growth, as they are known to interact with macromolecules such as DNA and RNA, both electrostatically and covalently, promoting different cellular effects (Minois, Carmona-Gutierrez et al. 2011; Lightfoot and Hall 2014). Polyamines carry positive charges on nitrogen at physiological pH and become "supercations"; yet they distribute the charge along the entire length of the carbon chain making them structurally unique and distinct from point-charges of other cellular bivalent cations. Polyamines represent phylogenetically old molecules; and they continue to provide new purpose for biology and diseases. For example, an alternative arginine metabolism pathway also leads to polyamine production through the putative neurotransmitter agmatine plus carbon dioxide via arginine decarboxylase (ADC) and subsequently to putrescine via agmatinase (Halaris and Plietz 2007; Bernstein, Derst et al. 2011). Agmatine binds several receptors notably imidazoline receptors (I_1R, I_2R) with high affinity in the CNS, alpha 2 adrenoceptors (α-2), NMDA receptors and 5-HT receptors (Molderings and Haenisch 2012). It also produces a variety of physiological and pharmacological effects. So, while this endogenous molecule has been known for 100 years,

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its biosynthesis and therapeutic potential remain relatively unexplored. It was only recently that decarboxylation of arginine was found in the brain. The controversy and neglect in agmatine research arose in relation to the difficulty of demonstrating ADC activity in mammalian systems compared to bacteria, plants and fish. It wasn't until 1994 that Ries and colleges demonstrated ADC activity and identified agmatine in the mammalian brain (Li, Regunathan et al. 1994).

Arginine metabolism and its by-products become essential in regards to the inflammatory challenge presented or the cytokines induced (proinflammatory versus anti-inflammatory). Two enzymes capable of regenerating the pool of L-arginine comprised of argininosuccinate synthetase 1 (*ASS1*) which converts citrulline (a NOS product) to argininosuccinate and argininosuccinate lyase (*ASL*), which converts argininosuccinate to L-arginine (Wiesinger 2001. Interestingly, proinflammatory stimuli can also induce both ASS1 and ASL concomitantly with NOS, suggesting a feedforward or recycling of citrulline and L-arginine in response to inflammation (Nagasaki, Gotoh et al. 1996). Furthermore, coinduction of NOS and arginase 1 with LPS has been observed (Sonoki, Nagasaki et al. 1997). Many reports show increased arginase 1 expression through the conical IL-4/ IL-13 JAK/ STAT pathway (Welch, Escoubet-Lozach et al. 2002; Mantovani, Sica et al. 2004; Nolan, Maher et al. 2005; Martinez, Helming et al. 2009; Varin and Gordon 2009) but it is also upregulated by proinflammatory stimuli and other insults (Rodriguez, Hernandez et al. 2005; El Kasmi, Qualls et al. 2008; Lee, Rizer et al. 2010; Lee, Ruiz et al. 2013; Fenn, Hall et al. 2014; Cherry, Olschowka et al. 2015). It remains clear that arginine metabolism will be governed by the inflammatory milieu, cell type, and combination of stimuli elicited. While arginine metabolism is historically considered to increase primarily in microglia, arginase is also expressed in neurons at lower levels and may compete with neuronal NOS (Yu, Iyer et al. 2001; Yu, Iyer et al. 2002; Yu, Yoo et al. 2003; Peters, Berger et al. 2013; Quirie, Demougeot et al. 2013) which may independently impact neural transmission and subsequent cognitive processing. Furthermore, by-products of the arginine metabolism that produce polyamines and nitric oxide can provide opportunities for post-translational modifications of proteins including polyamination and S-nitrosylation, which can modify aggregation-prone/disordered proteins including tau, (Tucholski, Kuret et al. 1999; Pevalova, Filipcik et al. 2006; Reynolds, Reyes et al. 2006; Reyes, Reynolds et al. 2008; Reyes, Fu et al. 2011; Reyes, Geula et al. 2012; Fontaine, Sabbagh et al. 2015), alpha synuclein (Kotzbauer, Giasson et al. 2004; Yu, Xu et al. 2010; Liu, Qiang et al. 2011) and Aβ (Kummer, Hermes et al. 2011). This suggests an interaction of polyamine metabolism, neuroinflammation and neuropathological markers associated with neurodegenerative disease states.

Altered Arginine and Polyamine Metabolism in Alzheimer's disease

Two independent studies show that modulating arginine metabolism and/ or the polyamine system in animal models of tau or amyloid deposition alter disease pathology (Hunt, Nash et al. 2015; Kan, Lee et al. 2015). Additionally, in the previous two studies, amyloid and tau pathology changed L-arginine levels. More specifically, viral-mediated tau overexpression reduced arginase 1 mRNA levels and rTg4510 tau transgenic mice showed increased levels of L-arginine. Several studies showed altered polyamines, polyamine metabolites and L-

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arginine metabolism in CSF, plasma, or brain tissue of patients with mild cognitive impairment (MCI) and AD (Morrison and Kish 1995; Inoue, Tsutsui et al. 2013; Trushina, Dutta et al. 2013; Liu, Fleete et al. 2014; Graham, Chevallier et al. 2015). A recent report using untargeted blood-based metabolic profiling revealed that L-arginine and polyamine metabolism was differentially disrupted between control patients, patients with MCI and AD converters, which could be used to predict converters for up to two years (Graham, Chevallier et al. 2015). As more evidence surfaces regarding altered arginine and polyamine metabolism in AD, it is tempting to speculate activation of the urea cycle in the brain under conditions of chronic inflammation or neurodegeneration. Although this idea has been previously suggested in AD (Hansmannel, Sillaire et al. 2010), the activation of the urea cycle and changes in arginine metabolism may be disease-specific (Patassini, Begley et al. 2015).

Impact of L-arginine and Polyamine on Autophagy and Inflammation

The impact on cell biology versus pathology likely depends on the interplay of arginase, NOS, and their downstream products, polyamines or nitric oxide, and the availability of L-arginine for neurons versus microglia. Recently, increasing autophagy has been suggested as a therapeutic strategy for aggregation-prone/disordered proteins. Mammalian target of rapamycin (mTOR) is a negative regulator of autophagy and sensitive to amino acid availability and inhibition of mTOR signaling has been shown to impact neuropathology in certain models of AD (Wullschleger, Loewith et al. 2006; Sancak, Peterson et al. 2008; Caccamo, De Pinto et al. 2014). Furthermore, recent evidence has shown that mTOR and components of the mTOR complex are reduced during continual arginase 1 overexpression (Hunt, Nash et al. 2015). Additionally, arginase 1 overexpression significantly reduced multiple aspects of the tau phenotype associated with inflammation and microglial/macrophage activation including Iba-1 and CD45 along with various cytokines (i.e. IL-1β, TNFα, IL-12, IL-10, KC-Gro) (Hunt, Nash et al. 2015), while conditional deletion of arginase 1 in myeloid cells showed increased phospho-tau pathology following adeno-associated viral tau truncated at D421 (a caspase cleaved site which is preferentially cleared through autophagy (Dolan and Johnson 2010) than mice with sufficient arginase 1 (Hunt, Nash et al. 2015). However, it remains uncertain as to which mediated the autophagy response, arginine deprivation or polyamine production. Yet, this is consistent with other reports demonstrating that L-arginine and L-arginine deprivation act as a sensor for autophagy arguably through mTOR signaling (Ban, Shigemitsu et al. 2004; Yao, Yin et al. 2008; Savaraj, You et al. 2010; Wu, Liang et al. 2011; Hsueh, Knebel et al. 2012; Banik, Renner Viveros et al. 2013; Angcajas, Hirai et al. 2014). However, making the association less clear, the longer chain polyamine spermidine also acts as an inducer of autophagy but may not occur through mTOR signaling (Eisenberg, Knauer et al. 2009; Morselli, Galluzzi et al. 2009; Morselli, Marino et al. 2011), yet it has been demonstrated to increase longevity, restore memory in various models through autophagy, and is elevated in centenarians (Eisenberg, Knauer et al. 2009; Morselli, Galluzzi et al. 2009; Madeo, Eisenberg et al. 2010; Marino, Morselli et al. 2011; Minois, Carmona-Gutierrez et al. 2011; Morselli, Marino et al. 2011; Gupta, Scheunemann et al. 2013; Minarini, Zini et al. 2013).

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Conclusion

Overall, increased metabolism of arginine through inflammation, aging, and/or injury may modulate various biological pathways that impact neurodegenerative diseases. Recent work in animal studies would suggest a role for arginase and NOS in amyloid and tau neuropathology (Hunt, Nash et al. 2015; Kan, Lee et al. 2015); however, lack of arginine metabolism or accumulation may also influence pathways including autophagy.

Systemic Inflammation and Alzheimer's Disease

Clive Holmes, Jessica Teeling

Introduction

In AD, inflammation is thought to be largely mediated by the CNS resident cells with a dominant role being played by cytokines. However, there is also a developing interest in the potential role for peripherally derived cells and other mediators of inflammation. Furthermore, there is increasing recognition that neuroinflammatory processes in the AD brain are markedly influenced by inflammation that occurs outside the CNS (Perry and Holmes 2014; Takeda, Sato et al. 2014; Goldeck, Witkowski et al. 2016)

Pre-clinical studies

In the past the parenchyma of the CNS was considered an 'immunologically privileged site' because the blood brain barrier (BBB) was thought to prevent the entry, or exit, of many molecules including antibodies from the periphery and was largely devoid of macrophages, neutrophils and lymphocytes. This historical concept has led to research on CNS inflammation being viewed in isolation and independent of systemic inflammation occurring outside the CNS. However, communication of the presence of systemic inflammation to the brain is clearly supported by the constellation of centrally derived symptoms that occur in animals following administration of TLR agonist to mimic an acute systemic bacterial or viral infection. This syndrome is known as 'sickness behavior' and includes anorexia; depression; somnolence; decreased social interaction; decreased concentration and fever, and is an evolutionary conserved, homeostatic mechanism that allows the body to adapt to an insult (Hart 1988). The state of the BBB in AD is still uncertain and, although controversial, it is possible that there may be, in limited circumstances, some direct entry of immune cells (monocytes, macrophages) from the periphery into the brain. Thus, it is known that sepsis (Lee and Slutsky 2010) and persistent peripheral inflammatory stimuli may compromise the BBB because of a breakdown of the intercellular tight junctions caused by lipopolysaccharide (LPS) and peptidoglycans (Nau, Sorgel et al. 2010). However, there are a number of other communication routes from the periphery to the brain that occur despite the presence of an intact BBB. These include:

1. Stimulation of peripheral nerves (e.g. the vagus) by cytokines and prostaglandin which signal to the medulla oblongata and are relayed to the hypothalamus (Ek, Kurosawa et al. 1998; Dantzer, Konsman et al. 2000).

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2. Direct actions of LPS or pro-inflammatory cytokines at brain areas lacking a BBB e.g. the circumventricular organs (Blatteis, Bealer et al. 1983).

3. Direct action of LPS or pro-inflammatory cytokines of perivascular macrophages in the neurovascular unit of the BBB (Matsumura and Kobayashi 2004).

In animals, unless the peripheral inflammatory event is extreme, sickness behavior induced by LPS is a relatively benign and transient phenomenon (Perry 2010). Damage limitation in the brain is in part due to the wide number of regulatory mechanisms that reduce the pro-inflammatory response to an acute challenge within the brain. Thus, following a peripheral signal the pro-inflammatory response in the brain appears to be suppressed by the increased production of a large variety of proteins from microglial cells including anti-inflammatory cytokines IL-10; TGFβ and other cytokine signaling protein suppressors (Rivest 2009).

Systemic inflammation induced by real life bacteria, for example *S. typhimurium,* leads to robust systemic inflammation and a delayed innate immune activation in the brain, with cerebral endothelial and microglial cells showing prolonged phenotype and functional changes, including enhanced sensitivity to a secondary challenge (Puntener, Booth et al. 2012).

With aging and in AD, microglial cells also show enhanced sensitivity to inflammatory stimuli with an upregulation of a range of cell surface receptors and an exaggerated microglial response to a second inflammatory stimulus. This phenomenon is called priming and its exact origins are unknown. It might be caused by microglial senescence, by prolonged exposure to the aged neuronal environment including the presence of Aβ protein, or by sustained systemic inflammation. The result is that in aged animal or AD mouse models, priming results in microglial cells that show a markedly increased production of IL-1β, and an increased production of reactive oxygen species by microglia to even a very modest peripheral inflammatory event. Thus, priming results in a microglial state that once activated cuts across the simple classification of M1 or M2 states by having elements of both (Perry, Nicoll et al. 2010; Perry and Holmes 2014).

This hypothesis is supported by animal studies showing an exaggerated inflammatory and oxidative stress response to peripheral stimuli in aged mice (Godbout and Johnson 2009), increased concentrations of IL-1β in the CNS and neuronal apoptosis in the ME7 prion mouse after peripheral challenge with the bacterial mimic LPS or the viral mimic polyinosinic-polycytidylic acid (Cunningham, Wilcockson et al. 2005; Cunningham, Campion et al. 2009; Field, Campion et al. 2010).

Clinical studies

Blood markers of peripheral inflammation

A number of studies have tried to establish differences in serum or plasma markers of inflammation between AD populations and aged matched control groups in cross sectional studies. A meta-analysis of these studies (Swardfager, Lanctot et al. 2010) suggests that overall there are increases in pro-inflammatory cytokines in AD compared with control groups, although clearly cross-sectional studies cannot establish whether this is cause or

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effect. A proteomics study with no a priori candidates reported the presence of a number of plasma inflammatory proteins including complement factors and clusterin to be associated with hippocampal atrophy and clinical progression in MCI and AD (Thambisetty and Lovestone 2010). In another AD cohort, evidence of acute and chronic systemic inflammatory diseases was associated with raised serum TNFα levels and an exacerbation of sickness behaviour like symptoms and increased cognitive decline (Holmes, Cunningham et al. 2009; Holmes, Cunningham et al. 2011).

Imaging studies

Functional magnetic resonance imaging studies of healthy human volunteers have shown that low dose *Salmonella typhi* LPS are associated with significant increases in systemic IL-6 with higher cytokine levels being associated with increased neuronal activity in the substantia nigra (Brydon, Harrison et al. 2008). PET imaging studies with direct examination of microglial activation using the Translocator Protein (TSPO) ligand have also shown preliminary evidence of increased ^{11}C-PK11195 binding in the brains of subjects with chronic aseptic systemic inflammation (Drake, Boutin et al. 2011). AD cross sectional PET studies support evidence of an up to 50% increased binding of ^{11}C-PK11195 in the Frontal and Temporal Cortex (Diorio, Welner et al. 1991; Cagnin, Brooks et al. 2001; Edison, Archer et al. 2008; Okello, Edison et al. 2009; Venneti, Lopresti et al. 2009; Wiley, Lopresti et al. 2009). Interestingly, AD studies that have examined both microglial and Aβ markers have found significant correlations between cognitive scores and both TSPO ligands ^{11}C-PK11195 and ^{11}C-PBR28 (Kreisl, Lyoo et al. 2013) but not with the amyloid ligand Pittsburgh compound B (^{11}C-PIB) binding (Edison, Archer et al. 2008; Yokokura, Mori et al. 2011) supporting the view that microglial activation rather than Aβ alone may be a key change leading to neurodegeneration.

Therapeutic strategies

Small trials in early AD with indomethacin suggested some evidence of reduced cognitive decline (Rogers, Kirby et al. 1993). However, this study was not replicated in a later follow up study (de Jong, Jansen et al. 2008). Large scale studies of other NSAIDs including naproxen (Aisen, Schafer et al. 2003) and rofecoxib (Reines, Block et al. 2004) in AD have also been unsuccessful. Randomised trials with a range of other anti-inflammatory drugs, including prednisone (Aisen, Davis et al. 2000), hydroxychloroquine (Van Gool, Weinstein et al. 2001), simvastatin (Simons, Schwarzler et al. 2002) atorvastatin (Sparks, Sabbagh et al. 2005; Feldman, Doody et al. 2010), aspirin (Bentham, Gray et al. 2008) and rosiglitazone (Harrington, Sawchak et al. 2011) have also shown no clinically significant changes in primary cognitive outcomes in patients with AD. More recently, we have conducted a small study of AD subjects using the TNF-α inhibiting agent Etanercept in AD subjects on the basis that a more targeted approach to completely block peripheral TNF-α may have a more beneficial effect. The study showed that the drug was well tolerated in the AD population and preliminary evidence of a reduction of decline on a number of clinical outcomes (Butchart, Brook et al. 2015). This study design is now being replicated in patients with early AD (amyloid positive PET scans in amnestic Mild Cognitive Impairment) as part of

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the INMIND consortium with an examination of the effects of Etanercept on neuroimaging markers of inflammation as the primary outcome.

Conclusions

Sepsis and persistent peripheral inflammatory stimuli may compromise intercellular tight junctions and subsequently lead to BBB breakdown. However, even with intact BBB, periphery-to-brain signaling may take place, e.g. through stimulation of peripheral nerves by cytokines and prostaglandin which signal to the medulla oblongata and are relayed to the hypothalamus; furthermore, LPS or pro-inflammatory cytokines may act directly at brain areas lacking a BBB such as the circumventricular organs; or in the neurovascular unit of the BBB. Microglial cells may fence the pro-inflammatory response in the brain by increasing production of anti-inflammatory cytokines IL-10; TGFβ and other cytokine signaling protein. Despite promising results in previous studies using NSAIDs, thus far no clinically relevant benefit in terms of cognitive improvement could be replicated on a large scale. However, further trials are underway using more targeted study designs, e.g. on the basis of TNF-α inhibition.

Long-term potentiation and neuroinflammation

Marina A. Lynch

Introduction

It is well documented that neuroinflammatory changes, that are characteristic of the aged brain and evident in neurodegenerative diseases, are associated with compromised cognitive function. This is also reflected in animal models where the deficit in cognitive function is accompanied by impaired synaptic plasticity. Specifically it has been consistently shown that a decrease in hippocampal long-term potentiation (LTP), which is perhaps the most studied form of synaptic plasticity, is coupled with a decrease in performance in hippocampal-dependent tasks. Neuroinflammatory changes result from loss of homeostatic control and, although several cells play contributory roles in driving neuroinflammation, activation of microglia, and also astrocytes, is crucial in the sense that they are the primary producers of inflammatory mediators, which impact negatively on synaptic plasticity (Figure 6).

Inhibitors of synaptic plasticity

It has been known for 2 decades or more that inflammatory cytokines, including IL-1β, IL-6, and both type I and type II interferons (IFN) inhibit synaptic plasticity, in particular LTP (Lynch 2015). Since learning and consolidation of memory are dependent on changes at the level of the synapse, it is not surprising that these inflammatory cytokines also inhibit various forms of memory (Donzis and Tronson 2014). Perhaps the cytokine that has elicited most interest is IL-1β and it has been shown by many groups that application of IL-1β to hippocampal slices or central administration of IL-1β inhibits LTP in the major afferent pathways in the hippocampus (Lynch 2015). The inhibitory effect of IL-1β has been shown to be blocked by the endogenous receptor antagonist, IL-1ra (Loscher, Mills et al. 2003). However, the effect of IL-1β on LTP is complex and probably dependent on concentration,

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the specific afferent pathway stimulated, the method of delivery of IL-1β and the timing (Yirmiya and Goshen 2011). Thus a deficit in LTP in IL-1R1$^{-/-}$ mice has been described (Avital, Goshen et al. 2003) while it has also been shown that IL-1ra can inhibit LTP (Schneider, Pitossi et al. 1998; Loscher, Mills et al. 2003).

Given that age-related neuroinflammatory changes are undisputed and a negative correlation between inflammation and LTP exists, it is not surprising that LTP is impaired in aged animals. It is similarly reduced in lipopolysaccharide (LPS)-treated animals and in Aβ-treated transgenic mouse models of AD, which trigger microglial activation and increased production of inflammatory cytokines (Lynch 2015). Inflammatory stimuli, such as Aβ, LPS and IL-1β exert a greater effect in aged, compared with young, animals (Minogue, Lynch et al. 2007; Patterson 2015) perhaps because the microglial activation state has shifted and the cells are primed. However, a recent study revealed that IL-1β acts to inhibit LTP because of a direct effect at the synapse and that there was an age-related increase in sensitivity to IL-1β that could be explained by an increase in the IL-1 receptor accessory protein, IL-1RAcP, which promotes inflammatory changes (Prieto, Snigdha et al. 2015). This study indicates that microglial priming is not the only factor in the age-related sensitivity to IL-1β, at least as far as LTP in synaptosomes is concerned. The authors argue that the increased expression of IL-1RAcP is driven by the age-related increase in IL-1β, so the question of the source of the IL-1β and the reason for its increased concentration remain to be addressed. One possibility is an increase in inflammasome assembly with the consequent increases in caspase 1 activation and IL-1β processing (Youm, Grant et al. 2013). Significantly, microglial activation, caspase 1 activation and hippocampal and cortical IL-1β concentrations were markedly decreased in aged NLRP3-deficient mice compared with wild type mice. These changes were associated with improved performance in hippocampal-dependent tasks providing further evidence of an inverse relationship between hippocampal IL-1β and cognitive function (Youm, Grant et al. 2013). Knocking out NLRP3 in a mouse model of AD (APP/PS1 mice) was shown to exert similar effects; the increases in caspase 1 activation and IL-1β concentration, and the deficits in hippocampal-dependent tasks and LTP that are characteristic of APP/PS1 mice, were all attenuated in NLRP3-deficient APP/PS1 mice (Heneka, Kummer et al. 2013). Interestingly, microglia in NLRP3-deficient APP/PS1 mice had characteristics of the M2-like phenotype and were more phagocytic, and consequently Aβ accumulation was reduced in these animals. Overall, these findings point to a detrimental effect of IL-1β on hippocampal function. Several factors can stimulate NLRP3 activation but the key factors that trigger this in age remain to be determined. Among the multiple possibilities are factors like HMGB1 and ATP that are released from damaged neurons and can act on TLR2/4 and purinergic receptors respectively, providing signals 1 and 2. HMGB1 has been shown to be increased in the hippocampus with age (Barrett, Costello et al. 2015) and its inhibitory effect on LTP has been described (Costello, Watson et al. 2011).

Microglia in the aged brain

The broader question of the factors that stimulate microglia to adopt a persistent inflammatory phenotype in the aged brain remains to be answered. These cells are maintained in a relatively quiescent state because of the suppressive microenvironment in

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the brain where there is a relatively high concentration of anti-inflammatory factors, where neurotransmitters like noradrenaline exert a calming influence on the cells and where microglia interact with other cells, for example neurons, through ligand-receptor interactions that also reduce cell activation. The interaction between neuronal CD200, CX3CL1, CD45 and their respective receptors on microglia prevents microglial activation. However, ageing is associated with a disruption in ligand-receptor interactions and age-related decreases in CD200 and CX3CL1, associated with microglial activation and inflammatory changes, have been described (Lyons, Downer et al. 2007; Lyons, Lynch et al. 2009; Lynch 2010; Fenn, Smith et al. 2013).

The impact of the immune system on the brain is profound (Ransohoff, Schafer et al. 2015) and the findings that peripheral immune cells access the brain under certain circumstances bring into focus the importance of understanding the impact of these infiltrating cells on resident cells in the brain. We have found that the age-related increase in blood brain barrier permeability is associated with increased infiltration of macrophages, especially in APP/PS1 mice (Minogue, Jones et al. 2014). Although macrophages that infiltrate the brain are efficient phagocytes, particularly of Aβ, our evidence suggests that macrophages from aged animals, and also APP/PS1 mice, are more responsive to inflammatory stimuli (Barrett, Costello et al. 2015; Barrett, Minogue et al. 2015). Therefore, when these macrophages encounter the inflammatory environment that exists in these mice, they have the potential to exacerbate the already-existing neuroinflammation (Costello et al. unpublished). Macrophages may also be the source of the increase in IFNγ observed with age and in APP/PS1 mice (Minogue, Jones et al. 2014). Significantly, IFNγ inhibits LTP (Kelly, Minogue et al. 2013) and synergizes with Aβ to increase microglial activation (Jones, Minogue et al. 2015). Infiltration of T cells also occurs with age, particularly in APP/PS1 mice, and our evidence indicates that Th1 and also Th17 cells activate microglia *in vitro* and *in vivo* while their presence in the brain of APP/PS1 mice negatively impacts hippocampal-dependent cognitive function (Browne, McQuillan et al. 2013; McManus, Higgins et al. 2014; McManus, Mills et al. 2015).

Conclusion

While there are multiple questions that remain to be answered with respect to the microglial activation states, the evidence overwhelmingly suggests that when the cells adopt an inflammatory phenotype, synaptic function and cognition are impaired. To make significant advances, clarification is needed with respect to the beneficial/damaging effects of microglial activation in age and in AD and this relies on a greater understanding of multiple activated cell subtypes and their functional characteristics. The likelihood that cells in different activation states co-exist needs to be addressed and an appreciation of how these cells interact is likely to provide answers to what currently presents as intractable issues.

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TBI and Alzheimer's risk: Targeting dysregulated glial activation and innate immunity

Linda J. Van Eldik, PhD and Adam D. Bachstetter, PhD

Introduction

Traumatic brain injury (TBI) is exceedingly common, with nearly 2 million people in the United States seen annually in emergency departments for head injuries (Faul, Xu et al. 2010), and many more who do not seek medical treatment. For those with a single mild TBI, the likelihood of a favorable recovery is high, and there may be no residual cognitive or neurological symptoms immediately evident. However, subclinical pathophysiological changes to the CNS caused by TBI persist, which increase the risk for later life neurologic impairments, including dementia (for review see Johnson, Stewart et al. 2010; Shively, Scher et al. 2012). The multifaceted mechanisms through which a TBI increases the risk for developing a neurodegenerative disease is not currently well defined. One potential mechanism is dysregulation of glial activation and a priming of the innate immune system.

Glial dysregulation and innate immune system priming as risk factors for dementia

The evidence suggesting involvement of aberrant glial activation and a priming of the innate immune system on increasing the risk of developing dementia largely comes from animal models. However, a few notable studies in humans have shown persistent signs of inflammation months-to-years after a person suffered a moderate-to-severe TBI. For example, a PET imaging study using a ligand that binds to activated microglia showed increased signal present up to 17 years after TBI, demonstrating an unresolved chronic inflammatory response (Ramlackhansingh, Brooks et al. 2011). Consistent with this, persistent elevated microglial activation has been documented in human autopsy brain by increased $CD68^+$ and $MHCII^+$ macrophage / microglia staining in long term (up to 18 years) survivors of head injury (Johnson, Stewart et al. 2013).

While the clinical literature is limited, extensive work has been done in preclinical models of TBI, describing the temporal onset of glial activation and innate immune response in a number of disease models and injury severities. Even with diverse models of TBI, differences in severities of injury, and differences in animal species, the acute inflammatory response to the injury is remarkably stereotyped (for review see Morganti-Kossmann, Satgunaseelan et al. 2007; Donnelly and Popovich 2008; Loane and Byrnes 2010). Figure 7, largely adapted from two recent studies in mouse models of diffuse brain injury (Bachstetter, Rowe et al. 2013; Webster, Van Eldik et al. 2015), summarizes the temporal pattern of the neuroinflammatory response to TBI. Within the first minutes to hours after the injury, cytokine / chemokine levels begin to rise, and subsequently peak 6-12 hours later. The cytokine / chemokine levels may return to baseline or remain chronically elevated over the sham levels (Bachstetter, Rowe et al. 2013; Webster, Van Eldik et al. 2015). Morphological changes in microglia begin within minutes and can clearly be seen by at least 6 hours after the injury (Bachstetter, Rowe et al. 2013; Roth, Nayak et al. 2014). The microglia response

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largely peaks by 1 week after the injury. Similar to human TBI, the microglia response in mouse models of TBI may stay elevated or worsen with time compared to sham-injured mice (Webster, Van Eldik et al. 2015). Interestingly, the recruitment of peripheral immune cells to the brain appears to occur in a narrow window between 1-7 days after the injury, and corresponds to increased levels of the chemokine CCL2. The CCL2-CCR2 pathway has been shown to be important for recruitment of peripheral immune cells to the brain after TBI (Hsieh, Niemi et al. 2014; Morganti, Jopson et al. 2015). Morphological changes to astrocytes follow a similar temporal pattern of activation post-injury, albeit slightly more delayed in onset (Bachstetter, Rowe et al. 2013; Webster, Van Eldik et al. 2015).

Microglial priming

Repetitive CNS stressors can alter this pattern of acute post-injury neuroinflammatory responses and resolution of the inflammation, leading to an exaggerated and unresolved neuroinflammatory response. Microglial priming, a process whereby a primary insult can sensitize, or “prime,” microglia to exhibit an exaggerated response to a subsequent injury, is increasingly being recognized as a potential mechanism by which a state of heightened microglial reactivity elicited by a prior insult can dramatically influence the responses to subsequent insults (for a review on microglia priming see Norden, Muccigrosso et al. 2015). Stressors which have been described to prime the neuroinflammatory response include a prior TBI, advanced age and neurodegenerative pathology. For example, a role for amplified and prolonged microglia activation has been postulated to contribute to the development of chronic traumatic encephalopathy, a progressive neurodegenerative disease seen in individuals with a history of repetitive brain trauma (Norden, Muccigrosso et al. 2015). Another example of how the neuroinflammatory response can be primed by exposure to multiple CNS stressors is a recent study combining a TBI with an amyloidosis mouse model of Alzheimer’s disease (AD) pathology (Webster, Van Eldik et al. 2015). The combination of AD pathology and brain injury resulted in a heightened and prolonged neuroinflammatory response to the injury (see top left panels in Figure 7), and worsened cognitive performance on a behavioral task compared to mice that had only a TBI or only AD pathology. A small molecule experimental therapeutic that selectively restores injury- or disease- induced overproduction of proinflammatory cytokines towards homeostasis was shown to rescue the cognitive deficits in the AD + TBI mice, providing evidence of a link between the dysregulated neuroinflammatory response and cognitive deficits in the AD + TBI mice (Webster, Van Eldik et al. 2015). These data suggest that selective targeting of abnormal neuroinflammatory responses may be a viable therapeutic approach to reduce the risk of neurologic impairment after TBI. As is indicated by the yellow boxes in Figure 7, there are multiple secondary injury mechanisms associated with dysregulated glial activation and innate immunity that could be points for therapeutic intervention following a TBI. Even within broad classes of secondary injury mechanisms, such as blood-brain-barrier (BBB) damage or immune cell trafficking, there are many specific molecular targets regulating these biological responses that could be explored for development of selective therapeutic interventions. An important concept for intervening in dysregulated glial activation and innate immunity following a TBI is that of therapeutic window: targeting a mechanism at the appropriate time after injury. For example, the acute CNS proinflammatory cytokine surge

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occurs in a treatable time frame (hours after injury), and has been shown to be amenable to therapeutic intervention with small molecule experimental therapeutics (Lloyd, Somera-Molina et al. 2008; Bachstetter, Webster et al. 2015). Suppression of the acute neuroinflammatory responses to TBI also provides an example of the concept of pharmacodynamics, i.e. the biochemical and physiological effects of a drug on the body. When an upstream process, such as proinflammatory cytokine overproduction, drives downstream detrimental events, then inhibition of the upstream process suppresses the downstream process whether the drug is still present or not (if the injury/activating stimulus is no longer present).

In the case of a neurodegenerative disease, where the activating stimulus (protein misfolding and aggregation, age, priming, etc.) remains, the situation is more complex and a more chronic administration of drug is likely to be necessary to target the chronically dysregulated glial activation and innate immunity. In animal models of AD, selective suppression of the proinflammatory cytokine overproduction has been shown to be an effective therapeutic strategy. In addition, dysregulated cytokines/neuroinflammation can be suppressed by targeting more than one intracellular signaling pathway: p38α-dependent and p38α-independent pathways (Bachstetter, Norris et al. 2012; Watterson, Grum-Tokars et al. 2013; Roy, Grum-Tokars et al. 2015). Targeting proinflammatory cytokines is only one example of a potential avenue to suppress dysregulated glial activation and innate immunity following CNS injury or in CNS disease. Selected other examples include: (1) pharmacological scavenging antioxidants to neutralize reactive oxygen and nitrogen species (Hall 2015); (2) CCR2 antagonist or CD11d blocking antibody to block the trafficking of macrophages, monocytes and neutrophils to the CNS (Bao, Shultz et al. 2012; Morganti, Jopson et al. 2015); (3) inhibition of the calcineurin/NFAT (nuclear factor of activated T-cells) signaling pathway to target astrogliosis (Furman, Sama et al. 2012).

A consideration in targeting dysregulated glial activation and innate immunity is the growing evidence for a remarkable heterogeneity in microglia. For example, a recent study (Bachstetter, Van Eldik et al. 2015) demonstrated variations in human microglial morphology that showed some degree of selectivity towards different neurodegenerative diseases, including AD, hippocampal sclerosis of aging, and dementia with Lewy bodies. The microglia heterogeneity includes the well-described hypertrophic and ameboid microglia which, in comparison to the ramified microglia, are generally associated with microglia “activation”. In addition, rod shaped microglia, which have also been found in animal models of TBI associated with diffuse axonal injury (Bachstetter, Rowe et al. 2013; Webster, Van Eldik et al. 2015), are seen in the hippocampus of aged individuals (Bachstetter, Van Eldik et al. 2015). The function of rod shaped microglia is an area of microglia biology that needs further exploration. Finally, as previously described (Streit 2006), dystrophic microglia with processes that are spheroidal, beaded, de-ramified or fragmented are commonly seen in the aged brain. In the case of hippocampal sclerosis of aging, dystrophic microglia are proportionally the most prevalent microglia phenotype in the CA1 region of the hippocampus (Bachstetter, Van Eldik et al. 2015). Similar to rod shaped microglia, the influence of dystrophic microglia on CNS pathophysiology is unknown.

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Conclusion

The diversity and heterogeneity of the dysregulated glial activation and innate immunity responses to CNS injury and disease provide a number of druggable pathways and opportunities for therapeutic intervention. However, the complexity of neuroinflammatory responses also highlights the critical need for continued research to better understand which responses are beneficial and which are harmful (Heneka, Kummer et al. 2014; Heppner, Ransohoff et al. 2015).

A novel 3D culture system that gives astrocytes and neurons the third dimension – a new tool for assessing (patho)physiological responses of cells *in vitro*

Milos Pekny and Till Puschmann

Introduction

Since the advent of cell culture systems, their advantages and shortcomings were obvious. The maintainance of live cells independent of the body to which they once belonged, involves attending to their preservation and propagation, and offers countless experimental opportunities that would often be difficult to achieve in experimental animals and impossible in human patients. However, cell cultures constitute highly artificial systems, in which the surviving cells have gone through multiple selection processes that have no *in vivo* counterparts. Moreover, cells in cultures have been removed from their natural context, lost the multitude of regulatory and feedback loop mechanisms, as well as cellular partners, with which they normally interact, and thus resemble a solitary player of a symphony orchestra who has lost the conductor, fellow musicians and whose musical scores got scrambled. One severe limitation of cell culture systems has been the lack of the third dimension: only a very limited number of cell types in any mammalian organ do not extend in the third dimension. Thus, the enforced planar tiling of adjacent cells in cultures is almost always highly artificial and imposes major cellular stress that changes the morphology, rheological and functional properties of such cells.

Astrocyte cell cultures

Astrocytes in the CNS fulfill a wide range of homeostatic functions, are important regulators of neuronal functions and, when made reactive by the disease process, profoundly affect both acute and later responses in brain diseases including neurodegenerative disorders (Pekny and Pekna 2014). Astrocytes also emerge as pivotal regulators of CNS inflammatory mechanisms with important implications for a whole range of neurological diseases (Sofroniew 2015). Astrocyte culture systems have long been used to maintain, propagate and experimentally challenge the brain or spinal cord astrocytes, enabling us to study interactions between astrocytes and other cell types (Booher and Sensenbrenner 1972; McCarthy and de Vellis 1980; Saura 2007). These systems advanced the understanding of the function of astrocytes in healthy brain and spinal cord, including their roles in neurotransmitter recycling, synaptogenesis, metabolism and signaling within the astrocyte

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network; they also allowed aspects of disease situations to be modelled, such as neurodegenerative diseases, hypoxia, or neurotrauma (Sullivan, Martinez et al. 1976; Cornell-Bell, Finkbeiner et al. 1990; Ellis, McKinney et al. 1995; Abramov, Canevari et al. 2003; Neary, Kang et al. 2003; Wyss-Coray, Loike et al. 2003; Abramov, Canevari et al. 2004; Garwood, Pooler et al. 2011; Lange, Bak et al. 2012; Schousboe 2012). However, the currently available two dimensional (2D) culture systems for astrocytes have many limitations, such as a highly reduced morphological complexity, enforced tiling of adjacent cells, and a prominent undesired baseline reactivity (Lange, Bak et al. 2012; Puschmann, Zanden et al. 2013).

Three-dimensional cell cultures

New methods for astrocyte preparation (Foo, Allen et al. 2011) and three-dimensional cell culture systems (Puschmann, Zanden et al. 2013) allow some of these limitations to be overcome (Figure 8). The undesired baseline reactivity of astrocytes in 2D cultures that exhibit prominent signs of cellular stress is overcome in the newly developed Bioactive3D culture system, a bioactively coated polyurethane nanofiber scaffold-based cell culture system which was previously developed for astrocytes (Puschmann, Zanden et al. 2013) (Figure 9). Levels of intermediate filament proteins GFAP, nestin and vimentin, and of the Hsp70 stress protein, signs of astrocyte reactivity and cellular stress, were several fold lower in astrocytes in Bioactive3D compared to standard 2D cultures (Figure 9). The filopodia of astrocytes grown in Bioactive3D were more motile, and those of adjacent astrocytes interacted much more with each other than in standard 2D culture systems (Puschmann, Zanden et al. 2013). Immunocytochemical and Western blot analyses of connexin-43 showed a large reduction in the number of gap-junction clusters per astrocyte in Bioactive3D, which by supporting their complex morphology allows cell–cell interactions only at certain areas of astrocyte processes, similar to what is seen *in vivo* (Puschmann, Zanden et al. 2013). This is in contrast to the enforced planar tiling of astrocytes in 2D cultures, which are extensively, but artificially, connected through gap junctions. Astrocytes cultured in Bioactive3D showed decreased proliferation (Puschmann, Zanden et al. 2013), a desirable feature for many *in vitro* experiments. Stimulation by heparin-binding epidermal growth factor (HB-EGF), which was previously proposed as a serum replacement for astrocyte cultures (Foo, Allen et al. 2011), was sufficient for cell survival, induced Mapk/Erk pathway activation, and supported cell proliferation in both culture systems, with a stronger HB-EGF-induced proliferative response of Bioactive3D grown astrocytes, possibly due to their lower baseline reactivity (Puschmann, Zanden et al. 2013; Puschmann, Zanden et al. 2013).

Transferability from 2D- to 3D culture

Astrocytes established and maintained for 7 days in a standard 2D system and then transferred to Bioactive3D behaved as those established in Bioactive3D, thus proving the usefulness of Bioactive3D in situations where the initiation or expansion of cultures in 2D culture systems are preferable or become obligatory (Figure 9). Astrocytes grown in Bioactive3D remained highly responsive to activation. Treatment with ATP (100 μM for 1h following serum deprivation for 4h) led to a comparable activation of the MapK/Erk1/2 signaling cascade in astrocytes grown in Bioactive3D and in a standard 2D system (Figure

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9). Thus, Bioactive3D makes astrocytes only minimally reactive under basal conditions and allows experiments in which activation of astrocytes is to be studied - alone or in co-cultures with other cell types - either in a pathological context (e.g. neurodegeneration, trauma or ischemia), or while evaluating possible new treatment strategies aiming at modulation of astrocyte activity. The Bioactive 3D culture system has already proven its usefulness for assessing (patho)physiological responses of astrocytes (Puschmann, Zanden et al. 2013; Puschmann, Zanden et al. 2013) and can be used for identification of clinically relevant targets and leads targeting astrocytes in disease.

The Bioactive3D culture system has now also been optimized to support 3D culture of neurons (Puschmann, de Pablo et al. 2014). Similar to astrocytes, morphologically complex neurons of the brain or spinal cord spread out in all three dimensions, and this opportunity is lost in 2D cell cultures. The Bioactive 3D was modified to support the formation of complex 3D neuronal networks allowing neurons to extend neurites into the z-direction, i.e. into the nanofiber scaffold (Puschmann, de Pablo et al. 2014). The neurite outgrowth was found to be the function of the nanofiber diameter and the pore size (Puschmann, de Pablo et al. 2014). Confocal image analyses of neurons labeled for neuron-specific beta-III-tubulin showed that neurons grown in the 3D system extended their neurites through the entire thickness of the poly-D-lysine coated nanofiber scaffold. Western blot and immunocytochemical analyses showed that despite the presence of a comparable number of glutamine synthetase positive contaminating astrocytes in 2D and 3D culture systems, the astrocytes in the 3D system expressed lower levels of the intermediate filament (nanofilament) protein GFAP, a marker of reactive astrocytes.

In vitro 5-ethynyl-2-deoxyuridine (EdU) labeling and Western blot analysis of the proliferation marker MCM2 demonstrated that the fraction of proliferating astrocytes that contaminated the 3D-grown neuronal cultures was lower than in 2D cultures. This shows that the contaminating astrocytes in 3D neuronal cultures are less reactive and less proliferative than in 2D-grown neuronal cultures. Western blot analysis of the pre-synaptic marker synaptotagmin showed that synaptogenesis, a key attribute of neuronal cell cultures, was not negatively affected by less reactive astrocytes in the 3D system.

Conclusion

As outlined above, the 3D cell culture system for neurons provides a highly useful platform for the growth of and experimentation on live neurons.

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Abbreviations

AD	Alzheimer disease
BBB	blood-brain barrier
CNS	central nervous system
CSF-1R	colony stimulating factor receptor
DR	delayed rectifier
EMPs	erythro-myeloid precursors
ESC	embryonic stem cell
iPSC	induced pluripotent stem cell
P2YR12	Purinergic Receptor P2Y, G-Protein Coupled, 12
TGF-β	transforming growth factor-β
YS	yolk sac

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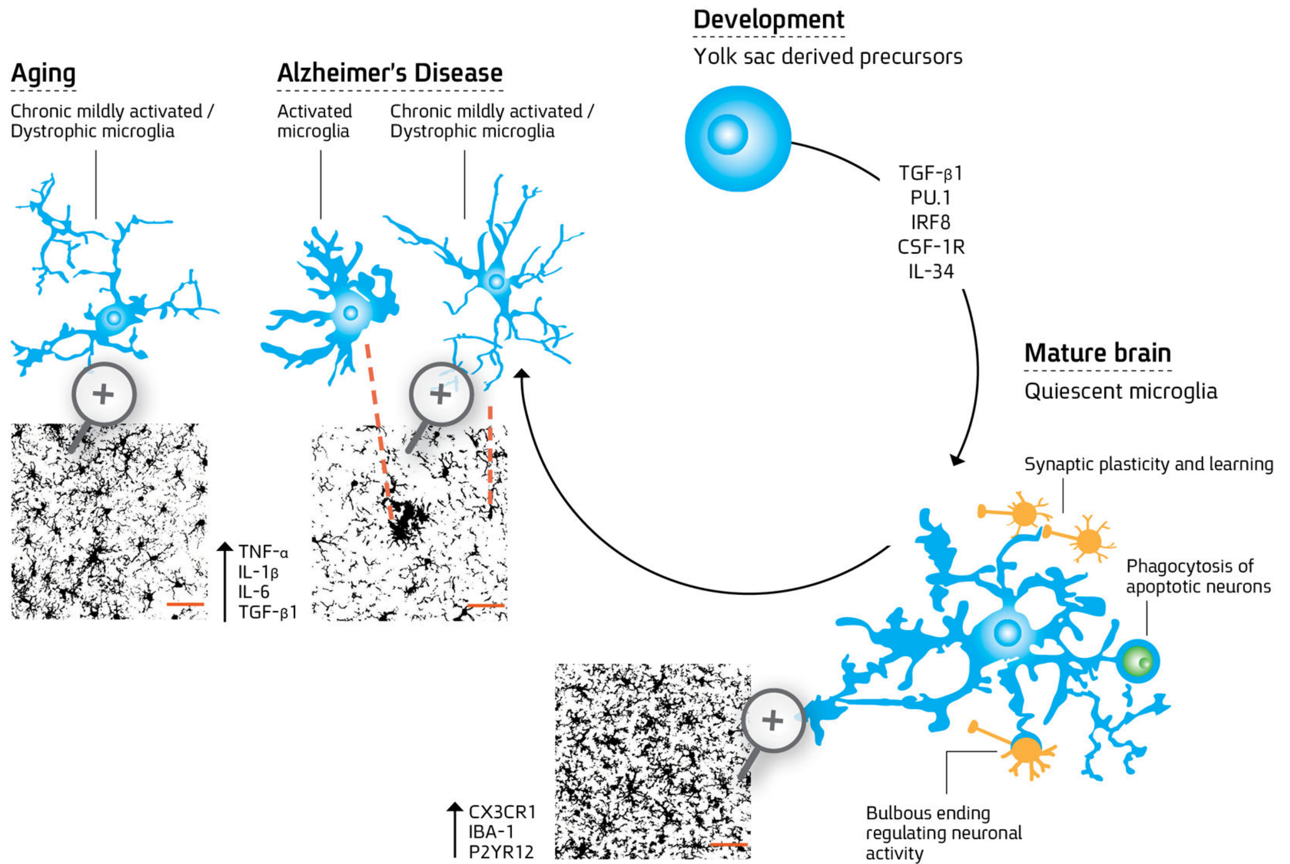

Figure 1. Microglial differentiation

Rounded yolk sac derived precursors invade the brain parenchyma in PU.1- and IRF8-dependent pathways and are regulated by CSF-1R, its ligand IL-34, and TGF-β to become fully differentiated microglial cells. During embryonic development, these cells progressively develop ramified morphology and constantly scan the brain microenvironment, eliminating neurons and pruning excessive synapses. In the mature healthy brain, surveilling microglia assist in maintaining brain homeostasis and function by regulating neuronal activity using special morphological features- bulbous endings, controlling synaptic plasticity by finger-like protrusions that wrap around dendritic spines, and engulfing cells undergoing apoptosis. These cells are highly ramified expressing CX3CR1, Iba-1 and P2YR12, are highly dynamic and dense to cover the tissue uniformly. During aging, microglia exhibit chronic mildly activated phenotype with increased expression of proinflammatory mediators, such as TNF-α, IL-1β, and IL-6 and exhibit reduced ramification with process shortening and thickening and some aberrant morphological features resembling dystrophy. Their coverage of the tissue is impaired. In Alzheimer's disease (AD), microglia proliferate and accumulate with activated and dystrophic phenotypes around plaques of amyloid β (Aβ). Microglia cells surround the plaque with distinct morphological phenotypes. The first layer of more amoeboid cells are found in close proximity to the plaque and resemble activated microglia and/or infiltrating monocytes. Additional layers of microglial cells are located on the plaque edges, exhibiting decreased morphological complexity compared to the healthy mature microglia. Microglia cell

coverage is impaired in APP-Tg mice, in which tissue space is left devoid of microglia processes. Considering the dynamic nature of the microglial processes, such a robust loss of branches during aging and disease may significantly impair the overall sensing capacity of microglia.

Confocal z-stack images showing microglia process densities and spatial coverage area were adopted from (Baron, Babcock et al. 2014). Bars represent 50 μm.

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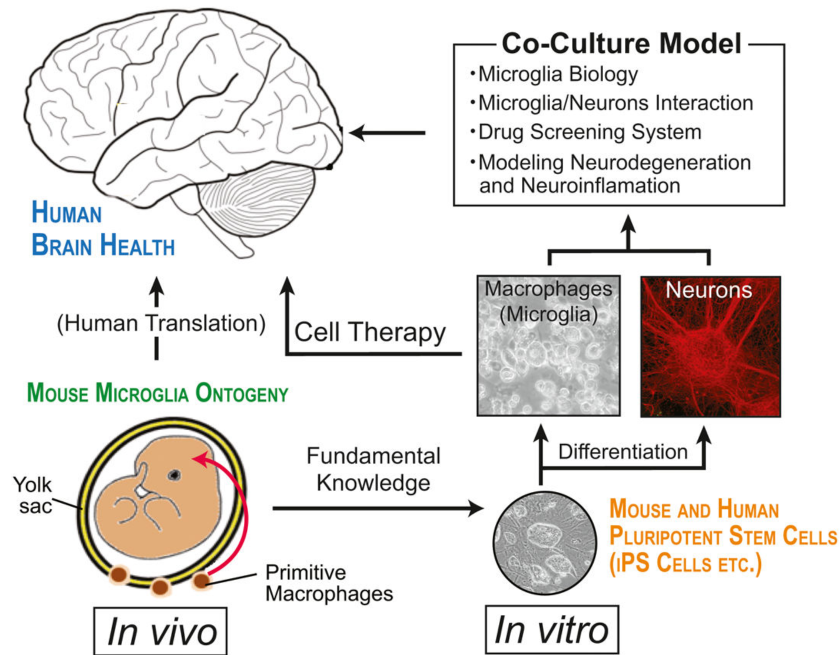

Figure 2. Microglial ontogeny and implications for the use of pluripotent stem cell-derived macrophages for therapy

A precise understanding of microglial ontogeny *in vivo* may provide adequate methodologies for *in vitro* microglial differentiation from induced pluripotent stem cells. Findings in such *in vitro* models using stem cells will complement the knowledge gained *in vivo*. The choice of an adequate cell source for microglia will further contribute to a better understanding of microglial biology in health and disease, and will facilitate the refinement of accurate developmental and pathophysiological study models as well as drug screening systems for new microglia-targeted therapies.

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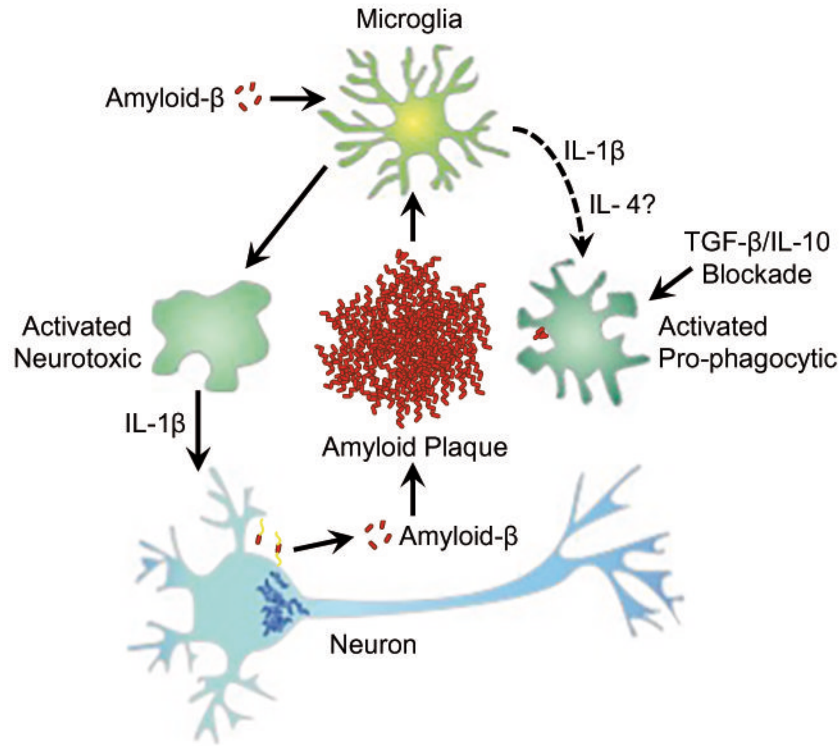

Figure 3. Paradoxical effects of cytokine signaling in amyloid-β clearance

Amyloid-β induced microglial activation can be modified in the presence of anti-inflammatory cytokines (e.g. IL-4) or by genetic/pharmacologic blockade of TGFβ and/or IL-10 signaling to shift microglia from pro-inflammatory neurotoxic cells to activated pro-phagocytic cells with the capacity to reduce amyloid-β plaque load. In experimental systems, increased IL-1β expression can drive both a neurotoxic response (e.g. increased tau phosphorylation, indicated by interneuronal fibrils) as well as create an environment that supports pro-phagocytic microglial activation.

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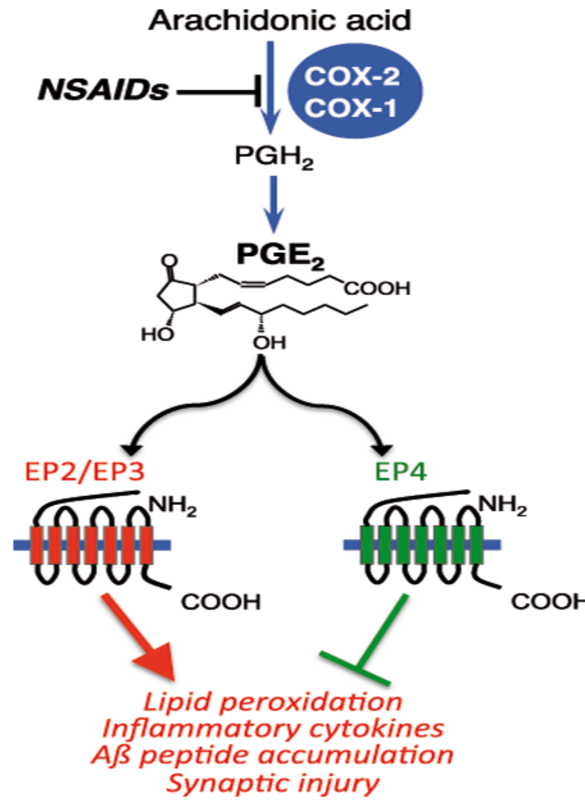

Figure 4. Mechanisms of action of inflammatory PGE_2 EP receptors in preclinical models of AD

The primary action of NSAIDs is inhibition of COX-1/COX-2, with one consequence being reduction of downstream PGE_2 production and signaling. The PGE_2 EP2 and EP3 receptors enhance inflammatory oxidative stress, pro-inflammatory gene expression and are pro-amyloidogenic. In contrast, EP4 signaling is anti-inflammatory and enhances Aβ peptide phagocytosis. Reduction of PGE_2 generation in response to NSAIDs may reduce deleterious EP2/EP3 signaling but also reduce beneficial EP4 signaling, which plays a beneficial role early in development of pathology in the APPSwe-PS1 E9 model of familial AD.

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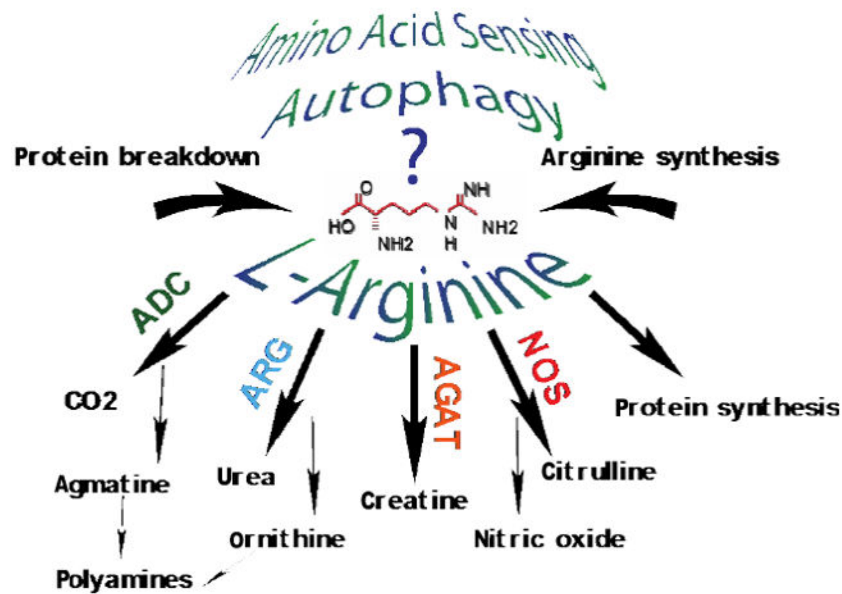

Figure 5. L-Arginine pathways

L-arginine represents a branch point of metabolic pathways including arginine decarboxylase (ADC), arginases (ARG), glycine amidotransferase (AGAT), and nitric oxide synthases (NOS). Arginine is essential for protein synthesis and amino acid turnover and may serve as a sensor for amino acid deprivation and autophagy activation.

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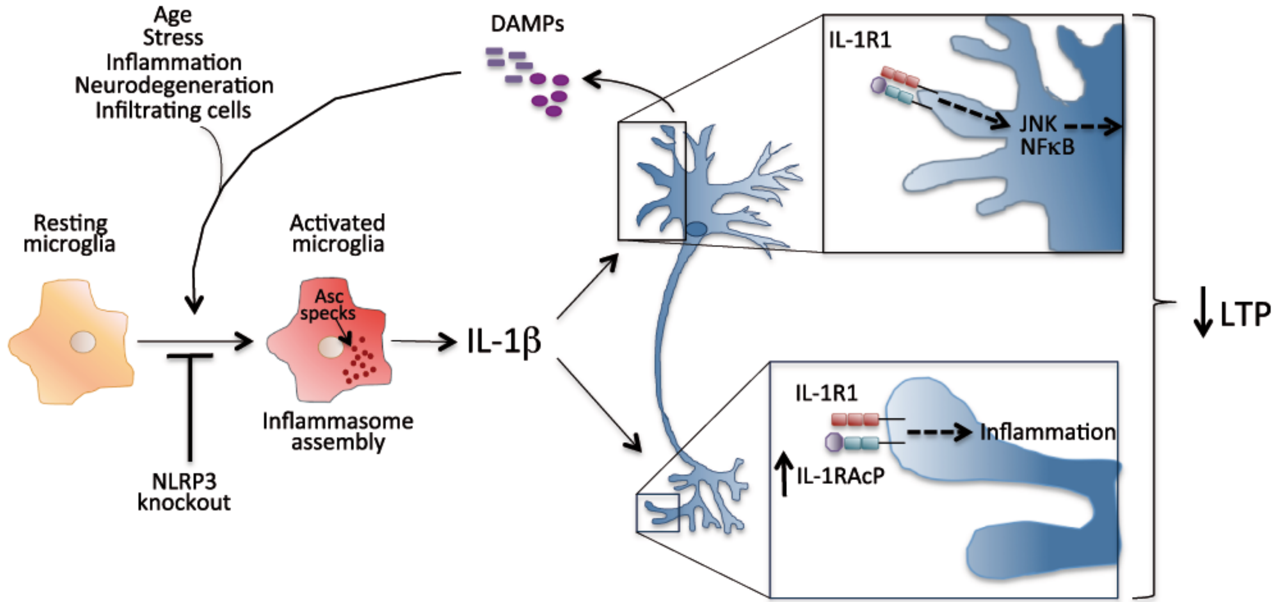

Figure 6. Microglia in LTP

Several factors stimulate microglia to adopt an inflammatory phenotype and, in most cases, inflammasome activation and the consequent production and release of IL-1β. IL-1β interacts with IL-1R1 activating signalling molecules like JNK and NFκB that exert an inhibitory effect on LTP. In addition, the age-related increase in IL-1RAcP at synapses results in an exaggerated effect locally that can also depress LTP. In age, neurons have been shown to release DAMPs that can feed back to enhance microglial activation, contributing to a damaging cycle of events.

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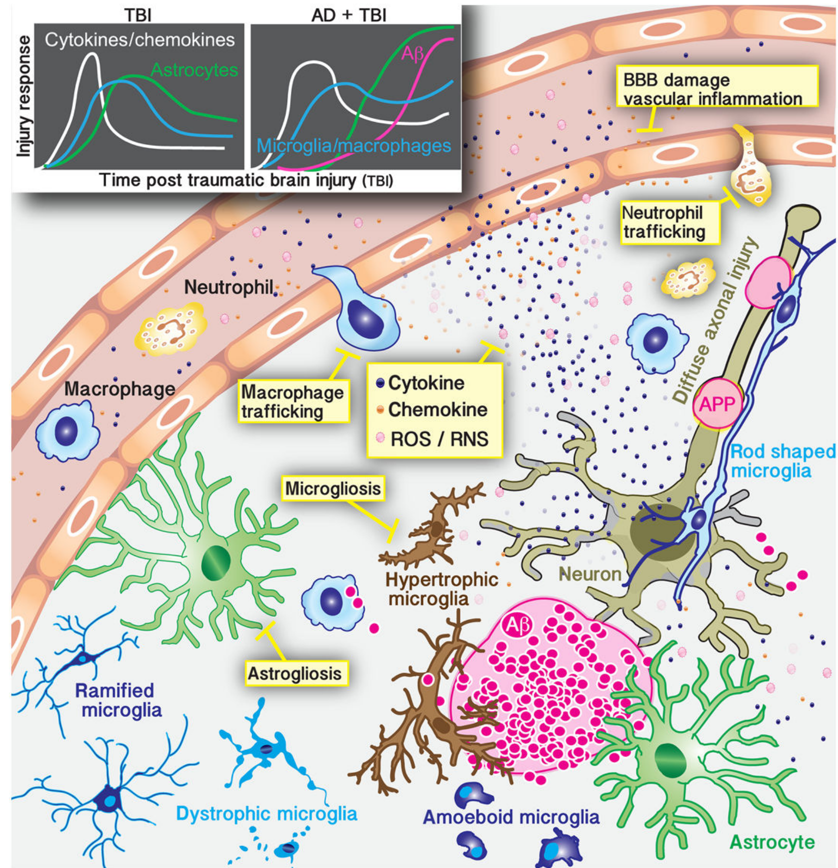

Figure 7. Targeting dysregulated glial activation and innate immunity in TBI and AD

Top left panels show a conceptualized profile of the temporal changes in cytokines / chemokines, astrocytes, and microglia / macrophages following TBI, and how the neuroinflammatory responses are heightened and prolonged in the presence of beta-amyloid (Aβ) pathology (Webster, Van Eldik et al. 2015). In the figure, potential areas for therapeutic intervention targeting glial activation and innate immunity are highlighted in the yellow boxes. APP=amyloid precursor protein, BBB=blood-brain barrier, ROS/RNS=reactive oxygen species/reactive nitrogen species.

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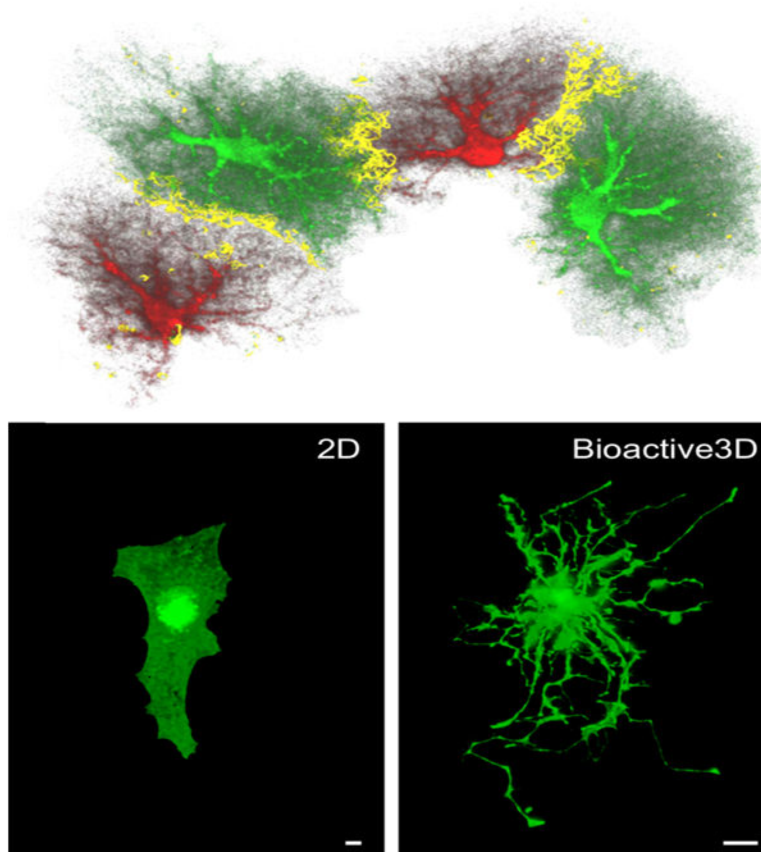

Figure 8. The Bioactive3D culture system supports the complex cell morphology of astrocytes and comes as a novel tool to study and modulate astrocyte activation in situations such as nerodegeneration, ischemia or neurotrauma

Upper panel. Astrocytes are highly complex cells with a multitude of cellular processes as demonstrated on this 3D reconstruction of adjacent astrocytes in the adult mouse hippocampus. Astrocytes were filled with either Alexa 568 or Lucifer yellow dyes. The gray matter is largely subdivided into individual astrocyte domains. Astrocytes are interconnected via gap junctions into a highly dynamic network, however in the healthy adult brain, astrocytes do enter domains of their astrocyte neighbors. Hence the limited overlap zone between adjacent astrocytes reflecting the interdigitation of fine cellular processes of adjacent astrocytes (shown in yellow). Reproduced from (Wilhelmsson, Bushong et al. 2006). Lower panel. Primary astrocytes cultured in the Bioactive3D system, which is composed of polyurethane nanofibers coated with poly-L-ornithin and laminin (Puschmann, Zanden et al. 2013), preserve some of the complex morphological and biochemical features of *in vivo* astrocytes that are normally lost upon 2D culture (compare the left and right panel). Space bar, 10 μm. The primary astrocytes were derived from mice expressing enhanced green fluorescein protein in their astrocytes (Nolte, Matyash et al. 2001).

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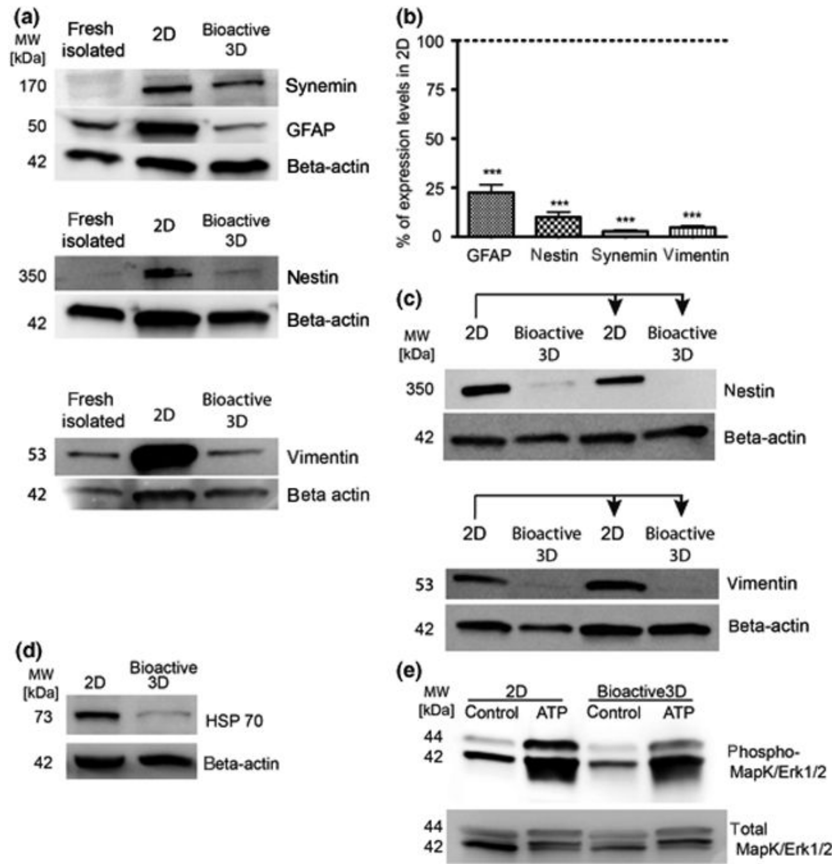

Figure 9. The Bioactive3D culture system highly reduces the cellular stress seen in conventional 2D cell culture systems

A) and **B)** Western blot analysis and levels of intermediate filament proteins GFAP, nestin, synemin and vimentin, which are known to be upregulated upon astrocyte activation and cellular stress, in freshly isolated astrocytes and astrocytes cultured in a standard 2D and Bioactive3D system. The undesired baseline activation of astrocytes seen upon 2D culture is absent in Bioactive3D; ***, $p<0.001$. **C)** The levels of intermediate filament proteins nestin and vimentin in astrocytes initially cultured in a standard 2D and then transferred to Bioactive3D, decrease correspondingly, indicating that astrocyte cultures can be established and expanded in 2D cultures and move to Bioactive3D just prior to experimental manipulations; days, days after transfer from 2D to Bioactive3D. **D)** The expression of HSP70, a cellular stress marker, is highly reduced in astrocytes cultured in Bioactive3D. **E)** Western blot analysis of phosphorylated MapK/Erk1/2 in astrocytes maintained in standard 2D cultures or in Bioactive3D and treated for 1 hour with 100 μM ATP shows that astrocytes grown in Bioactive3D are highly responsive to activation. Modified from (Puschmann, Zanden et al. 2013).