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Sexual differentiation of microglia

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ABSTRACT

Sex plays a role in the incidence and outcome of neurological illnesses, also influencing the response to treatments. Despite sexual differentiation of the brain has been extensively investigated, the study of sex differences in microglia, the brain's resident immune cells, has been largely neglected until recently. To fulfill this gap, our laboratory developed several tools, including cellular and animal models, which bolstered in-depth studies on sexual differentiation of microglia and its impact on brain physiology, as well as on the onset and progression of neurological disorders. Here, we summarize the current status of knowledge on the sex-dependent function of microglia, and report recent evidence linking these cells to the sexual bias in the susceptibility to neurological brain diseases.

1. Introduction

Microglia are a small minority of all the cells present in the Central Nervous System (CNS), about 10–15% of all brain cells (Del Rio-Hortega, 1965), and their function was believed to be restricted to the local immune defense of the CNS. More recently, a growing number of studies (Fig. 1) are demonstrating the large number of functions covered by these cells that are now considered to play a major role for brain maturation to maintain brain health.

Differently from the other cell types in the CNS, microglia do not originate from the ectoderm, but from myeloid precursors that, in early fetal development stages (embryonal day 8.5), migrate from the embryonic yolk sac to the brain (Kettenmann et al., 2011). Fate mapping analyses in mouse (Beers et al., 2006) and zebrafish (Herbomel et al., 1999) revealed that microglia originate from PU.1 + cells (Beers et al., 2006; Herbomel et al., 1999) and colonize the brain as CSF1R + erythro-myeloid progenitor cells (Ginhoux et al., 2010; Villa et al., 2016). The number of cells migrating to the CNS is relatively small (Herbomel et al., 1999), but their ability to proliferate is sufficient to colonize the whole brain. This proliferative potential is maintained all through the life span induced by colony stimulating factor 1 (CSF1). The CSF1 receptor is expressed in microglia also in the adult mice and deletion of the *Csfr1* gene or inhibition of the receptor activity results in a significant microglia loss (Elmore et al., 2014; Ginhoux et al., 2010). In the course of the embryo development, what is guiding microglia to the brain is not been clarified; in adults microglia may migrate to specific brain regions attracted by inflammatory stimuli. (Casano and Peri, 2015; Lenz et al., 2012; Schwarz et al., 2012). A characteristic of microglia is their ability to acquire different morphologies, each of them mirroring a specific function (Benedusi et al., 2017). In the adult brain, microglia are found in different shapes: when highly ramified, microglia are patrolling the brain parenchyma in search of microorganisms, cells debris or deposits of misfolded proteins to be eliminated by engulfment and digestion; all these elements act as inflammatory stimuli to which microglia react by retracting all branches to take the ameboid morphology characteristic of the inflammatory microglia. In this conformation microglia may engulf the noxious/toxic material and synthesize a vast array of inflammatory molecules (e.g. proinflammatory cytokines and chemokines, including tumor necrosis factor (TNF) and interleukin-1) aimed at opposing and destroying neurotropic microorganisms. Generally, this activated state is not maintained for long time, and microglia are able to revert to a deactivated state characterized by a novel change of morphology (towards the branched phenotype) and the synthesis and release of growth factors aimed at repairing the damage done in the course of the inflammatory reaction. We are still studying to identify the molecular mechanisms driving microglia through their differential functional roles and precise biomarkers for each stage of microglia activity.

Most relevant was the finding that microglia in their reactive form may trim neuronal dendritic spines; this suggested an involvement of these cells in the control of neuronal ability to communicate with each other in the adult brain (Kettenmann et al., 2013). In addition, it has been hypothesized that the double capacity of microglia to regulate neuronal communication and clear cell debris may be very relevant in the latest phases of brain development when the definitive neuronal circuitries are formed (Paolicelli et al., 2011). Indeed, different from what found in adult, mature brain, microglia morphology in the immature CNS is mostly ameboid and its gene expression includes both classical pro-inflammatory as well as alternative anti-inflammatory markers (Crain et al., 2013; Cunningham et al., 2013; Lenz et al., 2013). This phenotype, clearly

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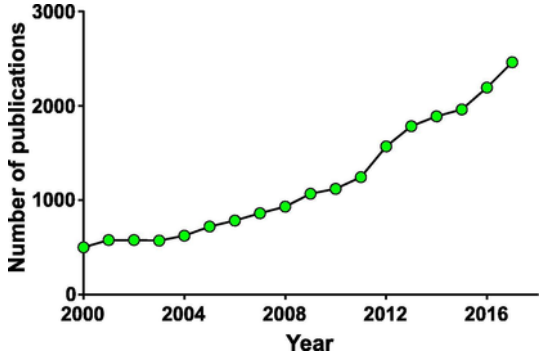

Fig. 1. Number of publications containing the word microglia in the title or in the abstract (). Source: dimensions.ai

different from that of the adult microglia, is likely associated with the specific functions exerted in the course of brain development and several authors demonstrated that in rodents, during the first week after birth, microglia eliminate redundant or apoptotic neurons and mold synaptic structures, participating in the shaping of the mature CNS (Mallat et al., 2005; Matcovitch-Natan et al., 2016).

The finding that, in addition to the specific immune mission, microglia play an active role in shaping brain circuitries and controlling neuronal activity raised questions concerning their relevance in the onset and progression of brain disorders.

2. Microglia in health and disease

A longstanding hypothesis has been that microglia had a detrimental role in selected neurological disorders (such as neurodegenerative diseases) because sustained neuroinflammatory processes can impair neuronal function and survival. Neurodegenerative diseases are generally characterized by an excessive production and accumulation of protein aggregates that constitute a trigger for microglia-dependent inflammatory processes; these deposits of aberrant proteins grow with the progression of the pathology and, in a long run, cause the recruitment of most microglia that - too busy in the removal of undesired material - cannot revert to the anti-inflammatory, reparatory, phenotype. The continuous and sustained release of neurotoxic molecules by microglia would damage the parenchyma as well as the other brain cells determining neuronal death (Rogers et al., 2002; Solito and Sastre, 2012). According to this hypothesis, McGeer et al. proposed the use of anti-inflammatory agents to limit the progression of these diseases (McGeer and McGeer, 1996). Afterwards, the growing awareness of the vast array of microglia functions in the brain helped in explaining the limited efficacy of anti-inflammatory therapies and compelled the research of novel therapeutic interventions aimed at maintaining or increasing microglia capabilities to repair damaged tissues and cells and to regulate aberrant neurotransmission (Akiyama et al., 2000; Hagino et al., 2004; Schenk and Yednock, 2002). However, the biology underlying the vast variety of microglia actions requires additional investigation because, as already mentioned, we start just now to understand that besides their anti-inflammatory actions, microglia serve a number of other functions not directly associated with inflammation, and possibly critical for the maintenance of neural functions (Salter and Beggs, 2014); the most relevant of such functions is the control of synaptic activity (Parkhurst et al., 2013; Tremblay et al., 2010). In the healthy CNS, microglia are not "resting" but are highly active in a "surveillance and rapid response" state (Tremblay et al., 2011). Microglia processes scan the parenchyma continuously to get in touch with synapses; once in the proximity of a synapse, microglia sense the molecules released by neurons, or located at synaptic compartments, through specific receptors, such as the fractalkine receptor and the complement receptor 3 (Kettenmann et al., 2011; Koizumi et al., 2013), and react with the release of a wide repertoire of soluble factors such as BDNF, glycine, L-serine, TNFα., which affect neuronal processes, including basal neurotransmission and synaptic plasticity (Schafer et al., 2013; Yamasaki et al., 2014). For instance in case of excitatory synapses (Pocock and Kettenmann, 2007), microglia, once become aware of the extent of neuronal firing and synaptic function respond by extending their processes to be in direct contact with the active synapses (Chen et al., 2014; Tremblay et al., 2010; Wake et al., 2009); *in vivo* time-lapse imaging showed a decrease of firing after microglia enwrapping of the soma of highly active neurons (Li et al., 2012). Thus, microglia may tame the neuronal damage consequent to excessive synaptic activity by either releasing repair factors that limit the damage caused by a prolonged release of excitatory neurotransmitter or by engulfing and eliminating overactive dendritic spines. Another mechanism by which microglia participate in neuronal activities is by regulating adult neurogenesis as described in the hippocampus (Gemma and Bachstetter, 2013; Sierra et al., 2010), and this event might result in improving learning/memory processes (Parkhurst et al., 2013; Rogers et al., 2011).

These microglia physiological functions may be very important for the healthy functioning of the adult CNS. This new awareness led to the hypothesis that apart or in addition to their inflammatory functions, aberrations in non-inflammatory functions of microglia may contribute to the onset of neurodegenerative diseases (Tremblay et al., 2011). In this context, a systems biology approach applied on large-scale human brain-tissue sampling (Zhang et al., 2013), identified a remodeling in the complement-dependent signaling of microglia as the functional category most strongly associated with the pathophysiology of neurodegeneration. The anomalous upregulation of complement signaling could hyperactivate the synapse elimination pathways, thus triggering synaptic loss, an early hallmark of neurodegenerative diseases (Tremblay et al., 2011).

The importance of microglia in a neurodegenerative disorder such as AD became indisputable when genome-wide association studies (GWAS) reported a consistent number of genetic susceptibility loci for late-onset AD (LOAD), which were associated with the inflammatory pathway (Mhatre et al., 2015). One of the most striking findings was that a mutation (R47H) in TREM2 – a gene involved in the microglial phagocytic process - leads to a 3- to 4-fold increased risk of sporadic AD (Guerreiro et al., 2013). Accordingly, a recent study where the human mutated TREM2 was integrated into the genome of the 5XFAD murine model of AD showed significant impairment of microglia phagocytic activity (Song et al., 2018), thus suggesting a link between this microglial loss of function and AD. Since these pioneering studies, a profusion of novel investigations shifted the focus on the relevance of loss of microglia homeostasis in neuronal disorders. Keren-Shaul and colleagues (Keren-Shaul et al., 2017) using single-cell RNA-seq on the 5XFAD model of AD, discovered a novel microglia phenotype associated with neurodegenerative diseases (named DAM) and identified specific markers, spatial localization (primarily in the brain regions affected by the disease), and pathways associated with these cells (Keren-Shaul et al., 2017). This specific phenotype seems to trigger a protective mechanism aiming at containing and removing the neuronal damage (Deczkowska et al., 2018). Most relevant is that many of the genes associated with DAM are the same identified by GWAS studies on AD, including TREM2 (Guerreiro et al., 2013), that participates in DAM differentiation (Keren-Shaul et al., 2017). Further studies identified the presence of DAM in other ND models, such as tauopathy (Friedman et al., 2018), ALS (Friedman et al., 2018; Krasemann et al., 2017), MS (Keren-Shaul et al., 2017; Krasemann et al., 2017), as well as in aging (Mrdjen et al., 2018). Moreover, the DAM expression pattern was detected also in postmortem brains from AD patients (Friedman et al., 2018; Keren-Shaul et al., 2017). These data suggest that microglia may acquire a specific phenotype in the presence of neurodegenerative cues necessary to slow down the progression the disease.

Therefore, despite most of the evidence regarding the involvement of microglia in neurodegeneration was collected for AD, their engagement in the etiopathogenesis of other neurodegenerative diseases is most likely. Thus, the finding of these non-inflammatory functions of microglia will provide novel sights on microglia involvement in brain pathologies that diverge from the strict association with a gain of inflammatory activity; rather, CNS disorders could result from loss of selected non-inflammatory microglia functions.

3. Are microglia involved in brain sexual differentiation?

We have been aware of the fact that the brain is sexually differentiated for a long time and several morphological studies proved the existence of differences in the brain of female and males; the understanding of the mechanism responsible for such differentiation originates from pioneering experiments done in the second half of twentieth century when it was observed that the exposure to androgens early in the development accounted for major brain sex differences in terms of structure and function. Roger Gorski and colleagues (Gorski et al., 1978) initially identified sex differences in the preoptic area (POA) of the rat hypothalamus, a brain region generally implicated in the control of male reproductive behaviour. A cluster of cells in the POA, defined as the sexually dimorphic nucleus (SDN), was shown to be much larger in males than in females and involved in neural mechanisms necessary for sexual behaviour (Swaab et al., 2001). Subsequent studies extended the number of sexually dimorphic regions (including the anteroventral periventricular nucleus of the hypothalamus (AVPV, (Bloch et al., 1987)), and the spinal nucleus of the bulbcavernosus (SBN, (Breedlove and Arnold, 1983) in rat spinal cord) and demonstrated that the testosterone synthesized by the testes during embryogenesis in humans and neonatally in rodents (Clarkson and Herbison, 2016) is responsible for the masculinization of selected brain circuits that control sexually differentiated behaviours and physiological processes (Gorski et al., 1978; Lenz and McCarthy, 2010). In the absence of such organizing action induced by steroid hormones, the brain remains feminine as default program (Gorski et al., 1978; Lenz and McCarthy, 2010).

The sex differences in the CNS include sex-specific neuroanatomical features; neurons show regional differences in volume, cell number, connectivity, morphology, neurite complexity, dendritic length and spine number, but also transcriptional and epigenetic changes (McCarthy et al., 2009). Thus in the adult animals the neuronal response to stimuli is sexually differentiated: for instance in the rat hippocampus, the morphology of pyramidal neurons and stellate cells in response to visual stimuli vary with sex, with female rats raised in an enriched environment showing increased dendritic branching relative to males housed in the same environment (Juraska et al., 1985). In the same brain area, following repeated restraint stress, the number of apical branch points and dendritic length of the CA3c pyramidal neurons decreases in male, but not in female rats (Galea et al., 1997). Moreover, a strong sex difference exists in the long-term potentiation (LTP), due to differential synaptic NMDA receptor activation at perforant path in male and female rats, resulting in a more enduring LTP in males (Maren et al., 1994). GABA-mediated stimulation of neurons from the *substantia nigra* of juvenile rats produces sex-biased physiological effects leading to depolarization in males, but hyperpolarizing the same cells in females (Galanopoulou, 2005). Neurons are not the only brain cells undergoing sexual differentiation, astrocytes show a clear sexual dimorphism in terms of distribution (Collado et al., 1995; Garcia-Segura et al., 1988; Suarez et al., 1991), differentiation, primary process length and number (Amateau and McCarthy, 2002, 2004; Johnson et al., 2008) and function (Garcia-Segura et al., 1995; Kuo et al., 2010; Mong and McCarthy, 1999; Suarez et al., 1992),

With regard to microglia most of the experiments done so far aimed at investigating sex differences in the course of CNS development. In the early postnatal development (P4), males have a higher number of microglia cells in brain regions involved in learning, memory and cognition processes (Schwarz et al., 2012); slight sex differences in cell size and phagocytic capacity were identified in the hippocampus across all developmental stages (Weinhard et al., 2018).

Studies in rodent showed that microglia maturation occurs slowly: just before birth microglia are close to be fully differentiated (Butovsky et al., 2014), but complete maturation is reached during the second postnatal week (Bennett et al., 2016; Matcovitch-Natan et al., 2016). Each stage in microglia development is associated with specific gene expression programs and regulatory networks (Matcovitch-Natan et al., 2016): in immature, embryonic microglia there is a major expression of proteins for the cell cycle and for chromatin remodelling, while in the adult microglia canonical transcription factors, such as EGR1 and SALL1, appear in the early post-natal stage and the expression levels rise with time. Other transcription factors, including JUN, FOS, MEF2A, and MAFB, are expressed in adult microglia only (Matcovitch-Natan et al., 2016). These transcriptional changes associated with developmental stages may be a reflection of the progressive changes occurring in the brain, as microglia cells are in constant communication with the other CNS cells through specific neurotransmitters, neurohormones, and neuromodulators, and are able to mold their function in response to the surrounding microenvironment (Crain et al., 2013). In the latest stages of CNS development, microglia morphology shifts from ameboid to the ramified, quiescent structure typical a healthy adult brain (Villa et al., 2016): this is one of the many changes in phenotype that characterize the transition between immature and adult microglia.

Hanamsagar and colleagues (Hanamsagar et al., 2017) profiled microglia transcriptome in the course of brain development and showed that from the transcriptional point of view, the maturation process has features resembling to the programs of pro-inflammatory activation typical of adult cells. Most relevant is that the temporal maturation steps follow distinct trajectories in males and in females. Starting from embryonic day 18 sex has a significant impact on microglia maturation that is delayed in males relative to females. In the presence of acute immune activation, such as following stimulation with LPS, it was observed an acceleration in male microglial development, while female microglia did not change its maturation stage (Hanamsagar et al., 2017). These data suggest that male microglia could be more sensitive to inflammatory events, which could be responsible for a faster aging of microglia and this could affect the risk of disorders (Hanamsagar et al., 2017).

We do not know how the genetic sex influences microglia maturation and the extent to which sex hormones are involved. Microglia expression of sex hormone receptors is relative to the stage of brain maturation (Villa et al., 2016): mRNA levels of the Estrogen Receptor α (ERα) are detectable in microglia obtained from P3 mice (Crain et al., 2013) and the content increases in adult mice (Crain et al., 2013). No sexual differences were observed in ERα microglia mRNA at any age (Crain et al., 2013; Sierra et al., 2008). ERβ expression was detected in primary microglia cultures from P0 newborns only (Saijo et al., 2011), and its expression becomes undetectable starting from P3 until adulthood (Crain et al., 2013; Sierra et al., 2008). Data on the expression of progesterone receptor (PR) and androgen receptor (AR) in the course of development show that microglia may express both (Quadros et al., 2007), although AR and PR do not appear to be expressed in microglia in adult mice (Sierra et al., 2008). Therefore, microglia may respond directly to the surge of testosterone that occurs perinatally and that could address microglia towards a male-specific pattern of maturation. The question to be raised here is whether microglia have a role in the shaping of neural interconnections occurring in brain development and whether the sex-specific differences in the maturation of microglia are relevant for the creation of the neuronal networks that characterize male and female brains. It is indeed increasingly recognized that throughout the development, the exposure to factors that permanently modify the function of microglia and immune system may severely impact on sexual behavior in adult life (Lenz et al., 2013). For instance, innate immune activation during development can lead to neurological outcomes in a sex-dependent manner (Schwarz et al., 2012), thus indicating the important role played by the endocrine-microglia communication in the structural organization of the brain during its maturation. A central role of microglia in the sexual differentiation of the brain was proposed by Lenz and colleagues (Lenz et al., 2013), which observed that perinatal treatments with minocycline – an inhibitor of microglial activity - prevented the masculinization of the brain that is normally induced by estradiol (Lenz et al., 2013). The pharmacological inhibition of microglia hampered the production of a pro-inflammatory molecule, the prostaglandin E2 (PGE2), which is synthesized in the POA following the neonatal testosterone surge, and is responsible for the establishment of male-specific neuroanatomical features in the brain (Amateau and McCarthy, 2004). A complete view of the mechanisms regulating brain sexual differentiation certainly requires more specific investigations; however, what we can conclude from the data reported so far is that during development microglia undergo a sex-dependent

of neonatal microglia may not provide information translatable to adult microglia.

4. Sex differences in adult microglia

Indeed, studies on the physiology of adult microglia have been limited so far; this is likely due to the difficulties of maintaining adult, fully mature, microglia cells in culture. The limited number of data available so far, however showed a sex-specificity in the expression of classical or alternative activation markers (Crain et al., 2013; Weinhard et al., 2018), the expression of purinergic receptors (Crain and Watters, 2015), cell numbers (Mouton et al., 2002), distribution into the CNS (Lawson et al., 1990), response to exercise (Kohman et al., 2013) and to stress (Bollinger et al., 2016; Bollinger et al., 2017; Fonken et al., 2018). Quite peculiar is the sexual dimorphic involvement of microglia in neuropathic pain signalling: while in male mice pain sensitivity is mediated by specific microglia-neuronal signalling pathways triggered by the activation of P2X4R on spinal microglia (Beggs et al., 2012), further experiments have shown that these cells do not participate in pain processing in female animals (Mapplebeck et al., 2017; Sorge et al., 2015; Taves et al., 2016). Similarly, social interactions in adolescents are influenced by microglia in males only, through a mechanism mediated by the complement C3, resulting in the engulfment and lysosomal elimination of spines expressing neuronal dopamine D1 receptors in the nucleus accumbens (Kopec et al., 2018).

These reports strongly support the idea that sex differences in microglia in the brain may be more extensive than just a difference in number, morphology, or specific functions, but may also be phenotypically distinct.

Recently, by taking advantage of the novel technique to isolate pure population of microglia from the brain of adult mice developed in our laboratory (Pepe et al., 2014; Villa et al., 2018), we carried out a whole genome RNA-seq analysis to compare the transcriptomes of microglia in male and female mice (Villa et al., 2018). When we compared the abundance of the transcripts of microglia from the two sexes (by applying a threshold of 0.01 to p-values) we found more than 500 differentially expressed genes (DEGs) (about 200 more expressed in males, and 350 more expressed in females). The functional analyses of the genes more expressed in males revealed a marked homogeneity: most of the DEGs showed a strict association with inflammatory processes, including regulation of cell migration and cytokine production; molecular signature analysis of transcription factors (TFs) identified NF-κB as the TF most involved in the regulation of the genes more expressed in males (Villa et al., 2018). Analogously, a subsequent paper reported for male microglia a transcriptomic profile skewed towards the pro-inflammatory activation (Guneykaya et al., 2018). The higher NF-κB activity was confirmed using the NF-κB-reporter mouse recently generated in our laboratory and carrying the luciferase gene under the control of a NF-κB-responsive synthetic promoter (Rizzi et al., 2017). In this mouse model, luciferase activity in microglia purified from the brain of both sexes was 2.4-fold higher in males (Villa et al., 2018). This male-specific grade of activation of the transcription factor seems to be a feature characteristic of microglia, since whole-body, *in vivo* imaging in unstimulated conditions showed a similar basal level of NF-κB transcriptional activation in males and females (Villa et al., 2018).

Less straight-forward phenotype resulted from the analysis of the microglia genes more expressed in females: functional analyses identified ontogenies associated with morphogenesis, development or cytoskeleton organization. More informative was the molecular signature analysis of TFs, that identified proteins related to the inhibition of inflammatory response and promotion of repair mechanisms (Villa et al., 2018). Taken together, these data support the idea of a differential reactivity in microglia originating from the two sexes, strengthened by morphological (Fig. 2.) and biochemical studies carried out in primary cultures of microglia isolated from adult male and female mice, grown *in vitro* with neuron/astrocyte mixed cells (Villa et al., 2018). Thus, microglia cells appear to be sexually differentiated and to maintain sex-specific features independently by the microenvironment: this vision was further supported by the finding that microglia retain their sex-related characteristics even after transplantation in the male brain (Villa et al., 2018).

The molecular mechanisms leading to this sex dichotomy are still uncertain; our current hypothesis, corroborated by preliminary results, is that epigenetic modifications take place shortly after birth, when the surge of testosterone occurs (Gorski et al., 1978; McCarthy et al., 2009). We believe that this is the case because we experimentally mimicked the masculinization process by treating female mice with repeated neonatal injection of E_2 (Wu et al., 2009). The microglia isolated from the adult masculinized females showed a sex-bias even if the results obtained were not conclusive as the microglia of adult, masculinized females did not show a signature of gene expression completely superimposable with genetic males (Villa et al., 2018). Of course several factors may influence microglia function in adult animals, therefore more studies are required to fully understand the mechanisms leading adult microglia to acquire the male/female phenotype; among these we believe that gonadal steroids as well as neurosteroids may influence microglia as demonstrated by our as well as other groups in male and female animals treated with inflammatory stimuli as LPS in the presence/absence of estradiol (Loram et al., 2012; Vegeto et al., 2006; Vegeto et al., 2001).

Most sex-dependent differences in mammals are associated with reproductive functions and maintained in evolution; it is premature to speculate whether this is the case also for microglia. Data mining of datasets reporting the human transcriptomes of male and female brains at different stages of development (including adulthood), such as The Human Brain Transcriptome (HBT) (Kang et al., 2011), or the more recent BRAINSPAN dataset (http://www.brainspan.org/static/download.html), led to the conclusion that in the postnatal male brain there is a high expression of genes associated with microglia phagocytic and immune function (Prilutsky et al., 2017; Werling et al., 2016); moreover, genes involved in synaptic pruning were upregulated in males at prenatal stages and are downregulated in males postnatally (Prilutsky et al., 2017). Likewise, the Bilbo group compared the development-associated gene expression patterns observed in murine microglia to the BRAINSPAN dataset: similar patterns were observed also in humans despite the heterogeneity of brain tissue, since the expression of microglia-specific group of genes increased with age (Hanamsagar et al., 2017). They also observed that male microglial transcriptome was more developmentally mature than female microglia. Interestingly, environmental factors seem to play a role in triggering the developmental programs of microglial cells in a sex-dependent manner, being the maternal microbiome likely to influence the transcriptional maturation of microglia in the fetal brain, especially in males (Thion et al., 2018). Conversely, female microglia are more prone to responding to microbiome changes in adulthood, showing profound changes in microglial transcriptomic signatures after acute and chronic microbiome depletion (Thion et al., 2018). The main sex-biased features of microglia identified so far are summarized in Fig. 3.

These findings may have relevance for the understanding of the sex-related differences in the susceptibility or progression to brain disorders as the lifespan exposure to infectious agents may have a greater effect on males than females; moreover, activation of microglia during prenatal development could lead to an increased pace in reaching maturity of male microglia (Hanamsagar et al., 2017). It is important to underline that more studies should be done to support these findings as transcriptomics analysis of purified human cortical microglia from *post-mortem* samples did not find significant sex-related differences (Galatro et al., 2017).

5. Microglia sex difference and brain disorders

A sex difference in the incidence, severity, and/or progression has been reported for several neurological diseases (Villa et al., 2016). For example, AD has a higher prevalence in women above 65 years old (1.6–3:1 ratio compared to men), and also progresses with a greater cognitive deterioration (Plassman et al., 2011; Seshadri et al., 1997). Men have a higher incidence of Parkinson's disease (PD) (3.5:1 compared to women) and the disease has a slower progression in women (Baldereschi et al., 2000; Elbaz et al., 2002). Females have a lower incidence of stroke (which depends on age as well), however they display poorer outcomes and suffer a more precipitous decline in function fol-

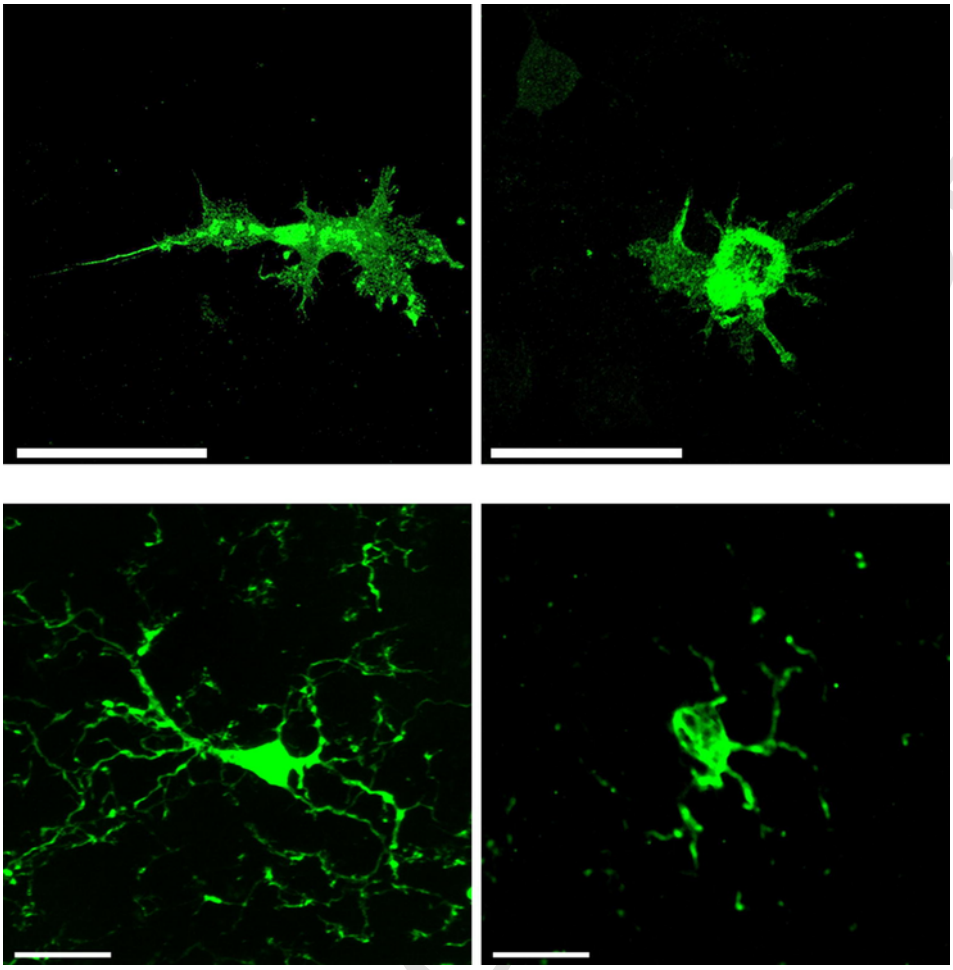

Fig. 2. Adult microglia isolated from CX3CR1-GFP transgenic mice. Female microglia *in vitro* (upper left panel) show, by and large, a phenotype reminiscent of unactivated microglia in brain tissue (lower left panel), while male microglia morphology *in vitro* (upper right panel) is more reminiscent of the activated microglia phenotype (lower right panel). Scale bar: 20 μm.

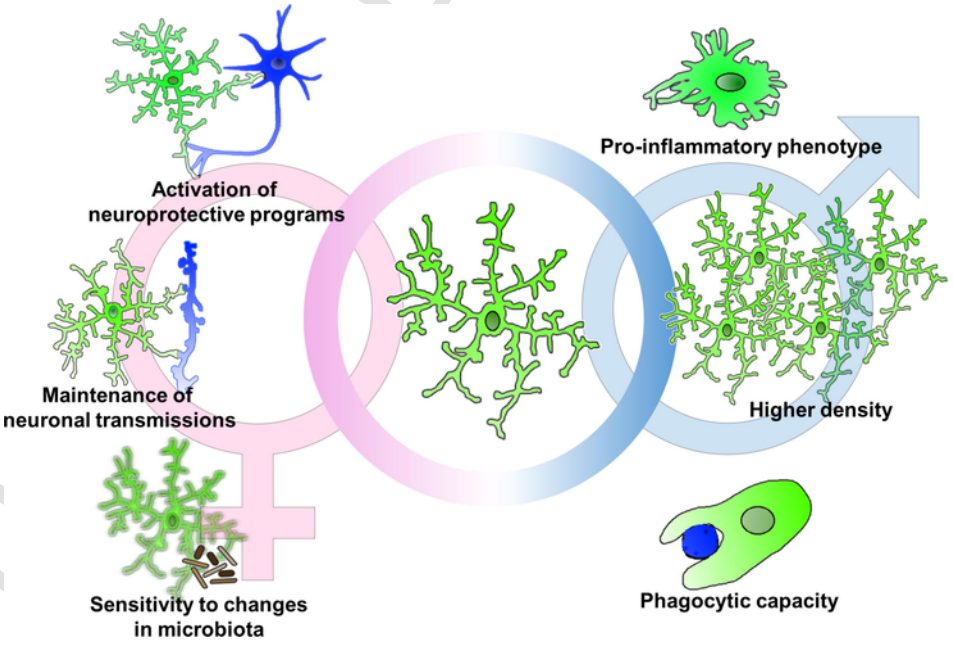

Fig. 3. Main sex-biased features of microglia. The schematic cartoon reports some of the most emblematic features of female (left side) or male (right side) microglia.

lowing stroke compared to males (Roy-O'Reilly and McCullough, 2014). Depression is prevalent in females (Altemus et al., 2014), while schizophrenia (McGrath et al., 2004) and autism (Werling and Geschwind, 2013) are prevalent in males. As a general rule, with some exception (e.g. Parkinson's disease), epidemiological studies indicate that disorders that emerge early in life are more common in males, whereas disorders that emerge later in life (at adolescence or beyond) are more common in females.

The reasons underlying these sex-related differences are still unknown as well as their origin could be genetic, associated to brain sexual differentiation and/or circulating sex steroids, all factors able to influence the activities of neurons, astrocytes, and microglia (Arnold, 2009; Gillies et al., 2004; Gorski et al., 1978; Joel et al., 2015; Joel and McCarthy, 2017; Li et al., 2014; Schwarz and McCarthy, 2008).

Even if it is plausible that microglia could be involved, it is premature to hypothesize the extent to which these cells could play a role. Initial studies are claiming a role for microglia in the sex prevalence of selected neurological diseases. A number of studies reported a pro-inflammatory activation of microglia following acute and chronic stress [extensively reviewed in (Calcia et al., 2016)], and the recent work of Bollinger and colleagues (Bollinger et al., 2016; Bollinger et al., 2017) highlighted a marked sex difference in microglial density, morphology, and immune factor expression across corticolimbic circuitry in stressed rats, which is possibly linked to the women's high vulnerability to stress-linked psychological disorders (Riecher-Rossler, 2017). Werling and colleagues (Werling et al., 2016) showed that autism spectrum disorder (ASD) is associated with a marked upregulation of sex-specific genes (genes generally more expressed in healthy males); among them there was a significant enrichment of microglia activation markers (Werling et al., 2016). The implication of these findings is that the sex-specific incidence of autism might be associated with the higher microglia reactivity, typical of males, responsible for alterations in neuronal connectivity leading to the manifestation of the disease prevalent in males. Parallel analyses on the same datasets showed that in the course of development microglia mature faster in males, leading to think that autistic individuals have an exaggerated development of microglia than healthy controls (Hanamsagar et al., 2017). A similar aberrant microglia phenotype was observed in AD tissue compared to controls (Hanamsagar et al., 2017) even if AD is prevalent in females. However, it is known that with ageing females experience a higher level of microglial activation compared to age-matched males, with possible implications on the development of neurodegenerative diseases (Mangold et al., 2017). Indeed, it is quite difficult to dissect the cellular and molecular mechanisms underpinning different brain pathologies when their development occurs in decades or, worse, it is rooted in brain development. The study of acute diseases (like stroke or brain injury) may provide a better understanding on the role played by the different neural cells, microglia included. Studies in traumatic brain injury (Acaz-Fonseca et al., 2015) or middle cerebral artery occlusion (Bodhankar et al., 2015), indicated that microglia from female mice show a lower degree of inflammatory activation following the insult.

In these studies, however, it is important to take into account the presence of circulating sex steroids known to influence microglia inflammatory response and progression though the different stages ensuing the inflammatory reaction (Vegeto et al., 2001; Villa et al., 2015). To circumvent this problem and to have a clearer vision of the role of sex of microglia in the neuroinflammatory process, we recently set up a methodology by which we can transplant adult microglia and evaluate its behaviour in a setting (physiological or diseased) different from the original. Using this methodology, we investigated the relevance of microglia sex in acute stroke. After permanent middle cerebral artery occlusion (pMCAO) (Villa et al., 2018) naïve male mice develop a larger injury than females, modelling stroke outcomes seen in men and women (Murphy et al., 2004; Villa et al., 2018). However, we observed that when microglia isolated from female mice was transplanted in males prior to pMCAO, there was a significant reduction of the lesioned area and a higher expression of the anti-inflammatory marker Ym1 around the damaged area. This clearly demonstrated the major role of microglia in containing the hypoxic damage and provided a strong evidence that this feature is more efficacious in female than in male microglia, independently from the levels of circulating estrogens (Villa et al., 2018).

6. Conclusions

This review intended to summarize the progress of the recent years in the understanding of microglia biology and its major role in healthy as well as diseased brain. The results obtained so far highlight microglia as major players in the brain physio-pathology and the complexity of activities that these cells may display.

We believe that one of the major progresses is associated with the possibility to study adult microglia with methodologies enabling 1. to identify and label these cells with specific genetic markers, 2. to isolate from the adult brains highly (> 95%) pure populations of these cells (Pepe et al., 2014; Villa et al., 2018). This latter is particularly relevant considering the complexity of functions covered by these cells and their phenotypic evolution in the course of life. These studies are demonstrating the major limitations of the classical *in vitro* studies carried out with microglia obtained from immature brains and not stratified for sex, as the stage of maturation of these cells is very relevant for their final functions. Major efforts should therefore be done to improve the methodologies to grow mature microglia *in vitro*: some progress has been recently done from our group by using co-cultures of microglia and neurons where neurons appear to enable microglia to maintain their specific phenotype (at least with regard to the sex of origin) (Villa et al., 2018), but of course more studies should be done and more biomarkers should be generated to enable us to correctly stage microglia both *in vitro* and *in vivo*. Our prediction is that transplantation studies combined with animal genetics will have a major impact on defining the role of microglia in sustaining/containing the progression of neurological and neuropsychiatric diseases: we will be able to observe the fate and activity of cells extracted from diseased brains in healthy brains and *vice-versa* in young, adult or aged animals. The advances obtained so far in our understanding of microglia functions might only be the tip of a big iceberg and this must be an incentive for further studies on microglia particularly for their potential major role as novel target for the numerous brain disorders waiting for a cure.

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