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MICROGLIA-NEURON INTERACTIONS IN THE ELECTROPHYSIOLOGICAL DOMAIN: CAN MICROGLIA RESPOND DIRECTLY TO ELECTRICAL SIGNALS?

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MICROGLIA-NEURON INTERACTIONS IN THE ELECTROPHYSIOLOGICAL DOMAIN: CAN MICROGLIA RESPOND DIRECTLY TO ELECTRICAL SIGNALS?

Thesis presented to Escola Superior de Biotecnologia of the Universidade Católica Portuguesa to fulfill the requirements of Master of Science degree in

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by

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Dedication

I want to dedicate this dissertation to my parents, for the unconditional love and support during the writing of this work as well as my whole life, without you nothing of this would be possible, thank you for always believing in me. I would also like to dedicate it to my boyfriend who had to endure my venting sessions, and frustrations when multiple experiments didn't go according to plan, but specially to my brother, that besides the life setbacks, he taught me through his examples, how to be a fighter and what is truly worthy of giving value to. This thesis is for you, to give you strength to finish yours when the time comes, knowing that I will be by your side to return the advices and motivation you gave me this last months.

Resumo

A microglia é um tipo de célula com um comportamento dinâmico, que está constantemente a analisar a sua vizinhança e a alterar a sua morfologia para se adaptar ao microambiente onde está inserida. Apesar de serem conhecidas como células eletricamente não excitáveis, as funções da microglia parecem estar extremamente coordenadas com o estado funcional das redes neuronais, o que sugere a existência de uma íntima comunicação com os neurónios. Embora esta comunicação, através de sinais químicos, já ter sido alvo de muitos estudos, ainda não é claro se a microglia tem a capacidade de monitorizar outro tipo de sinais, nomeadamente sinais elétricos.

In vivo, a microglia estabelece contactos regulares e transientes (durante 4-5 min) com as estruturas pré- e pós-sinápticas neuronais, sendo a duração destes contactos regulada pelo perfil de atividade neuronal. Além disso, a sua morfologia e o perfil de expressão genética também são modulados pelo nível de atividade neuronal. Estes factos indicam que a comunicação microglia-neurónio é sincronizada de modo a manter a funcionalidade e a homeostasia do SNC. Um elemento relevante, é que a microglia expressa diferentes tipos de canais iónicos dependentes de voltagem, tendo alguns destes canais, funções focadas na regulação do potencial de repouso da membrana e concentrações iónicas intracelulares. Estes canais iónicos dependentes de voltagem deixam em aberto a possibilidade de deteção direta de potenciais elétricos neuronais. Esta interação elétrica continua, porém, inexplorada. Portanto, este trabalho tem como objetivo investigar de que forma as propriedades elétricas do microambiente, para além da sinalização química, afetam a dinâmica da microglia. Por outras palavras, pretende-se decifrar se a microglia consegue detetar a atividade elétrica neuronal, e explorar os mecanismos envolvidos nessa deteção.

Para investigar essa possível capacidade, foram utilizadas câmaras de galvanotaxia, para estudar as mudanças de comportamento da microglia quando sujeitas a campos elétricos compatíveis com as condições fisiológicas. Campos elétricos de 40 e 400 V/m foram aplicados à microglia durante 6 ou 24 horas, e as mudanças morfológicas observadas na microglia foram quantificadas através de análise de imagem. Para o campo elétrico de 40 V/m, observou-se um aumento da área e uma diminuição da irregularidade da membrana da microglia, ao longo do tempo. Por sua vez, o campo elétrico de 400 V/m, em ambos os períodos de tempo estudados, causou um aumento das protusões citoplasmáticas e nenhuma alteração na área da microglia.

O impacto de compostos bioativos (secretoma) resultante de diferentes perfis de atividade neuronal na microglia foi igualmente investigado. O meio condicionado neuronal (NCM) foi extraído de culturas neuronais funcionalmente ativas, em diferentes estados de maturação in vitro, e foi exposto à microglia durante 24 ou 48 horas. O tratamento com NCM resultou num aumento da área da microglia e numa tendência para um aumento da ramificação destas células.

Em conclusão, este estudo mostrou que a microglia, tem a capacidade de detetar campos elétricos. Os mecanismos que levam à sua alteração morfológica na presença de campos elétricos ainda está por ser investigada. Trabalhos futuros poderão explorar estes mecanismos através do estudo da expressão de marcadores de ativação.

Palavras-Chave: interação microglia-neurónio, atividade elétrica neuronal, campos elétricos, morfometria, análise de imagem, câmaras de galvanotaxia

Abstract

Microglia are highly dynamic cells that constantly scan their surroundings and undergo changes in their morphology to adapt to their microenvironment. Although known as non-excitable cells, microglia functions seem to be highly coordinated with the neuronal activity levels, suggesting the existence of a very close signaling crosstalk with neurons. This communication in terms of chemical signals, has been the focus of many studies but, it is still unknown if microglia have the capacity to detect other kind of signals, namely electrical signals.

In vivo, microglia regularly establish transient contacts (for 4-5 min) with pre- and postsynaptic neuronal structures, being the frequency and duration of such contacts dependent on neuronal activity profile. Their morphology and gene expression profile are also shaped by neuronal activity. This indicates that microglia-neuron work in synchrony to maintain the functional and structural CNS homeostasis. Moreover, microglia express different types of voltage-gated ion channels. Some of these channels have the function of regulating the membrane rest potential and intracellular ionic concentrations**.** These voltage-gated ion channels open the possibility of microglia to be able to sense directly the neuronal electrical activity. Yet, this interaction remains unexplored in what regards its electrical dependence. Therefore, this work aims to investigate how the electrophysiological microenvironment, beyond the chemical signaling, impacts microglia dynamics. In other words, one aims to decipher if microglia can perceive the electrical activity per se and, explore the mechanisms by which it may occur.

To investigate this capacity, it were used galvanotaxis chambers to study the microglia behavior changes when exposed to electric fields (EF) compatible with the physiologic conditions. EFs of 40 or 400 V/m were applied to microglia for 6 or 24 h and the microglia morphological changes were quantified by image analysis. For the 40 V/m applied EF, one observed an increase in microglia area and a decrease in cell membrane irregularity, with time. The EF of 400 V/m, at both time points, resulted in an increase of the cytoplasmatic protrusions in the microglia but no change in the microglia cell area.

The impact of bioactive compounds (secretome) resultant from different neuronal network activity profiles on microglia behavior were also investigated. Neuron-conditioned medium (NCM) was extracted from functionally active neuron cultures, at different stages of maturation in vitro, and exposed to microglia for 24 or 48h. NCM

treatment resulted in an increase in microglia area and a tendency to these cells to become more ramified.

In conclusion, this study showed that microglial cells are capable of detecting EFs. The mechanisms by which microglia change their morphology in the presence of EFs are still under investigation. Future work may explore these mechanisms by evaluating the expression of microglia activation markers.

Keywords: microglia-neuron interaction, neuronal electrical activity, electric field, morphometry, image analysis, galvanotaxis chambers

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Chapter 1

Introduction

The central nervous system (CNS) is responsible for receiving and sending information throughout the body. The main cell types characterizing the CNS are essentially neurons and glial cells. Neurons are responsible for receive, integrate and transmit signals to other cells. Glial cells, which outnumber neurons within the CNS, have many roles, including the support of neuron's function, the maintenance of CNS homeostasis, the involvement in nervous tissue repair and, even the participation in signal transmission, among others. There are three major types of glial cells in the CNS: astrocytes, oligodendrocytes and microglia [1, 2].

Microglial cells are an integral and functional part of the CNS network and, the most abundant mononuclear phagocyte within the CNS. They are considered the most sensitive sensors of brain homeostasis, due to their continuous and active surveillance of the surrounding environment [2, 3]. During CNS development, microglia help to shape neural circuits by modulating the strength of synaptic transmission and sculpting neuronal synapses [4]. These and other microglia functions, have been described to be modulated by neurons, via the neuronal release of soluble factors like chemokine C-X3-C motif ligand 1 (CX3CL1; also known as fractalkine) and neurotransmitters, or via the expression of neuronal transmembrane glycoproteins, such as the Cluster of Differentiation 200 (CD200), Cluster of Differentiation 47 (CD47) and, Cluster of Differentiation 22 (CD22) [5]. Nevertheless, it has been hypothesized another way of microglia-neuron communication – the electrical signaling [6]. This type of signaling has been connected to the basics of embryonic development and wound healing [7]. Indeed, recent studies with microglia cell lines have shown the capacity of microglia to detect and respond to electric fields (EFs). Yet, there are no studies confirming this ability in primary microglial cells – the ones that present behavior more closest to the microglia in vivo [8].

Understanding the different ways of microglia-neuron interaction is the key to get insight of the functioning of the healthy brain and its intrinsic protection mechanisms to keep the homeostasis. This knowledge can be also a powerful tool to unravel the regulation of microglia functions in acute and chronic neurological disorders [9].

1.1 Motivation

For the past 15 years, microglial cells functions within the CNS have evolved from being the immune cell of the brain to be a key player in many other functions required for nervous system homeostasis. Nowadays, microglial cells are recognized to perform a role in several processes during nervous system development and maturation, such as synaptogenesis, synapses modulation, promotion of neuronal proliferation, influence the development and remodeling of the CNS vasculature, among others [10].

The contribution of microglia to each of these processes seems to be in straight dependence of a very close microglia-neuron interaction. Soluble factors released by neurons at synapses as well as the expression of complementary receptor-ligand pairs between these cells have been described to mediate the regulation of this microglia-neuron intimate communication [11]. In addition to the well-established chemical signaling, a number of recent studies suggest that microglia can also directly sense the level of neuronal activity [12, 13]. These studies suggesting an electrical modulation of microglia functions have been performed at the in vivo setting, where the neuronal network activity has turn to hyper- or hypoactive states. But, in such complex scenario it is difficult to isolate the real cause-effect on the basis of the microglia behaviors.

Therefore, in this dissertation one proposes to study the electrical dependence of microglia, in a well-controlled in vitro setting. This will be explored by establishing a physiological in vitro model, in which microglia behavior can be evaluated from the electrophysiological point of view.

To elucidate if microglial cells can indeed sense electrical activity per se, the application of physiological EFs to microglia cultured under optimized conditions will be explored. The cellular effects of EFs have been studied in many nervous system cell types [14-20], including microglia cell lines [6, 21]. But, given that microglia are very dynamic cells and, that microglia cell lines are known to not entirely reproduce the native properties of microglia in vivo, the data obtained from previous studies lack some degree of confidence. For these reasons, there is a strong need in the field to establish the proper in vitro conditions that can aid in deciphering the impact of electrical signals in microglia behavior.

1.2 Objective

The main goal of this dissertation project is to explore, in vitro and under a controlled electrophysiological environment, the capability of primary microglial cells to sense and react to electrical activity. The effect of direct application of two physiological EFs will be quantified and compared to the effect of differently active neuronal networks secretome, known to include soluble substances responsible for the reported microglia-neuron interaction in vivo. To this aim, microglia phenotype, in terms of morphological changes, will be monitored and characterized under each experimental condition.

In order to achieve this main goal, a truly multidisciplinary approach was taken combining experimental, computational and bioengineering components. The following specific sub-goals were defined:

- Optimize the in vitro culture conditions to cultivate primary microglial cells under a "resting"-like phenotype.

  To understand the potential impact of a specific stimuli, microglial cells should present an in vitro phenotype closer as possible to its in vivo phenotype at steady state. Moreover, microglia population cultured in vitro should be phenotypically homogenous as possible, in order to one be able to distinguish subtle alterations in their morphological phenotype after treatment. Therefore, the three most used microglia culture media reported in the literature will be evaluated and compared in terms of microglia morphological phenotype.

- Design, develop and validate a stimulation system for the application of physiological EFs to microglia cells.

  The efficient evaluation of the effect of an EF on cellular behavior requires a platform that can be simple and adaptable to the experimental conditions in study. Therefore, with the help of Computer Aided Design (CAD) and a 3D-printer, a stimulation platform will be designed and printed. A galvanotaxis chamber for the EF application will be also developed following the examples of previous reports. The stimulation system will be validated, prior to its application to microglia cells, using primary astrocytes, which response to EFs are already very well documented.

- Define image analysis workflows for morphometric assessment to be applied in the characterization of microglia morphological changes arising from experimental treatments.

  Microglia are very dynamic cells that actively change their morphology in response to homeostatic or reactive stimuli. The main morphological features that have been used to describe their homeostatic versus reactive/active state are their soma area as well as the level of ramification. From the morphometric parameters available within the free imaging analysis software ImageJ/Fiji, one will select the best in defining the main properties that allow the morphological characterization of microglia.

- Apply the developed and optimized protocols and methodologies to explore the effect of EFs and active neuronal network secretomes on microglia behavior.

  Established the proper primary microglia cell culture conditions, microglial cells will be exposed to two different physiological EFs as well as to secretome from neuronal networks at different in vitro maturation days. The effect of each experimental condition (electrical versus chemical signals) on microglia behavior will be assessed and quantified by the optimized image analysis protocols.

1.3 Document overview

This dissertation is divided into six main chapters. Chapter 1 provides an overview on the subject as well as the motivation and the objectives of the present work. In the chapter 2 is presented a literature review regarding microglia physiology and mechanisms for interaction with neurons. The main topics of this chapter include a brief description of microglia origin, properties and dynamics; microglia functions during embryonic development and adult CNS; ways of microglia-neuron communication; interfaces used to study microglia behavior; and, a brief description of the used of EF to study cell properties. Chapter 3 presents a detailed description on the reagents, materials and methods used in this work. The main findings achieved in this work are then described in Chapter 4. In Chapter 5, the main results of this work are discussed and compared with what is described in the literature. Finally, the main conclusions of this research are presented in Chapter 6, followed by considerations about future work/directions in the Chapter 7.

Chapter 2

Literature review

2.1 Microglia discovery and origin

Microglia are the resident immune cells of the CNS, representing 5-12% of the adult murine brain cells [22] and 0.5-16.6% of human brain cells [23], with some brain region variability. They are widely distributed throughout the CNS but with higher abundance in the gray matter.

In 1899, Franz Nissl, a German neuropathologist, found in a human brain with a severe neuropsychiatric condition that leads to a cerebral atrophy – syphilitic paralysis – a set of rod-shaped/ bipolar cells, which he described as reactive glial elements with migratory, phagocytic and proliferative potential. Nowadays, it is believed that these nameless cells were microglia [24, 25]. In 1913, Santiago Ramón y Cajal, a Spanish neuroscientist, pathologist and Nobel prize winner, also described a set of cells with this morphology, using a gold chloride staining method [26, 27]. However, due to the method limitation in detecting cell ramifications, Cajal could only observe microglia cell bodies. Thus, it was impossible to distinguish these cells from neurons and astrocytes. Following Cajal research, Pio Del Rio Hortega, a Spanish neuroscientist, revolutionized the study of neuroglia by developing and improving metallic impregnation techniques that aid to visualize the cell bodies as well as their ramifications [28, 29]. With his silver carbonate staining technique, Hortega was able to identify two kinds of cells and to unveil their origin: microglia with a mesodermic origin and, oligodendroglia with an ectodermal origin [30-32]. Hortega was the first to provide a morphologic and functional characterization of microglia, which allowed a long lasting and controversial debate about these cells' origin through years [31].

During the embryonic development, the most crucial phase is the gastrulation. Gastrulation consists in the migration of epiblast cells to create a trilaminar structure, which will develop into specific structures in the embryo. The most internal germ layer is the endoderm, which is responsible to form the epithelial lining of the gastrointestinal tract, liver, pancreas and, the respiratory tract. The cells from the second layer – the mesoderm – originate the skeleton, muscles, connective tissue, heart, blood vessels and kidneys. The ectoderm, the third and most exterior germ layer, gives rise to cell lineages

that differentiate to become the central and peripheral nervous systems, sensory organs, skin and its appendages. This layer undergoes an additional subdivision, generating the neuroectoderm, that gives rise to the nervous tissue [33, 34].

Between the latter 19th and early 20th century, various scientists such as Nissl, Robertson, Cajal and Hortega, believed that microglial cells had mesodermal origin. By the second half of the 20th century, researchers start to believe that microglial cells could share the same origin as neurons and other glial cells and, therefore, derive from pluripotent glioblasts from the neuroectoderm [35-37]. This hypothesis was motivated by in vitro studies showing that rodent microglial cells could be generated by the neuroepithelium cells of the ectoderm layer or astroglial cultures [38]. Additional evidences were provided by bone marrow transplantation studies in mice, showing that macrophages derived from the implanted bone marrow did not contributed for the adult microglia population [39].

In the same era, another theory was debated arguing that microglial cells derived from the first site of hematopoiesis – the extra-embryonic yolk sak (YS) – akin to primitive macrophages and, then migrate to the brain, in the early weeks of gestation [25, 35]. After the circulatory system formation, macrophages from the YS travel through the entire embryo, as they can be found spread all over the embryo, at early stages of the development, although they are more persistent in the CNS, where they differentiate into microglia cells. This differentiation is pending on the presence of TGF-b, a growth factor that is described in the adult human and rodent, as responsible to define the molecular and functional signature of the microglia, although, equal fundamental to the differentiation and maintenance of the microglia during the embryonic development [40]. Nowadays, based in the gene and surface proteins expression, is accepted that microglia arise from erythromyeloid progenitors (EMPs) in the YS and populate the neuroepithelium by embryonic day 9.5, in the murine brain [41-44]. Since embryonic day 10.5 until birth, microglia colonize the CNS by rapidly proliferating and mature into ramified microglia [45] – processes highly conserved across vertebrates [46-48]. In the first two weeks after birth, microglia number increase followed by a decrease to a constant level – the homeostatic level [4]. After birth, the increase in microglial cell number results from the proliferation of resident microglia, being their number in healthy nervous system dynamically regulated at the cellular level by coupled apoptosis and proliferation [48-50]. The turnover of a whole microglia long lived population over 96 days in the murine brain [50].

Since its discovery, microglial cells were known as the phagocyte of the CNS. But, in the recent years many other roles within CNS have been attributed to this glial cell type [22, 51]. Microglia have been shown to interact with neurons and other glial cells and, thus contribute to important processes that support the development, maintenance and repair of the CNS [52]. Some of these microglia contributions include, the regulation of neuronal survival and death [53-56], the maturation and formation of neuronal circuits [57, 58], synaptogenesis [59, 60], the coordination and managing of the patterning and wiring of networks [61], among others. In the following subchapters, a summary of the main microglia functions that help to establish and maintain the overall homeostasis of the nervous system during embryonic and postnatal development will be presented.

2.2 Microglia properties and dynamics

Microglial cells are extremely sensitive to the surrounding environment [22, 57, 58, 62]. In the healthy CNS, microglia continuously monitor their microenvironment, with their highly motile processes, for detection signals of injury and/or infection. This microglia phenotype, described as "resting", is characterized by cells displaying a small cell body with ramifications – multiple branches and processes – that terminate with bulbous endings. Although their cell body remains stationary within their environment, these resting microglia are actually very dynamic cells that reorganize their processes, by protruding and retracting, in a constant and rapid way to efficiently scan their surroundings [57]. These processes usually establish contacts with other glial cells [63, 64], blood vessels [47, 65] and neurons [66, 67]. In the presence of an insult (e.g. physical or chemical), microglia change their morphology towards an enlarged cell body with an amoeboid shape [68, 69]. This phenotype is described as "activated" microglia and, is usually associated with increased phagocytic and pro-inflammatory [68]. Given their mesodermal origin, microglia present similarities with other myeloid cells, such macrophages and monocytes. One of these resemblances is the expression of macrophage surface markers, as the glycoprotein F4/80, the colony-stimulating factor 1 receptor (CSF1R), the Cluster of Differentiation 11b (CD11b), the cell surface glycoprotein CD200 receptor (CD200R), the signal regulatory protein alpha (SIRPα or CD172a), the fractalkine receptor CX3CR1 and, the Ionized calcium Binding Adapter molecule 1 (Iba-1) [70-72]. Microglia is accounted as the main neuromodulator at stages during the development but also the adulthood. Therefore, these cells must have multiples ways of communication with the neurons.

Microglial cells express an astonishing amount of different ion channels [73]. There are evidences confirming that microglia membrane present H^+ channels, Na^+ channels, voltage-gated Ca^{2+} channels, Ca^{2+}-release-activated Ca^{2+} channels, voltage-dependent and -independent Cl^- channels, and K^+ channels [74]. The majority of these channels have been reported to be expressed in human microglia, with some exceptions that are more common in other species [74]. The H^+, Na^+ and some K^+ channels, display a voltage-dependent gating, therefore, their opening and closing depend on the membrane potential. In the CNS, the neuronal activity can lead to changes in the pH and ion concentration [75]. For instance, during the neuronal activity, in the extracellular spaces, the levels of the potassium ions increase, what also happens in the course of pathologic conditions, such as epilepsy, inflammation and spreading depression [76-78]. Microglia as a non "excitable" cell, at least not in the neuronal sense, lean in this different channel types to regulate most of the important functions, such as activation, secretion of factors, proliferation, chemotaxis, the respiratory burst [79, 80] and, the phagocytic ability [81], through surrounding changes triggered by neuronal activity.

2.3 Microglia – neuron interaction and functions

Microglia and neurons interact with each other mainly via ligand-receptor mechanisms as well as by different soluble factors, which allow the bi-directional regulation of each cell [82]. An example is the inhibitory immune complex between neurons and microglia via the membrane glycoprotein CD200 expressed by neurons and its receptor CD200R expressed by microglia [83]. CD200/CD200R signaling is developmentally regulated and has been shown to keep microglia immunologically inactivated (i.e. in a quiescent state) [84-86]. Another example is the expression of the fractalkine by neurons and its receptor CX3CR1 by microglia. This CX3CL1/CX3CR1 signaling is key in the regulation of microglial activation, both in the developing and adult brain.

In this chapter, it will be described in more detail when and how microglia interact with neurons to exert its functions, during the embryonic development, early postnatal age and adult CNS.

2.3.1 In the CNS development and early postnatal age

A number of recent studies have reported central function of microglia in specific aspects of brain development, namely in the regulation of neuronal apoptosis, growth, migration, survival and, synaptic development, among others (Figure 1 for an overview).

Microglia have been related with the regulation of neuronal numbers. Indeed, more than half of the neurons present during CNS development are eliminated by apoptosis before adulthood [53, 54]. As the phagocytic cell of the CNS, microglia play an important role in the removal of such apoptotic neurons. But, microglia may not be the solely responsible for phagocytosis of apoptotic neurons, since animals with microglia deficiency have not shown defects in the apoptotic neuron's phagocytosis [87]. But, apart from the clearance of cellular debris it has been shown that microglia can also induce neuronal apoptosis [87]. Microglial cells are commonly found nearby several neurogenic niches in the rat brain and close to apoptotic neurons [88]. In vivo studies have shown that microglial cells can promote cell apoptosis and that, as the microglia population decrease, the Purkinje cells apoptosis are also diminished [89]. This ability is believed to be related with the increase of oxidative stress, due to the superoxide ions release, which induces neuronal cell damage and/or death. This release is regulated by the integrin CD11b and the immunoreceptor DNAX activation protein 12 (DAP12) [89, 90]. After interaction with a target neuron, microglia release the CD11b a protein involved with numerous adhesion-related associations between cells such as macrophages and DAP12 – a transmembrane protein recognized as a key signal to induce cell death – triggering neuronal apoptosis without an inflammatory reaction [90, 91].

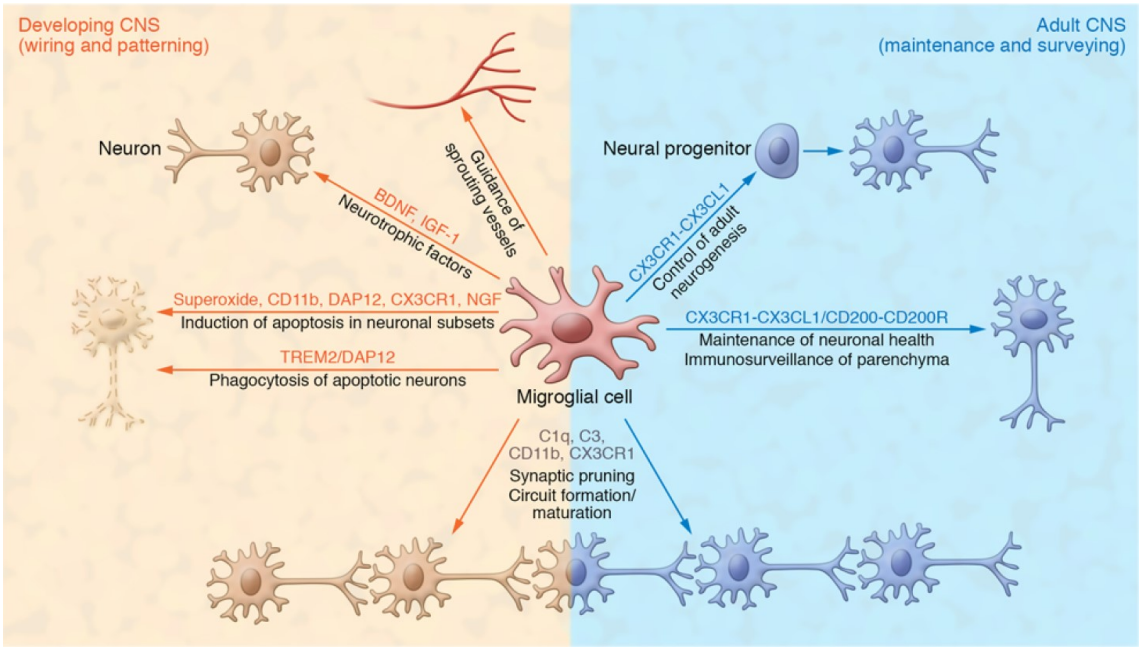

Figure 1. Homeostatic function of microglia in the developing and adult CNS. In addition to their function as a resident immune cell in the CNS parenchyma, microglia display a variety of other functions to maintain tissue homeostasis. Microglia modulate wiring and patterning in the

developing CNS by regulating apoptosis of neuronal subpopulations, releasing neurotrophic factors, and guiding sprouting vessels in the parenchyma. They also are important for circuit formation and maturation of neuronal networks, and for regulating adult neurogenesis and maintaining neuronal health in the adult CNS. Figure reused from [61] with the permission of The American Society for Clinical Investigation (ASCI).

During development and postnatal periods, microglia participate in the regulation of early brain connectivity assembly by promoting the outgrowth or fasciculation of axonal tracts via the CX3CL1-CX3CR1 pathway [92, 93]. Indeed, microglial cells were found to accumulate in the crossroads for axonal fiber tracts and neuronal migration during development, regulate the outgrowth of dopaminergic axons for instance [92].

As glial cells are a major source of trophic factors, it is not surprising that microglia are also a critical regulator of neuronal migration and survival during development. During development, microglia have been implicated in the promotion of neurons proliferation and survival, by the release of neurotrophic factors, such as brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and glial-derived neurotrophic factor (GDNF) [94]. It was also demonstrated an increase in the survival of layer V pyramidal neurons as a result of microglia production of insulin-like growth factor 1 (IGF-1) and CX3CR1/CX3CL1 signaling, at the early postnatal period [55]. At the same development period, in the cortical subventricular zone (SVZ), in the absence of the IGF-1, the neurotrophic support is claimed by the release of the interleukin 1β and 6 (IL-1β and IL-6), tumor necrosis factor-α (TNF-α) and interferon gamma (IFN)-γ [95, 96]. Through the release of IL-1β and IFN-γ, microglia support neurogenesis and, with IL-1β and IL-6 they sustain the oligodendrogenesis [95, 97]. Interestingly, the microglia cytokine production, migration and phagocytosis functions can be modulated by the release of vascular endothelial growth factor (VEGF) and C-X-C motif chemokine 12 (CXCL12; also known as SDF-1) from neural progenitors [96, 98]. Therefore, these studies suggest the existence of a bi-directional communication between microglia and the developing neurons [98, 99].

During CNS development, an excessive amount of synaptic connections are established. In the early postnatal period, many of these immature and unnecessary synapses are removed through engulfment of presynaptic inputs, to guarantee the proper functioning of neuronal networks – synaptic pruning. Synaptic pruning is triggered by the

CXCR1/CX3CL1 signaling and through the detection (by microglia) of the complement cascade initiating protein q (C1q), which localizes to developing synapses [100]. It has been proposed that C1q expression at synapses in need of elimination is regulated by immature astrocytes by the secretion of TGF-β [100, 101]. These C1q-tagged synapses are then phagocytosed by microglia in a complement component 3 (C3) dependent manner [4, 100]. Indeed, previous studies have shown that CX3CR1 knockout mice display defects in the synaptic pruning, leading to behavioral deficits and formation of synapses with immature connectivity [102, 103].

In parallel with these functions regulated by their interaction with the developing neurons, microglia also interact with other glial cells that modulate neuronal properties and function. Microglia have been implicated in the direct regulation of oligodendrogenesis and myelination [104]. In the mouse brain, during the first postnatal weeks, microglia with an amoeboid morphology or highly metabolically active are present in myelinating regions, suggesting their importance in the oligodendrocyte precursor cells (OPCs) maintenance [104]. Additionally, microglia have been reported to contribute to the development and remodeling of the CNS vasculature, by assisting in the connecting between sprouting vessels. This is accomplished by a two-way communication between the vasculature and the microglial cells, resulting in the release of soluble angiogenic factors that guide the vessels tips towards other vessels [105] . In mice and zebrafish models, it has been shown that microglia promote the vessels branching, being the branching points decreased when a reduction in microglia population is observed [106].

2.3.2 In the adult CNS

The bidirectional communication between microglia and neurons is not only appropriate for neuronal development but also to support the homeostasis of neuronal networks in the adult CNS. Therefore, this intimate microglia-neuron interaction is maintained in the adulthood, both at physiological and pathological conditions. Nonetheless, this chapter will only describe the microglia-neuron interactions in the healthy CNS.

In the adult healthy CNS, the main microglia functions are related with the modulation of synaptic properties and maintenance of synaptic plasticity [60] as well as the regulation of neurogenesis in the adult brain.

In vivo studies have shown that microglial cells make regular and direct contacts, for 4 to 5 minutes, with the synaptic terminals [12]. These contacts seem to be regulated by the

neuronal activity. Studies with zebrafish larvae confirmed that the frequency of such microglia contacts reduces as the neuronal activity also decreases, while the duration of such contacts increases. But this regulation is bidirectional, since after microglia contact neuronal activity is also altered [107]. The establishment of microglia contacts with synaptic terminals has been reported to be dependent on extracellular adenosine triphosphate (ATP) [58, 107, 108]. In brief, the neuronal activity triggers the neuronal Pannexin-1 hemichannels activation that consequently secrete the ATP molecules, which in turn bind to the microglial P2Y12 purinergic receptors. Therefore, given the chemoattractant property of ATP, microglia processes move to and wrap the neurons with higher ATP signaling [107]. This intimate contact may result in synapses pruning, depending on the level of neuronal activity. As described for the embryonic period, in the adulthood synapses with lower pre-synaptic activity are eliminated [102, 109] in a C3 dependent manner [100, 101, 109]. Yet, microglial cells may also be involved in the formation of new spines, a process that is mediated by the release of the neurotrophic factor BDNF [67, 110] and the direct contact with the dendrites, which leads to filopodia formation, Ca^{2+} currents and actin accumulation [66].

Another key microglia function in the adult CNS is the mediation of the synaptic plasticity, which covers the reinforcing or weakening of the synapses accordingly to its activity. Learning and memory functions are related with the strengthening of the synapses, which is regulated by the microglia CX3CL1/CX3CR1 signaling [111]. Microglia deletion in the adult CNS, resulted in a reduction of synaptic structural plasticity associated with the capacity to learn something new as well as in a reduction of synapses elimination, which interfere with the motor learning skills. These evidences clearly elucidate the role of microglia in the regulation and modulation of synapses [112].

In the adult CNS, microglia is responsible to regulate the neurogenesis in the SVZ of the hippocampus [113-117].

Although this function is affected negatively by the microglia aging, there are factors that can counteract that effect. As shown in rodents with access to a running wheel, exercise and environmental improvement lead to a significantly enhancement in cell proliferation and neurogenesis as well as improved performance in spatial memory and learning activities [118].

The microglia receptor CX3CR1 has been described as a crucial intervenient in the support and regulation of the adult neurogenesis [119]. Previous studies have shown that CX3CR1 deficiency or interruption induced a proinflammatory response by microglia, that consequently resulted in a significant reduction in the adult hippocampal neurogenesis [111]. Besides CX3CR1, factors such as IGF-1, IL-1β and TNF-α signaling via TNF receptors 1 and 2, have been also implicated in the regulation of adult neurogenesis [114]. A malfunction on the microglia regulation of neurogenesis results in a lack of limited ability to learn new memories [119]. Apart from promoting the neurogenesis in the SVZ, microglia also control the cellular density in the SVZ, by removing neurons still viable, but that have already apoptotic markers, that can be tracked by phagocytes [120].

Altogether, these findings show that this very close microglia-neuron interaction along development and adult life contributes to the modulation of both microglia and neurons functions. Through the controlled action of soluble factors or by the expression of membrane receptor/ligand pairs between this cellular partnership works towards the nervous system homeostasis. But, besides these well-established chemical/molecular signaling, a number of recent studies suggest that microglial cells may also be capable of directly sense the level of neuronal activity [10, 11]. This hypothesis is yet to be clarified.

2.4 Interfaces used to study microglia behavior/dynamics

The varied microglia roles within the CNS put microglial cells as a central target in different areas of research. Therefore, a number of interfaces and tools have been developed to broadly enable studies of microglia function in health and disease.

Microglia are the macrophages of the CNS. Due to the shared lineage of microglia and macrophages, many markers are common to both cell types, which turns difficult to find a set of markers able to differentiate these cells. Similar to macrophages, microglia express Iba1, CX3CR1, the mouse macrophage-restricted F4/80 glycoprotein, CD11b, CD40, CD45, CD68 and CD115 [121, 122]. A combination of CD11b and CD45 labeling has been frequently used to distinguish microglia from macrophages, since microglia express high levels of CD11b but low levels of CD45, whereas macrophages express high levels of both markers. However, this expression profile may change with disease or injury [123]. Besides the relative marker expression, traditional methods used to distinguish both cells include the morphological distinctions (ramified versus ameboid).

To overcome the inherent limitations of the traditional methods used for microglia identification, new tools to study microglia in vivo and in vitro have been developed. For example, one of the models useful for fate-mapping studies is the usage of transgenic animal models. The Cre-LoxP system is a powerful tool for genetic manipulation in vivo, allowing excellent spatial and temporal control of gene expression. This technique is commonly used to create gene insertions, inversions and translocations in animal DNA [124]. The transgenic animal is therefore designed based on the monocyte/macrophage markers such as LysM [125-127], colony-stimulating factor 1 receptor (CSF1R or CD115) [128, 129], CD11b [130-133], F4/80 [134] and CX3CR1 [135-141]. These transgenic animal models are excellent models for tracking, in time and space, microglial cells in vivo. Therefore, they have been used for lineage tracing, fate-mapping, characterization of inflammatory responses and, characterization of the systemic functions of endogenous macrophages studies.

Besides the added value of transgenic models to examine the microglia role in the CNS, they lack the ability to mimic the human microglia variability [142]. To resolve this handicap, it has been used bone marrow radiation chimeric mice – chimera animals. This model is used in studies aimed to distinguish the intrinsic versus extrinsic effects of determined mutations. These chimera models have been explored to study the role of CX3CR1 in microglia, by using CX3CR1-deficient mice as the donor and wild type mice as the recipient. The microglia motility, inflammatory response, proliferation and other aspects can then be analyzed using the resulting chimeric mice.

The communication between neurons and microglia has been a topic of great interest within the scientific community, since it seems to be key to understand the healthy brain functionality. Therefore, another technique worthy to mention is the optogenetics, which allows the manipulation of neuronal activity with temporal and spatial precision [143]. By manipulating the activity of neuronal networks, researchers have been exploring the reactions of microglia. There are no reported studies using optogenetics to control microglia activity. However, it is believed that it is possible, since this technique relies on the ability to control the release of intracellular calcium, so potentially leading to the expression of activation factors, cytokines and chemokines, like TNF-α and ROS. Recently, a series of studies suggested the possibility of applying this cutting-edge technique to manipulate glial cell activity [144].

The majority of studies exploring microglial cells properties, behavior and interactions are made in vivo. However, in vivo studies have inherent limitations such as the lack of detail in what regards cell-to-cell communication, for instance. In these cases, the isolation of the cause-effect factors is uncontrollable. In such conditions, it is indispensable to work with each cell type in isolated conditions (i.e. using in vitro models).

In vitro studies represent an important tool due to their greater efficiency, lower cost and ability to investigate an isolated cell population. To study microglial cells researchers developed various in vitro models based on the use of microglia cell lines, stem cell-derived microglia and primary microglia cultures, each of them with advantages and limitations [8]. The majority of microglia cell lines are derived from the spinal cord and immortalized by a viral transduction with oncogenes. There are cell lines from multiple sources: mouse, rat, macaque and even human. The main advantages of using a cell line model is the higher cell proliferation capacity and the easy culture maintenance. But, in the other hand, this type of cells has more probability of dedifferentiation, which means these cells can lose their original form or function and, therefore, the immortalization procedure may alter the microglia phenotype. Studies comparing microglia cell lines and primary microglia found genetic and functional differences [40, 145]. Consequently, microglia cell lines may not be representative of the microglia in vivo behavior.

The stem-cell derived microglia model has the advantage of having an unlimited availability of cells for the in vitro studies. There are two stem cell types used in the microglia research context: the embryonic stem cells (ESCs) and the induced pluripotent stem cells (iPSCs). ESCs are obtained from the inner cell mass of a blastocyst and the iPSC from a reprogramed adult cell, by the overexpression of four transcription factors [146, 147] . The advantage of using iPSCs relies on the fact that these cells can replicate the genetic background of the derived glial cells. Therefore, this model allows a fair comparison of the results between healthy donors and patients with neurological conditions. Still, due to the difficult process to differentiate microglia from stem cells, it was only recently possible to apply this model in microglia in vitro studies [148]. Even though this seems a good model to be used for the in vitro studies, the stem-cell derived microglia model is still very recent in the field, requiring more consensus in the methodology used and more validation studies to understand better the reliability of the model.

Lastly, the primary microglia culture models can be obtained from mice, rats, non-human primates and humans. Most of the methods used to isolate microglial cells consist in a mechanical and enzymatic dissociation of brain tissue, followed by a density gradient centrifugation [149, 150]. There are other methods using: i) antibody-coated magnetic beads to separate microglia from others brain cells with a magnetic-activated cell sorting [151], ii) fluorescent antibodies followed by fluorescence activated cell sorting (FACS) [8] or, iii) microglia isolation from mixed glial cell cultures by the shaking technique [152]. The most common primary microglia cultures used are obtained from rodent, which brings the disadvantage of not being a fair comparison with the human microglia, due to the evolutionary differences and lack of heterozygosity resultant from the inbreeding characteristics and the aseptic living environment. However, primary microglia cultures hold the advantage of retaining the majority of genomic and proteomic profile found in the in vivo setting [151].

2.5 In vitro electrical fields study

In 1786, Luigi Galvani was the first acknowledging the nerves response to long standing direct current gradients [153]. His experiment showed that a frog sciatic nerve connected with lighting rods, during a lightning storm, expressed a muscular response to the electric stimuli by displaying contractions in frog's leg muscles. Since then this technique is used to diverse kind of studies. Nowadays, the EF studies have been mainly used to explore the basics of embryonic development, wound healing and limbs regeneration, as the endogenously EF generate bioelectric currents ,which play crucial roles in these important biological processes [154-156]. There is still uncertain if the EF affect directly the cell or indirectly through chemical or physical changes in the extracellular environment, although it is known that it leads to variations in the cell charge distribution.

The EF nowadays are used to control diverse cell functions, for example, cell growth, adhesion, differentiation, proliferation, the reorganization of the cytoskeleton, secretion of proteins or gene expression [157-163]. This control is a result of the influence of the native EF in the extracellular regions though the cell membrane [164, 165]. This way of regulating the cell functions has been used in many different cell types, such as myoblasts, neurons, osteoblasts, endothelial cells, fibroblasts, muscle cell and epithelial cells [14-20]. These cell are able to communicate using receptors and ion channels present in the

cell membranes, that are able to broadcast and receive mechanical, chemical, and electrical signals intra and extracellularly.

Normally the cell membrane is impermeable to compounds with electrical charge, however, these channels, the transmembrane proteins, help the passage of ions. Ion channels are voltage, mechanic or ligand dependent, therefore when applied an EF this channels open and allow the change in the cell ions charge distribution [166]. The electromagnetic field interaction with the live cell is acquired through the alteration in the ions flow, influenced by ion channels and the membrane receptors, alternatively to the direct contact between the EF with the cell membrane, causing changes in the cell membrane charge [167-169].

The cell behavior under the effect of an EF can be evaluated and tested under in vitro conditions, using a galvanotaxis chamber. Usually, the galvanotaxis system is composed of a galvanotaxis chamber, in which the cells are plated, and two reservoirs on either side of the galvanotaxis chamber that are filled with an electrolytic solution and also contain the conductive electrodes connected to an external power supply. Bridging the galvanotaxis chamber and the electrolytic solution reservoirs are two agarose-based bridges that allow to complete the circuit [170]. In vitro studies, there are Different types of electrical stimulation have been applied in the in vitro studies, namely the direct, pulsed or alternated current. From these, the direct current is the one that consistently showed the ability to induce changes in cells morphology, migration and proliferation [21].

In vitro studies aiming to understand the EF effect in glial cells have shown that an exogenous EF can lead to changes in the cell behavior. Schwann cells from embryonic chicks, displayed a migration toward the anode, as a response to an EF of 3 V/m [171]. Astrocytes from newborn rats, responded to EFs between 50 and 500 V/m by aligning perpendicularly to the EF [172]. Microglia cell lines were also exposed to EFs between 40-400 V/m and, responded by presenting altered morphological properties, namely in what regards cell area and number cytoplasmic projections [6]. Still, there are no studies on primary microglia behavior under the influence of an EF. Based on the described published results is reasonable to assume these cells can also display a behavior response to EFs.

Chapter 3

Materials and Methods

3.1 Reagents

Deoxyribonuclease I from bovine pancreas (DNAse I), trypsin from porcine pancreas, poly-D-lysine (PDL), laminin from Engelbreth-Holm-Swarm murine sarcoma basement membrane and, the Triton X-100 were obtained from Sigma-Aldrich. Dulbecco's Modified Eagle Medium (DMEM) was acquired from Corning Inc. The penicillin-streptomycin solution (P/S) and the heat-inactivated fetal bovine serum (hiFBS) were purchased from Biowest. Neurobasal medium (NB), neurobasal plus medium, trypsin (1:250), L-glutamine, B-27™ Supplement (50X) and, B-27™ Plus Supplement (50X) were purchased from Gibco, ThermoFisher Scientific. The polyclonal rabbit anti-Iba1 antibody was purchased from Fujifilm Wako Chemicals; the polyclonal rabbit anti-glial fibrilarry acidic protein (GFAP) was obtained from Dako; the polyclonal goat anti-rabbit IgG (H+L) Alexa Fluor® 488 and the Hoechst 33342 dye were acquired from Invitrogen, ThermoFisher Scientific. Polydimethylsiloxane (PDMS, SYLGARD® 184 Silicone Elastomer Kit) and silicone vacuum grease were purchased from Dow Corning.

3.2 Cell Culture

All procedures involving animals were performed according to the European Union (EU) Directive 2010/63/EU (transposed to Portuguese legislation by Decreto-Lei 113/2013). The experimental protocol (0421/000/000/2017) was approved by the ethics committee of both the Portuguese Official Authority on animal welfare and experimentation (Direção-Geral de Alimentação e Veterinária - DGAV) and of the host Institution.

3.2.1 Mixed glial cell cultures

In order to obtain primary rat microglia cells, cultures of primary rat mixed glia cells (MGC) were first established from cerebral cortices of newborn Wistar rats, at postnatal day 2 (P2), following the protocol described by Chen Y et al [173], with minor modifications.

Briefly, after brain dissection and removal of meninges, the cortices from P2 rat pups were dissected in HEPES-buffered Hank's Balanced Salt Solution (H-HBSS) containing

1% (v/v) P/S, on ice. Collected cortices were mechanically dissociated with a 10 mL pipette and subsequently passed through a 25-gauge syringe. Then, tissue was enzymatically digested with 0.001 mg/ml DNase I and 0.0025% (w/v) trypsin from porcine pancreas for 15 min, at 37°C. After trypsin inactivation with DMEM containing 10% (v/v) hiFBS, cell suspension was centrifuged at 500 x g for 10 min and re-suspended in DMEM containing 10% (v/v) hiFBS and 1% (v/v) P/S. Cell suspension was filtered through a 40 µm nylon strainer (Falcon) to remove remaining large cell clusters and plated in 0.01 mg/ml PDL-coated 75 cm^2 flasks (cortices from 2 pups per flask) and maintained in DMEM containing 10% (v/v) hiFBS and 1% (v/v) P/S for 10 days, at 37°C in a humidified incubator with 5% CO_2. Culture medium was partially replaced at day 4, and totally replaced every 2 days in culture.

3.2.2 Rat cortical microglia culture

After 10 days in culture, the MGC reach confluence and are ready to be shaken. To obtain microglia cells, MGC cultures were shaken at 200 rpm in an orbital shaker for 2 h, at 37ºC. This shaking protocol results in microglial cells detachment from the flask. The medium containing the microglial cells in suspension was collected and centrifuged for 10 minutes at 1200 rpm. The supernatant was discarded, and the cellular pellet was suspended in DMEM with 10% (v/v) hiFBS and 1%(v/v) P/S. Microglial cells were counted and plated at the desired cell density, accordingly with the experiment to be performed.

After the shaking protocol, the removed MGC culture medium (containing detached microglia cells) was replaced by DMEM with 10% (v/v) hiFBS and 1%(v/v) P/S. MGC cultures were maintained at 37°C in a humidified incubator with 5% CO^2, for at least 4 h, before being exposed to another shake at 220 rpm overnight, at 37ºC (to remove the OPCs). MGC cultures can be used as source of primary microglial cells for three consecutive times (once per week).

3.2.3 Rat cortical astrocytes culture

Astrocytes were obtained after the third shake of MGC cultures. After collecting the microglia from the third shake, the MGC-containing flasks were shaken overnight at 220 rpm to detach the OPCs from the astrocyte layer. The OPCs in suspension were discarded and the astrocyte layer washed with 1X PBS. Afterward, astrocytes were detached from the flask with 0.25% (w/v) trypsin from porcine pancreas by incubating the flasks for 5

min, at 37°C. After trypsin inactivation with medium containing 10% (v/v) hiFBS, the astrocyte suspension was centrifuged for 5 minutes, at 500 g; re-suspended in 3 ml of medium and distributed into three other T75 flasks. Astrocytes cultures were maintained in DMEM medium containing 10% (v/v) hiFBS and 1% (v/v) P/S. Medium was renewed at every 3 days and cells were split when confluence was reached. Astrocytes were used at passage 4, when these cultures become highly enriched in astrocytes (Figure 8).

3.2.4 Embryonic rat cortical neurons culture

Rat cortical neurons were harvested from E18 Wistar rat embryos. The day before neurons harvesting, microelectrode arrays (MEA) were prepared for culture. MEAs used (i.e. 256MEA100/30iR-ITO, Multichannel Systems) are composed of composed of 254 titanium nitride (TiN) recording electrodes, organized in a 16 x 16 square grid, and 4 internal reference electrodes. Each recording electrode is 30 µm in diameter and interspaced by 100 µm. MEAs were sterilized with 70% (v/v) ethanol, air-plasma cleaned for 3 minutes (0.3 mbar) and coated with 0.01 mg/ml PDL, overnight in the incubator at 37°C. PDL excess was removed by three washes with sterile water and The MEAs were left to dry inside the laminar hood. Therefore, MEAs were coated with 5 µg/ml of adhesion protein laminin diluted in NB (not supplemented), for at least 2 h, at 37ºC. Laminin coating was removed immediately before cell seeding.

For each embryo, prefrontal cortex was dissected in cold H-HBSS and enzymatically digested with 0.7 mg/ml trypsin (1:250), diluted in H-HBSS (without Ca^{2+} and Mg^{2+}) for 15 min, at 37°C. After trypsin inactivation with H-HBSS containing 10% (v/v) hiFBS, tissues were washed twice with H-HBSS and re-suspended in NB plus supplemented with 2% (v/v) B27 plus, 0.5 mM L-glutamine and 1% (v/v) P/S. Subsequently, tissues were mechanically dissociated with a 5 mL pipette and filtered with a 40 µm nylon strainer (Falcon). Cells were counted, seeded at 1 x 10^5 cells/cm^2 onto MEAs and kept at 37°C in a humidified incubator with 5% CO^2. Medium was partially renewed once a week.

3.3 Characterization of microglia culture purity

To determine microglia purity resulting from the isolation method described in section 3.2.2, cells from shake 1 and 2 were seeded at 6 x 10^4 cells/cm^2 in 35 mm cell culture dishes (µ-Dish 35 mm high glass bottom, Ibidi) and cultured for 24 or 48 h. Cell culture dishes were pre-coated with 0.01 mg/ml PDL diluted in sterile 1X PBS for at least 2 h, at 37ºC. Cells were maintained in DMEM medium containing 10% (v/v) hiFBS and 1%

(v/v) P/S and fixed with 4% (w/v) paraformaldehyde (PFA), at the defined time points. Microglia cell cultures were then immunostained using the microglial marker Iba1, as described in section 3.10. Triplicates (n=3) were made for each time point.

3.4 Microglia culture medium selection

To provide cultured microglia with the best conditions to remain as similar as possible to the resting state found under physiological in vivo conditions, three culture media were tested: i) DMEM containing 10% (v/v) hiFBS and 1% (v/v) P/S; ii) DMEM with 2% (v/v) of hiFBS and 1% (v/v) P/S; and iii) NB supplemented with 2% (v/v) B27, 0.5 mM L-glutamine and 1% (v/v) P/S.

Microglial cells from shakes 1 and 2 were seeded onto 0.01 mg/ml PDL-coated µ-slide 8 well (Ibidi) at a density of 1 x 10^5 cells/cm^2. At 24 h later, initial medium culture was replaced for each culture medium formulation up to test. Cells were maintained in culture for 24 or 48 h, at 37°C in a humidified incubator with 5% CO^2. Cells were fixed with 4% (w/v) PFA at the defined time points and, immunostained with the marker Iba1, as described in section 3.10. Triplicates (n=3) were made for each condition/time point.

3.5 Galvanotaxis system

3.5.1 3D stimulation platform design

A custom made 3D stimulation platform was designed using the CAD software Onshape™ and printed using the slicer Repetier-Host software (version 1.6.2) and a 3D printer (Prusa i3). This platform, made of polylactic acid (PLA) filament (1.75 mm, BQ), includes a reusable structure for the agar salt bridges, an electrodes holder and, a triple dish holder, compatible with 35 mm cell culture dishes (Figure 2). The agar salt bridge structures were built in a U-shape with lateral openings that allow the visual confirmation of bubbles absence (Figure 2A). The electrodes holder was designed to fix the electrodes in such a way that prevent electrodes from lifting off the solution while transporting the stimulation platform (Figure 2B). The triple culture dish holder aids in the transport of the entire stimulation platform (Figure 2C). The final 3D stimulation platform presented in Figure 2 is a result of many adjustments and improvements.

3.5.2 Electric field chamber preparation

The EF chamber was designed in a way that a constant EF could be applied to a predefined area. The EF chamber is composed by a 35 mm cell culture dish (µ-Dish 35 mm high

glass bottom, Ibidi), two PDMS layers and a glass coverslip. The cell culture dishes were air-plasma treated for 2 minutes and coated with 0.01 mg/ml PDL for at least 1h, at 37ºC. After washing with 1X PBS, coated dishes were left to air-dry inside a flow hood. PDMS layers were prepared by mixing in a 10:1 ratio the PDMS and the curing agent. After complete degassing in a vacuum desiccator, the PDMS mixture was poured in a flat surface and cured for 90 minutes, at 70ºC. Cured PDMS layer was then cut into semilunar-like shape layers, cleaned with vinyl tape (471, 3M^{TM}) and sterilized with 70% (v/v) ethanol. Inside a flow hood, PDMS layers were glued with silicone vacuum grease to the bottom of the culture dish, in order to create a rectangular 10 × 21 mm seeding area. The glass coverslip (18 x 22 mm) was sterilized by 70% (v/v) ethanol, dried and glued to the top of the PDMS layers, creating a central chamber (Figure 3A).

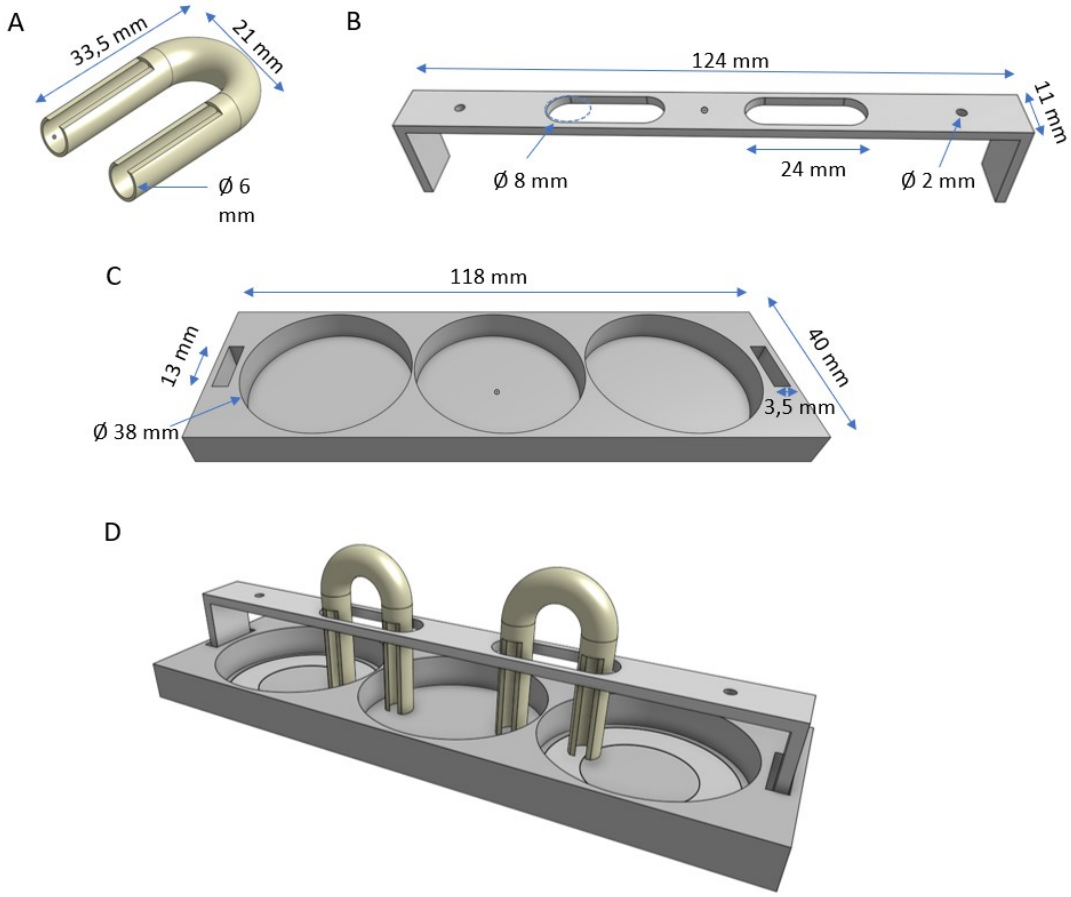

Figure 2. 3D stimulation platform. The stimulation platform designed and printed is composed by A) two agar salt bridge structures, B) and electrodes holder and, C) a triple culture dish holder. D) When assemble together these structures form a 3D stimulation platform.

3.5.3 Galvanotaxis system assembly

Prior to the galvanotaxis system assembly, the agar salt bridges and the conductive electrodes were prepared for use. In brief, the agar salt bridges structures previously developed were sterilized with 70% (v/v) ethanol and allowed to air-dry inside a flow hood. When completely dried, the cast ends were closed with parafilm and gently filled with the sterile and warm agar salt solution made of 2% (w/v) agarose diluted in Steinberg's solution (54 mM NaCl, 0.7 mM KCl, 1.6 mM $MgSO_4$, 0.4 mM $Ca(NO_3)_2$ and 1.4 mM Tris, adjusted to pH 7.4 with HCl, [174]). The agar salt solution was left inside the flow hood until complete solidification. Silver chloride (Ag/AgCl) electrodes (0.4 mm of thick, Science Products GmbH) were used in this study. Prior to its use, the silver chloride electrodes were hot glued to the electrode's holder (Figure 2B) and sterilized by ultraviolet light exposure (15 min).

To assemble the galvanotaxis system (Figure 3B), the EF chamber was placed in the middle of the dish holder, sided by two petri dishes filled with 4 mL of the electrolyte solution (i.e. Steinberg's solution). Then, the electrodes holder (with the Ag/AgCl electrodes) was fixed to the dish holder in a position that guarantee the submersion of electrodes in the electrolyte solution. Finally, a pair of agar salt bridges were placed in the vicinity of EF central chamber lateral ends to make the electrical connection between the EF chamber and the electrolyte solution reservoirs. The distance between agar salt bridges in the EF chamber was 21 mm (Figure 3A).

3.6 Galvanotaxis system validation

The custom made galvanotaxis system was validated prior to the cell experiments in two ways. First, the EF crossing the central channel of the EF chamber was confirmed using a voltmeter. In brief, after the galvanotaxis system was connected to the voltage-controlled power supply, the tip of the voltmeter was placed in different locations of the EF chamber to register the voltage crossing the EF chamber. When necessary the voltage imposed from the system was adjusted to fulfil the EF strength desired for the study.

Second, the feasibility of the custom made galvanotaxis system to study the cellular response to EFs of interest was further validated using primary astrocytes – a cell type widely explored and characterized in this type of studies. When exposed to EF of 400 V/m for 12-24 h, astrocytes align perpendicularly to the EF lines [112, 172]. In brief, astrocytes, at passage 4, were seeded at 7 x 10^4 cells/cm^2 in the central channel of the EF

chamber and allowed to adhere for 24 h, at 37 ºC in a humidified incubator with 5% CO_2. The EF was then applied at a strength of 400 V/m, for 12h. Control samples (unstimulated) missed the application of EF treatment. At 24 h of EF treatment, astrocytes were fixed with 4% (w/v) PFA and immunostained against GFAP (Section 3.10). Triplicates (n=3) were made for each condition.

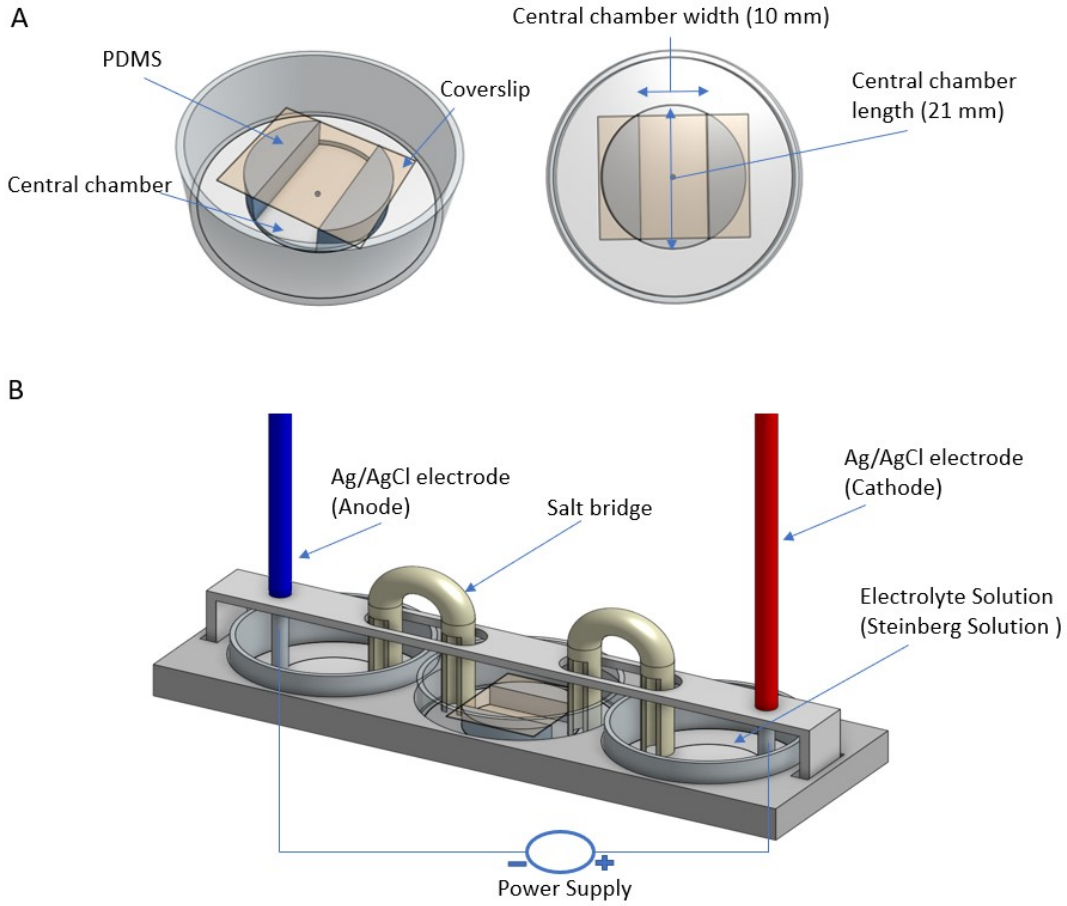

Figure 3. Galvanotaxis system overview. A) The electric field (EF) chamber, used to seed the microglia cells, was prepared by creating a central channel with two PDMS layers a glass coverslip place on top of the PDMS layers. B) 3D-view of galvanotaxis system assembly. The EF chamber is placed in the middle of the dish holder and is connected to the power supply, which drives a redox reaction at each electrode converting the electrical current into a ionic current. The ionic current is conducted, via the agar salt bridges, through the EF chamber by the movement of cations towards the cathode (the negatively charged electrode) and ions moving towards the anode (the positively charged electrode).

3.7 EF application

To determine effect of EF exposure on microglia, cells from shake 1 and 2 were seeded at 7.5 x 10^4 cells/cm^2 in the central channel of the EF chamber and allowed to adhere for 24 h, at 37 ºC in a humidified incubator with 5% CO_2. Afterwards, the EF chamber with microglial cells was placed in the middle of the dish holder and, the galvanotaxis system was assembled as described in section 3.6.

The galvanotaxis system, placed inside a humidified incubator at 37ºC and with 5% CO_2, was connected to a voltage-controlled power supply (UTP3305 DC Power Supplier, Uni-Trend Technology, Ltd) that imposed a pre-defined constant voltage to create a constant EF across the EF chamber. For this study, it was selected EFs of strength 40 V/m or 400 V/m. The voltage imposed by the power supply was determined by multiplying the desired EF (in mV/mm) by the distance (in mm) between each agar salt bridge. Prior to begin the experiment, the voltage applied to the EF chamber was confirmed with a multimeter in different locations of the EF chamber, as described in section 3.6.

The effect of the selected EF strengths on microglial cells behavior was evaluated after 6 or 24 h of EF exposure. Control samples (unstimulated) missed the application of EF treatment. At the defined time points, microglial cells were fixed with 4% (w/v) PFA and immunostained against Iba1 (Section 3.10). Triplicates (n=3) were made for each condition/time point.

3.8 NCM collection and microglia treatment

The impact of bioactive compounds (secretome) resultant from neuronal network activity on microglia behavior were investigated by exposing microglial cells to the NCM from neuronal cultures at different maturation days in vitro. At 4, 7, 14 and 21 days in vitro (DIV), 0.7 mL of NCM from primary cortical neurons cultures was collected, centrifuged at 1200 rpm for 5 min to precipitate out cell debris and, immediately frozen at -20ºC.

To determine effect of NCM exposure on microglia, cells were seeded onto PDL-coated µ-slide 8 well, in DMEM with 10% (v/v) hiFBS and 1% (v/v) P/S, at 1 x 10^5 cells/cm^2 and allowed to adhere for 24 h, at 37 ºC in a humidified incubator with 5% CO_2. Then, 80% of the microglia culture medium was removed and replaced by each NCM (same volume). In the control condition, 80% of microglia culture medium was replaced with new microglia medium DMEM with 10% (v/v) hiFBS and 1% (v/v) P/S). At 24 and 48 h

of NCM exposure, microglial cells were fixed with 4% (w/v) PFA. Triplicates (n=3) were made for each condition/ time point.

3.9 Monitoring of neuronal network activity recording

Primary cortical neuron cultures electrophysiological activity was monitored along time by using the MEA2100-256 recording system and the integrated temperature controller (Multichannel Systems) settled to 37ºC. Extracellular activity of neuronal networks was recorded at 4, 7, 14 and 21 days after cortical neurons seeding on MEAs and before the collection of NCM (see section 3.8). In brief, at defined DIV, MEAs containing neuronal cultures were placed on the MEA2100-256 recording system and left to equilibrate for 5 min before recording. Then, neuronal activity was recorded at a sampling rate of 10 kHz for a period of 5 min. After recording, raw data was converted to HDF5 file format, using the Multi Channel Analyzer software (Multichannel Systems), and analyzed using a custom-made MATLAB script. For data analysis, the recorded data was firstly filtered using a high and low pass filter of 200 and 3000 Hz, respectively. This allows to exclude from analysis any reading artifact. Next, after selecting the entire electrode matrix, the mean firing rate was calculated and the bursts characterized. These parameters, used to characterize the neuronal activity at each DIV, were exported as histograms. The obtained data was used to characterize the levels of neuronal activity of cultures used for NCM collection.

3.10 Immunocytochemistry

Cultured microglia and astrocytes were fixed with 4% (w/v) PFA in 1X PBS for 15 min, at room temperature (RT), permeabilized with 0.5% (v/v) Triton X-100 in 1X PBS for 10 minutes at RT, and blocked with 5% (v/v) hiFBS in 1X PBS for 30 minutes at RT. Cells were then incubated, overnight at 4ºC, with the primary antibodies raised against Iba1 or GFAP(both rabbit, 1:500) diluted in the blocking solution. After 3 washes with 1X PBS, cells were incubated with a secondary antibody Alexa Fluor 488 anti-rabbit IgG (goat, 1:1000) diluted in blocking solution for 1 h, at RT. Cells nuclei were stained with 0.1 µg/ml Hoechst 33342 dye for 10 minutes, at RT.

3.11 Image acquisition

Immunostained cells were imaged using a laser scanning confocal microscope Leica TCS SP5 II (Leica Microsystems) with the HCX PL APO CS 40×/1.3 oil objective. Laser lines

at 405 and 488 nm were used for Hoechst 33342 or Alexa Fluor® 488 excitation, respectively. For each sample, a minimum of 15 (purity, culture media test and NCM treatment experiments) or 20 (EF experiments) fields of view (1024 x 1024 pixel) were randomly selected and imaged for analysis. In EF experiments, for each region (anode, center and cathode) were acquired at least 6 fields of view. When necessary, image z-stacks were acquired at 0.5 µm steps. Images were acquired from three independent experiments.

3.12 Morphometric analysis

For microglia morphometric analysis, three parameters were employed and measured with ImageJ/Fiji (National Institute of Health, USA) [175] using the "Analyze" function: area, roundness and solidity. These parameters were chosen due to their significance in the characterization of the main microglia morphological properties. In the "resting" state, microglia are characterized by small cell bodies with ramifications – multiple branches and processes – that terminate with bulbous endings. In the presence of an insult, microglia change progressively their morphology towards an enlarged cell body with an amoeboid shape. The cell area parameter was select to characterize microglia cell body expansion or retraction – properties dependent on microglia activation status. The cell area is calculated by the total number of pixels in the filled shape of the cell image [176]. The roundness and solidity parameters were select to characterize the shape of microglia cell body and the level of microglia ramification, respectively. Roundness, a relatively recent parameter first introduced in 2016 [177], which measures how round is the shape of an object. An object is described as round if all points of a cross section are equidistant to a common center. Roundness is calculated as $(4\times \pi \times \text{cell area})/(\text{cell perimeter})^2$, being corrected by the aspect ratio. This parameter is helpful to evaluate if microglial cells present an amoeboid or more round-like morphology [178]. Cells with equal area values but with more ramifications will have larger perimeter values and therefore, decreased values for roundness. Solidity is calculated by the ratio of an object's area and its convex area. When an object shape deviates from a closed circle (i.e. presents irregular boundaries), its convex area increases and the calculated solidity decreases. Solidity value equals 1 for a solid object and, is below 1 for objects that contain holes or an irregular boundary. With this parameter, it is possible to determine the level of microglia ramification [176].

For morphometric analysis, acquired images were processed with a custom-made ImageJ macro designed to perform a semi-automated analysis of immunostained microglia cells, following the workflow illustrated in the Figure 4. Prior to any image processing, the image was converted to 8-bits, in order to use most of the tools available in the ImageJ software. Next, images were preprocessed with a medium filter with a radius of 2 pixels, to remove noise that could exist in the acquired image. Afterwards, the segmentation was performed through an automatic threshold (i.e. Default option), which is a variation of the IsoData algorithm. This method divides the image in two parts, the objects and the background, by taking an initial threshold. Then it calculates the average of the pixels, the pixels that are below or above are computed. Successively, the average of this values are calculated and the threshold is incremented (this procedure suffers interactions) until the threshold is bigger that the composite average [179]. Subsequently, the binary filter "Close" was applied, which consists in a dilation operation followed by an erosion to smooth the objects and fill small holes. Next, the binary filter "Fill holes" was applied to fill bigger holes in objects by filling the background. Lastly, using the tool "Analyze particles", the measurements were taken in the size range of 300 to infinity pixels. For the analysis, highly ramified cells with complex shape (Figure 5B), were not taken into account due to the macro inefficiency in processing analysis of such complex cell membrane.

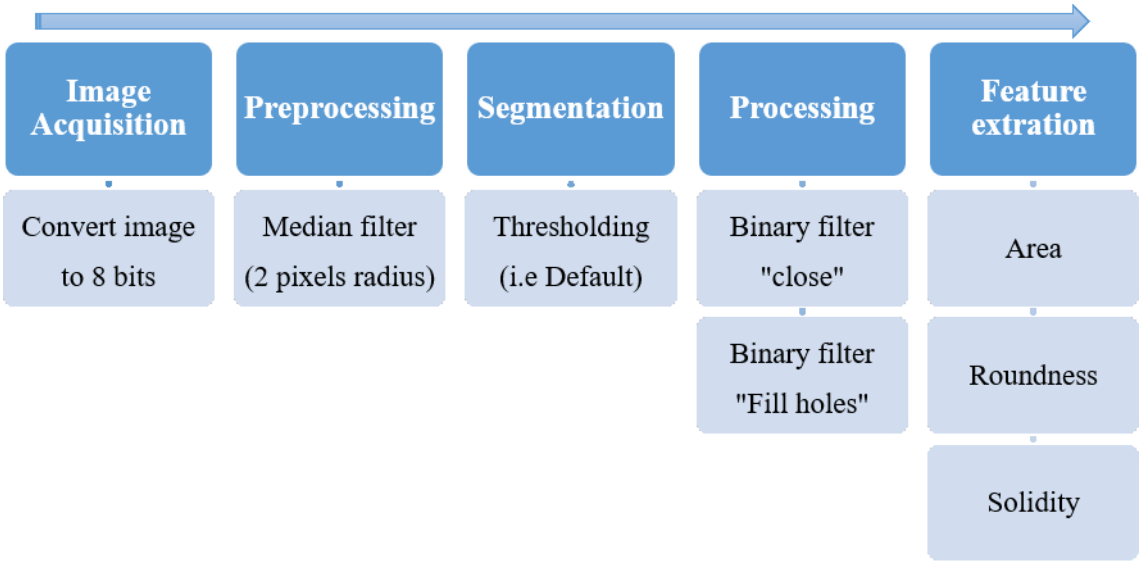

Figure 4. Image processing workflow of the custom-made ImageJ macro. Schematic flowchart detailing each step implemented during microglial cells segmentation for features extraction (area, roundness and solidity).

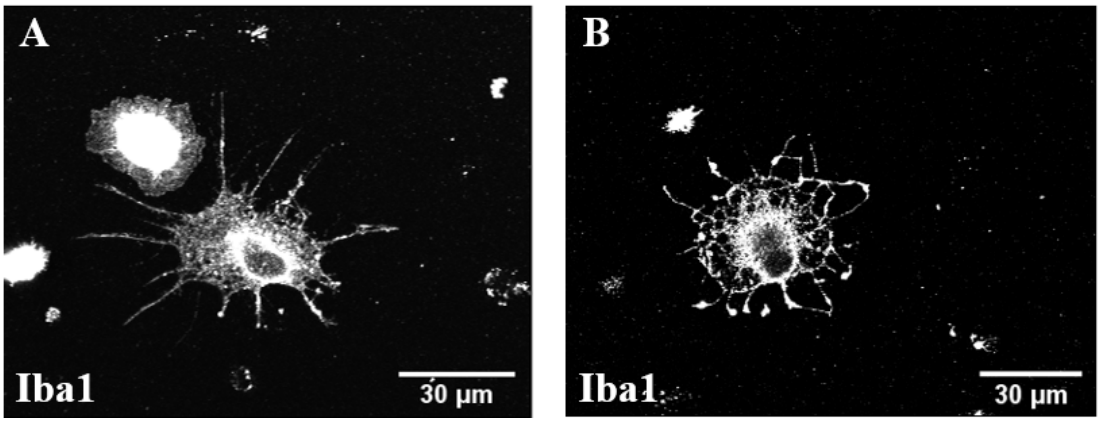

Figure 5. Representative images of the cell type found during the morphologic analyze. A) Image of a microglia cell able to be analyzed with the macro developed, despite the ramifications displayed. B) Example of a microglia cell with ramifications connected not allowing the macro to work, due to the fact that these protrusions led to a false perimeter.

3.13 Statistical analysis

The interquartile range method was used to select and remove the outliers from the data pool, prior to the statistical analyses, using the Microsoft Office Excel. Statistical analysis was performed using GraphPad version 5.0 software. Data's normality was tested using the d'Agostino and Pearson normality test. Since data did not follow a Gaussian distribution, the Kruskal-Wallis test followed by Dunns Multiple Comparison test was used to compare the median values between experimental groups (experiments for microglia culture media selection and NCM treatment experiments). The non-parametric Wilcoxon–Mann–Whitney test was used to compare the median values between experimental groups (EF exposure experiments). Significance was determined at p-values < 0.05.

Chapter 4

Results

4.1 Microglia cell culture characterization

Microglial cells used in this work were isolated from primary MGC cultures using the shaking method [173]. This is one of the faster and easiest methods that allow to obtain high amounts of microglia cells, but also other glial cells such as oligodendrocytes and astrocytes. Therefore, in this way one can considerably reduce the number of animals needed for these in vitro experiments. The purity of microglia cultures was determined at 24 and 48 h after seeding, by labelling these cells with the specific microglia marker Iba1. Immunocytochemistry data indicates that these cultures are highly enriched in microglial cells (> 86%), at both time points (Figure 6C). Microglial cells isolated by the shaking method presented enlarged cell body with cytoplasmatic processes (Figure 6A-B).

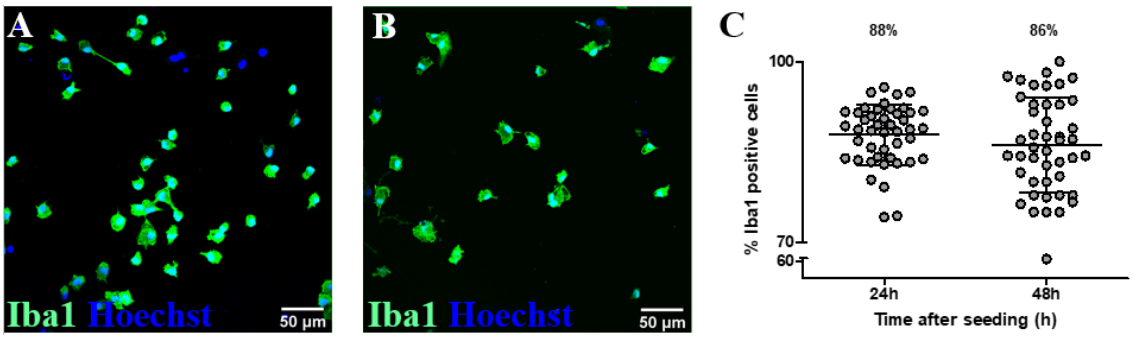

Figure 6. Microglia culture purity isolated from mixed glial cell cultures by the shaking method. Microglial cells were identified via immunocytochemistry using the specific marker for microglia Iba1. A) Representative images of microglial cells cultured for 24 or B) 48 h after isolation by shaking. Scale bar = 50 µm. C) The percentage of Iba1 positive cells was determined at 24 and 48 h after seeding (n = 3 for mixed glial culture isolation procedures).

4.2 Microglia culture media selection

In vitro models aiming to study the impact of a treatment on microglia behavior, under homeostatic conditions, should ideally recapitulate the "resting"-like phenotype of microglia in vivo. This will allow to expose microglia to different stimuli and characterize their response towards it. Unfortunately, at the time they are brought in culture, primary microglia become deprived of CNS microenvironmental cues that guarantee their resting

state in vivo. In an attempt to create an in vitro environment that can offer to microglia the conditions to be near a "resting"-like state, we have tested three different cell culture media: a serum-free, a low-serum and an intermediate-serum media. Microglia phenotypic changes are usually accompanied by morphological modifications, which is widely used to categorize different microglia activation states. In general, ramified resting microglia converts to an activated state by displaying larger cell bodies and decreased cytoplasmic processes, progressing to a full amoeboid morphology [69, 178]. Therefore, microglial cells cultured in each media formulation for 24 or 48 h were characterized in terms of morphological changes using the following morphometric parameters: area, roundness and solidity.

At 24 h in culture, microglia presented a homogenous area frequency distribution that range from 43.76 to 756.9 μm^2. Still, microglial cells cultured within 10% and 2% hiFBS showed significant differences in their population means and medians (Figure 7A and Table 1). No differences were observed in comparison with the NB culture medium at this time point. At 48 h in culture one could observe some differences in microglial cells area according to the media formulation used. Cells cultured in the presence of 10% hiFBS displayed a regular area distribution that range from 43.48 to 666.2 μm^2. Contrarily, in low and free-serum conditions microglial cells presented a significantly increased mean and median area values (Figure 7B and Table 1). In low-serum condition, microglia population presented a bimodal area frequency distribution, ranging from 45.05 to 325 μm^2 and from 325 to 886.1 μm^2, respectively. Microglia cultured in serum-free conditions presented a wider area frequency distribution that ranged from 49.21 to 1707 μm^2. These cells presented significantly higher mean and median area values in comparison with the other cell culture conditions (Figure 7B and Table 1).

Regarding the roundness and solidity parameters evaluation, no significant differences were observed between microglial cells cultured for 24 h in each culture media formulation (Figure 7C, E and Table 1). The roundness frequency distribution was very similar between treatments ranging from 0.13 to 0.99, with similar population means and medians (Table 1). The solidity frequency distribution was right-skewed with identical population means and medians between treatments, ranging from 0.47 to 0.96 (Table 1). At 48 h in culture, microglial cells cultured in medium containing 10% hiFBS showed significantly increased mean and median roundness/solidity values in comparison with the other media formulations (Figure 7D, F and Table 1).

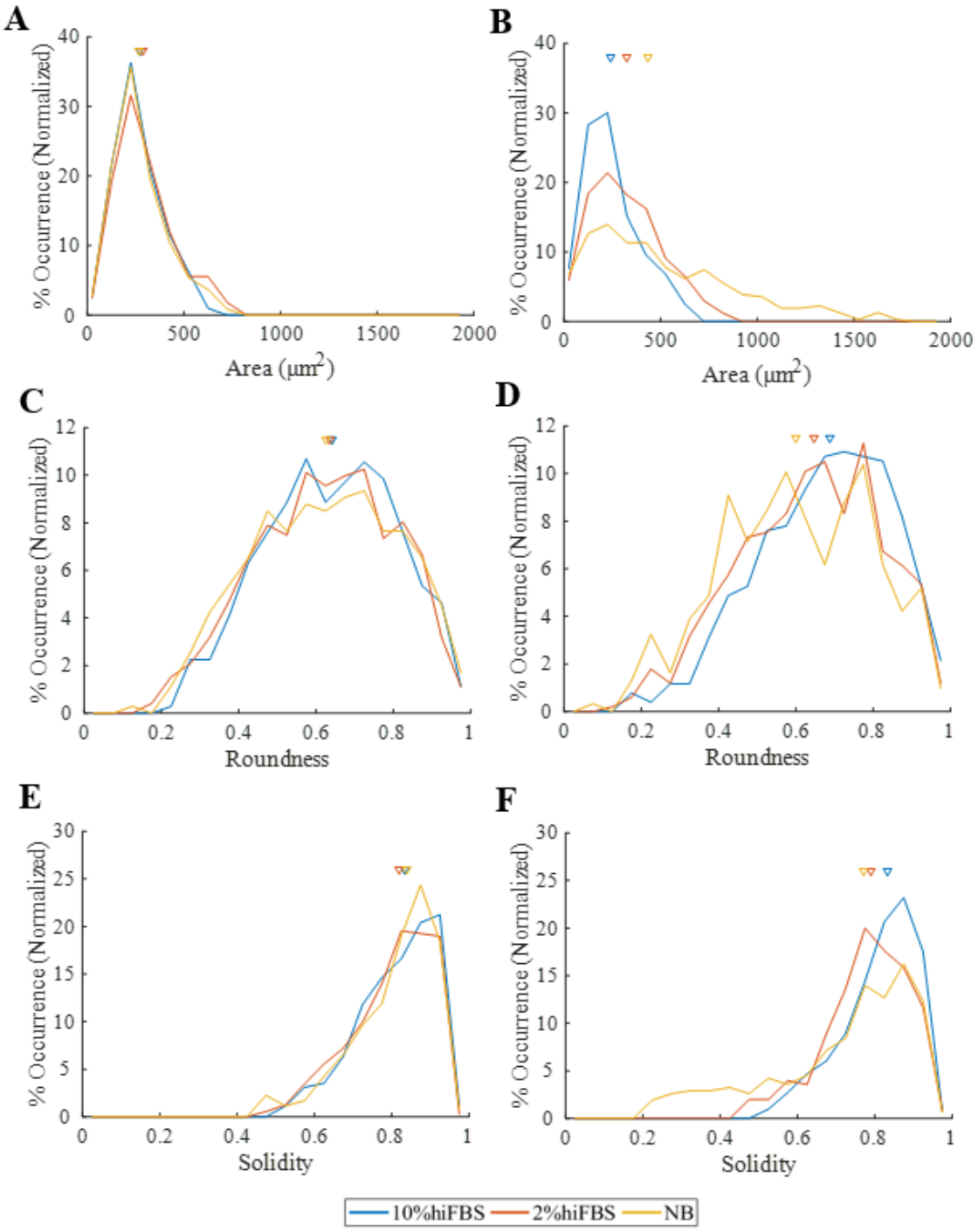

Figure 7. Frequency distribution of area, roundness and solidity, in microglial cells cultured in different media. Graphs illustrating the morphologic parameter in microglia after 24 (A, C, E) and 48 h (B, D, F) in culture with each culture media. ▼ Color-matched median values (blue: 10% hiFBS; red: 2% hiFBS, yellow: NB medium).

Together, these results showed that microglial cells cultured in medium containing 10% hiFBS were the smaller rounded ones, with less irregular boundaries. Still, these were the

cells presenting a more homogenous frequency distribution in all three parameters evaluated, thus with added value when aiming to distinguished subtle differences between treatments. Moreover, the 10% hiFBS medium formulation showed to be a better condition in terms of cell viability in culture (Table 1), especially in comparison with the NB medium (the one with lower roundness and solidity values). For all these reasons, the culture medium containing 10% hiFBS was chosen as the control culture medium for the remaining experiments developed and presented in this thesis.

Table 1. Summary of the descriptive statistics for the selection of microglia culture medium. The area, roundness and solidity parameters were quantified after 24 and 48 h in culture with each media formulation. N, number of cells analyzed; Min, minimum value; Max, maximum value; SD, standard deviation; CI, confidence interval.

Parameters		N	Min	Max	Median	Mean ± SD	95% CI of mean
Area							
24h	10% hiFBS	711	45.17	634.9	271.4	289.8 ± 120.7	[281.0:298.7]
	2% hiFBS	722	44.31	752.4	286.9 #	319.0 ± 51.00	[308.0:330.0]
	NB	353	43.76	756.9	267.6	299.0 ± 142.3	[284.1:313.8]
48h	10% hiFBS	513	43.48	666.2	240.9	271.4 ± 141.4	[259.2:283.7]
	2% hiFBS	505	45.05	886.1	324.8 ###	346.3 ± 182.5	[330.4:362.3]
	NB	308	49.21	1707	434.2	536.5 ±378.5	[494.0:578.9]
Roundness							
24h	10% hiFBS	711	0.23	0.99	0.64	0.64 ± 0.17	[0.62:0.65]
	2% hiFBS	722	0.19	0.99	0.64	0.62 ± 0.18	[0.61:0.63]
	NB	353	0.13	0.98	0.63	0.62 ± 0.18	[0.60:0.64]
48h	10% hiFBS	513	0.17	0.98	0.69 ##	0.67 ± 0.17	[0.66:0.69]
	2% hiFBS	505	0.12	0.97	0.65 ###	0.63 ± 0.18	[0.62:0.65]
	NB	308	0.09	0.97	0.60	0.60 ± 0.19	[0.58:0.62]
Solidity							
24h	10% hiFBS	711	0.50	0.96	0.83	0.81 ± 0.10	[0.80:0.82]
	2% hiFBS	722	0.47	0.96	0.82	0.80 ± 0.10	[0.80:0.81]
	NB	353	0.47	0.96	0.84	0.80 ± 0.11	[0.80:0.82]
48h	10% hiFBS	513	0.52	0.96	0.83 ###	0.81 ± 0.10	[0.80:0.82]
	2% hiFBS	505	0.46	0.96	0.79 #	0.78 ± 0.11 ###	[0.80:0.79]
	NB	308	0.22	0.97	0.77	0.71 ± 0.20	[0.70:0.73]

# p<0.05; ## p<0.01; ### p<0.001; Kruskal-Wallis Test followed by Dunns Multiple Comparison Test

4.3 Impact of EFs in microglia cells

To evaluate if microglial cells are responsive to electrical activity per se (i.e. without chemical cues released during neuronal activity), microglia were exposed to EFs using a galvanotaxis system designed and optimized in the framework of this thesis (see Figure 2 and 3). Prior to use for application of EFs to microglia cells, the developed galvanotaxis system was validated with primary cultures of astrocytes, well-known to react to EFs by changing their orientation in relation to the EF [172]. Following previous reported studies, an EF of 400 V/m was applied, for 12h, to astrocytes cultured in the custom-made galvanotaxis system. As reported in other studies, astrocytes aligned perpendicularly to the EF when using the custom-made galvanotaxis system (Figure 8). Therefore, the effect of EFs in microglial cells was evaluated using this system. To the best of our knowledge, this is the first study evaluating the influence of EF in primary microglial cells behavior. Thus, one have decided to explore the EFs previously reported in studies with immortalized murine BV-2 microglia cell lines [6] – 40 and 400 V/m. Microglial cells were treated with each EF for 6 or 24 h and, their behavior were characterized in terms of morphological changes (i.e. area, roundness and solidity).

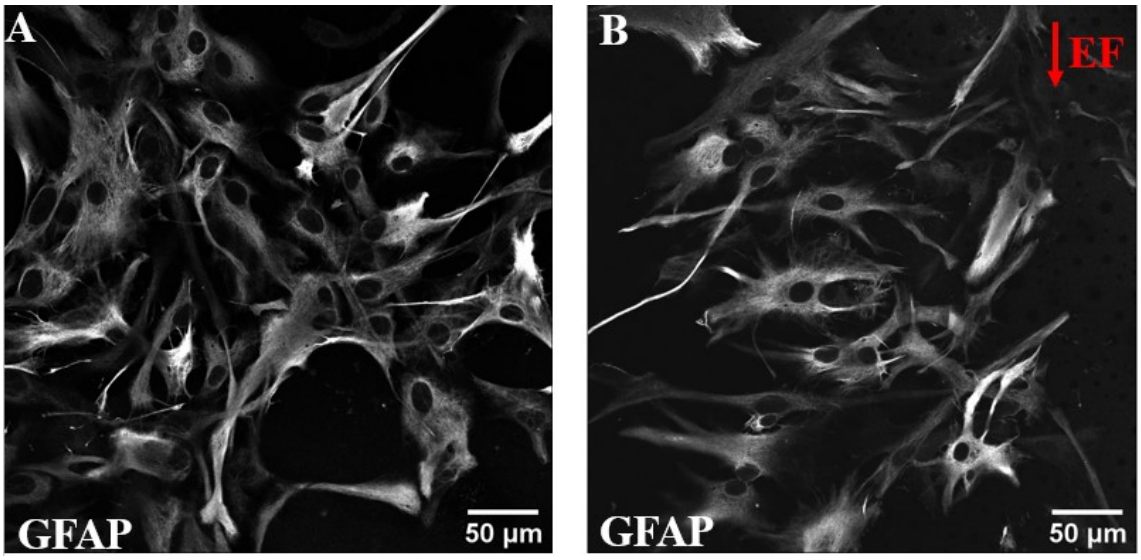

Figure 8. Astrocytes alignment. A) Primary astrocytes cultured in the absence of EF showed a random distribution of their cytoplasmic processes in space, while B) astrocytes exposed to an EF of 400 V/m, for 12h, displayed a perpendicular alignment (in relation to the EF) of their processes. Scale bar = 50 µm.

Microglial cells exposed to an EF of 40 V/m presented significant changes in mean and median area values in relation to the control (Figure 9A-B, Table 2). After 6 h of EF application, microglial cells significantly decreased their area. At 24 h after stimulation, it was observed a significant increase in the area of the treated cells and a 30% decrease in cell number, in comparison with the control condition.

In terms of roundness and solidity, no differences were observed between stimulated and control microglia cells, after 6 h of EF application (Figure 9C, E and Table 2). However, after 24 h of EF application, both roundness and solidity mean/median values were significantly different from the control condition: roundness values decreased while solidity values increased (Table 2).

Microglial cells exposed to a 400 V/m EF for 6 h presented a significant decrease of mean and median area values in relation to the control (Figure 10A, Table 3). No differences were observed between treatment and control condition after 24 h of EF exposure (Figure 10B, Table 3).

Regarding the microglia roundness, only the exposure to 400 V/m for 24 h caused a significant decrease in the mean values of microglia roundness (Figure 10C-D and Table 3). However, a significant decrease in solidity mean/median values, in relation to the control condition, were observed at both 6 and 24 h of EF exposure. (Figure 10E-F and Table 3).

Together, these results showed that each EF tested impact microglial cells differently. The 40 V/m EF treatment lead to variable microglia morphological changes, accordingly with the time of stimulation. Contrarily, the 400 V/m EF resulted in small microglial cells with more irregular boundaries.

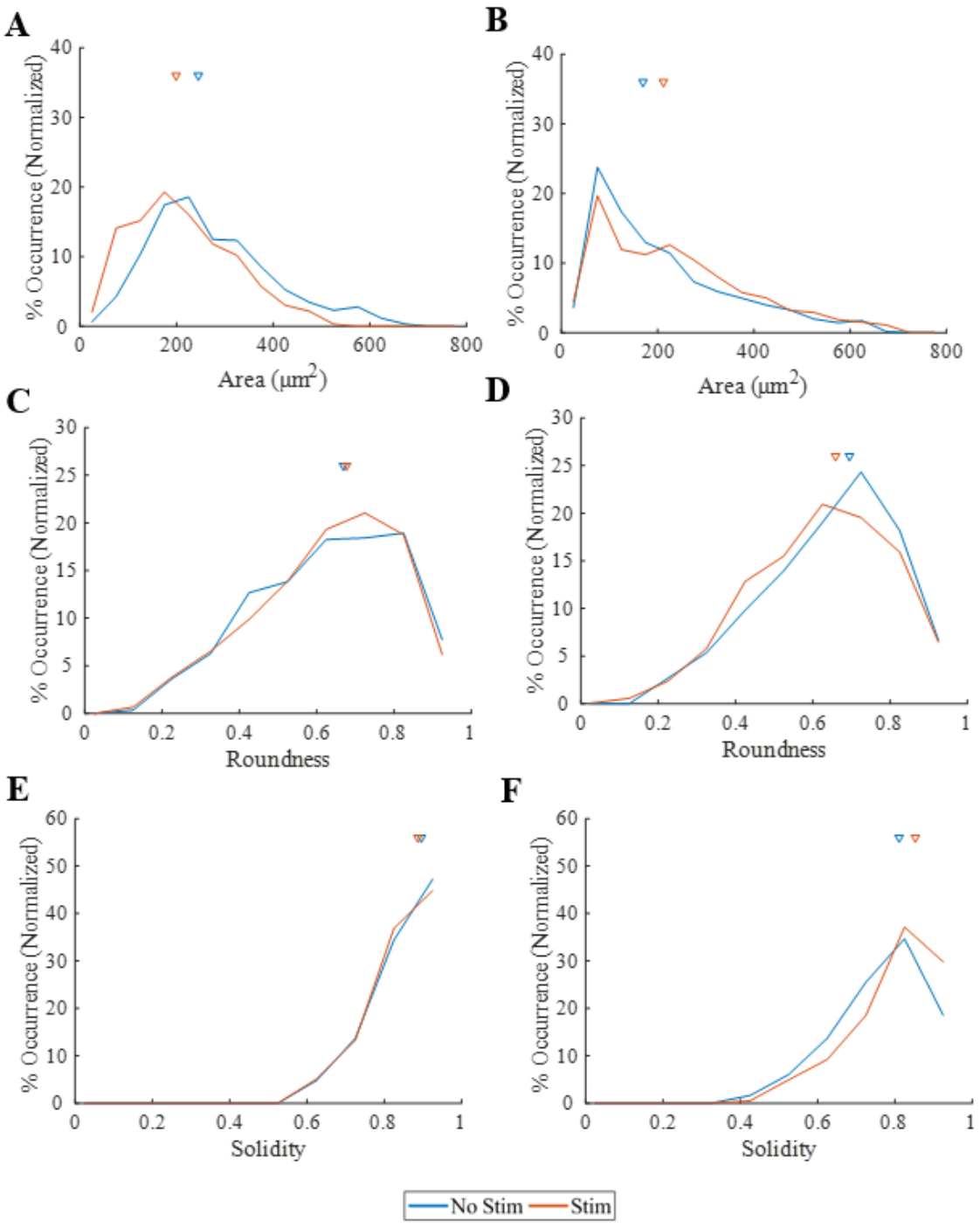

Figure 9. Frequency distribution of area, roundness and solidity in microglial cells treated with the 40 V/m EF treatment. Each morphologic parameter was evaluated at (A, C, E) 6 h and (B, D, F) 24 h after EF exposure. ▼ Color-matched median values (blue: Control group, no stimulation; red: Treatment group, 40V/m stimulation).

Table 2. Summary of the descriptive statistics for the 40 V/m EF treatment of microglia cells. The area, roundness and solidity parameters were quantified after 6 and 24 h under EF application. CTRL, control treatment; N, number of cells analyzed; Min, minimum value; Max, maximum value; SD, standard deviation; CI, confidence interval.

Parameters		N	Min	Max	Median	Mean ± SD	95% CI of mean
Area							
6h	CTRL	608	45.05	652.3	245.1	271.7 ± 126.1	[261.6:281.7]
	40 V/m Stim	922	20.12	510.4	199.1###	212.8 ± 106.4	[205.9:219.6]
24h	CTRL	2129	43.04	656.1	169.6	210.0 ± 144.4	[203.9:216.1]
	40 V/m Stim	1433	43.04	1111	211.9###	235.5 ± 155.3	[227.4:243.5]
Roundness							
6h	CTRL	608	0.20	1.00	0.67	0.66 ± 0.19	[0.64:0.67]
	40 V/m Stim	922	0.15	1.00	0.68	0.65 ± 0.18	[0.64:0.67]
24h	CTRL	2129	0.21	1.00	0.70	0.67 ± 0.17	[0.66:0.68]
	40 V/m Stim	1433	0.10	1.00	0.66###	0.65 ± 0.18	[0.64:0.66]
Solidity							
6h	CTRL	608	0.61	0.97	0.90	0.87 ± 0.08	[0.86:0.88]
	40 V/m Stim	922	0.62	0.97	0.89	0.87 ± 0.08	[0.86:0.87]
24h	CTRL	2129	0.44	0.97	0.81	0.80 ± 0.12	[0.78:0.79]
	40 V/m Stim	1433	0.48	0.97	0.85###	0.82 ± 0.11	[0.82:0.83]

$p<0.001$ versus Control; Mann-Whitney test

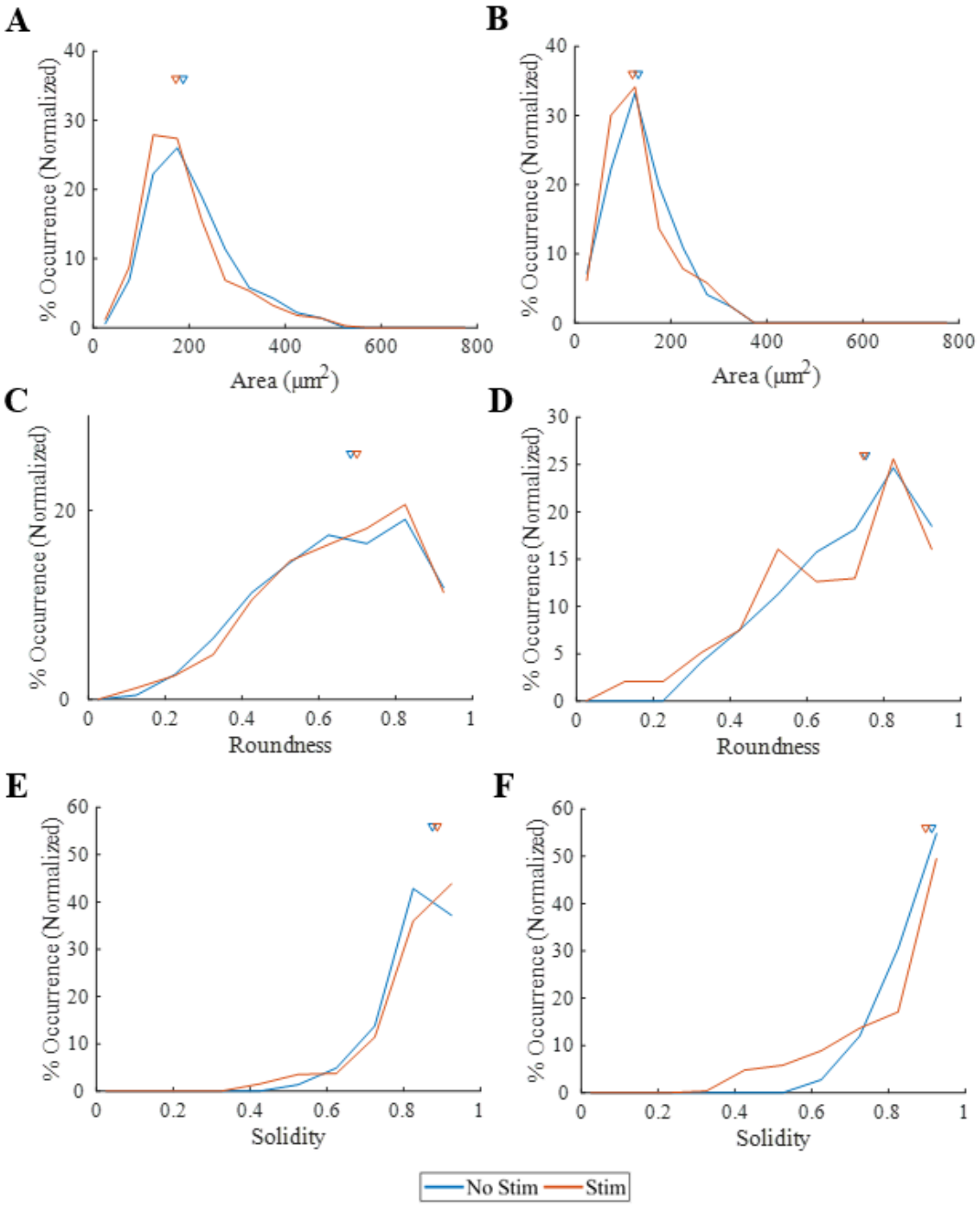

Figure 10. Frequency distribution of area, roundness and solidity, in microglial cells treated with the 400 V/m EF treatment. Each morphologic parameter was evaluated at (A, C, E) 6 h and (B, D, F) 24 h after EF exposure. ▼ Color-matched median values (blue: Control group, no stimulation; red: Treatment group, 400V/m stimulation).

Table 3. Summary of the descriptive statistics for the 400 V/m EF treatment of microglia cells. The area, roundness and solidity parameters were quantified after 6 and 24 h under EF application. CTRL, control treatment; N, number of cells analyzed; Min, minimum value; Max, maximum value; SD, standard deviation; CI, confidence interval.

Parameters		N	Min	Max	Median	Mean ± SD	95% CI of mean
Area							
6h	CTRL	1329	43.19	496.2	187.4	204.1 ± 87.6	[199.4:208.8]
	400 V/m Stim	990	27.41	510.4	172.0###	189.3 ± 87.8	[183.8:194.8]
24h	CTRL	292	43.48	324.4	132.0	138.8 ± 63.8	[131.4:146.1]
	400 V/m Stim	293	43.76	340.1	119.9	132.8 ± 66.8	[125.1:140.4]
Roundness							
6h	CTRL	1329	0.12	0.99	0.68	0.67 ± 0.19	[0.66:0.68]
	400 V/m Stim	990	0.12	0.99	0.70	0.68 ± 0.19	[0.67:0.69]
24h	CTRL	292	0.32	0.99	0.75	0.73 ± 0.17	[0.71:0.75]
	400 V/m Stim	293	0.12	0.99	0.75	0.70 ± 0.20	[0.67:0.72]
Solidity							
6h	CTRL	1329	0.56	0.97	0.88	0.86 ± 0.08	[0.85:0.86]
	400 V/m Stim	990	0.45	0.97	0.89##	0.85 ± 0.11	[0.85:0.86]
24h	CTRL	292	0.64	0.96	0.91	0.88 ± 0.07	[0.87:0.89]
	400 V/m Stim	293	0.38	0.96	0.90#	0.83 ± 0.15	[0.81:0.84]

# $p<0.05$, ## $p<0.01$, ### $p<0.001$ versus Control; Mann-Whitney test

4.4 Impact of NCM in microglia cells

To evaluate the impact of neurons secretome (in the absence of electrical signals) in microglial cells behavior, these cells were exposed to NCM from neuronal cultures at different maturation days in vitro (DIV 4, 7, 14 and 21).

At each DIV, neuronal cultures presented different levels of activity, which progressively increased with time. At DIV 4, neuronal networks were yet to be established and, therefore no activity was detected (data not shown). As shown on Figure 11, since DIV 7 neuronal cultures start to display detectable levels of spontaneous activity, with increased mean firing rate values and burst events.

The results obtained showed a significant increase in microglial cells area treated with any of the NCM in study when compared with the control condition, both at 24 and 48 h (Figure 12 and Table 4). The area frequency distribution of microglia cultured in control

conditions was unimodal, ranging from 45.17 to 634.90 μm^2 and from 43.48 to 666.20 μm^2, at 24 or 48h respectively. Contrarily, the area frequency distribution of microglia cultured in contact with NCM from different DIV were all multimodal, with several small populations (Figure 12). Although at 24 h there was no clear correlation with area values and NCM from increasing DIV, after 48 h microglial cells showed a significant decreased area when in contact with NCM from longer DIV (i.e. DIV 14 and 21) in comparison with the NCM from shorter DIV (i.e. DIV 4 and 7). The higher mean/media area values were observed for NCM from DIV 7 while the lower values were observed for NCM from DIV 14, at both time points. Regarding the microglia roundness after NCM treatment, one can observe that the roundness frequency distribution of microglia cultured under NCM treatment was wider in range and multimodal as compared to the control condition, at 24 and 48 h (Figure 12C-D and Table 4). Except for the cells treated with NCM from DIV 4, all NCM treatments resulted in a significant decrease in the microglia cell roundness values in relation to the control condition, both at 24 and 48 h (Figure 12C-D and Table 4). The NCM responsible for creating the lowest roundness value in microglial cells was the one obtained from neuronal cultures with 21 DIV, both at 24 h and 48 h (Table 4).

Microglia treatment with NCM from neuronal cultures at different DIV showed to exert also a significant impact in microglial cells solidity values. After 24 h in contact with the NCM, microglial cells presented significant lower mean/median values of solidity as related to the control condition. The same was observed for the 48 h time point, with the exception of NCM from cultures at DIV 14, which showed similar values to the control treatment (Figure 12E-F and Table 4). The NCM responsible for the lowest solidity value in microglial cells was the one obtained from neuronal cultures with 21 DIV, both at 24 h and 48 h (Table 4). As observed for roundness, the solidity frequency distribution of microglia cultured under NCM treatment was wider in range (left-skewed) as compared to the control condition, at both 24 and 48 h (Figure 12E-F).

Together, these results showed that microglial cells cultured in presence of NCM from older neuronal cultures showed significantly less irregular boundaries. Still, while one could observe a ~ 40% increase in microglia cell number when treated with NCM from DIV 14, the present results showed around 25% decrease in cells number for NCM treatments from DIV 4 and 21 (Table 4).

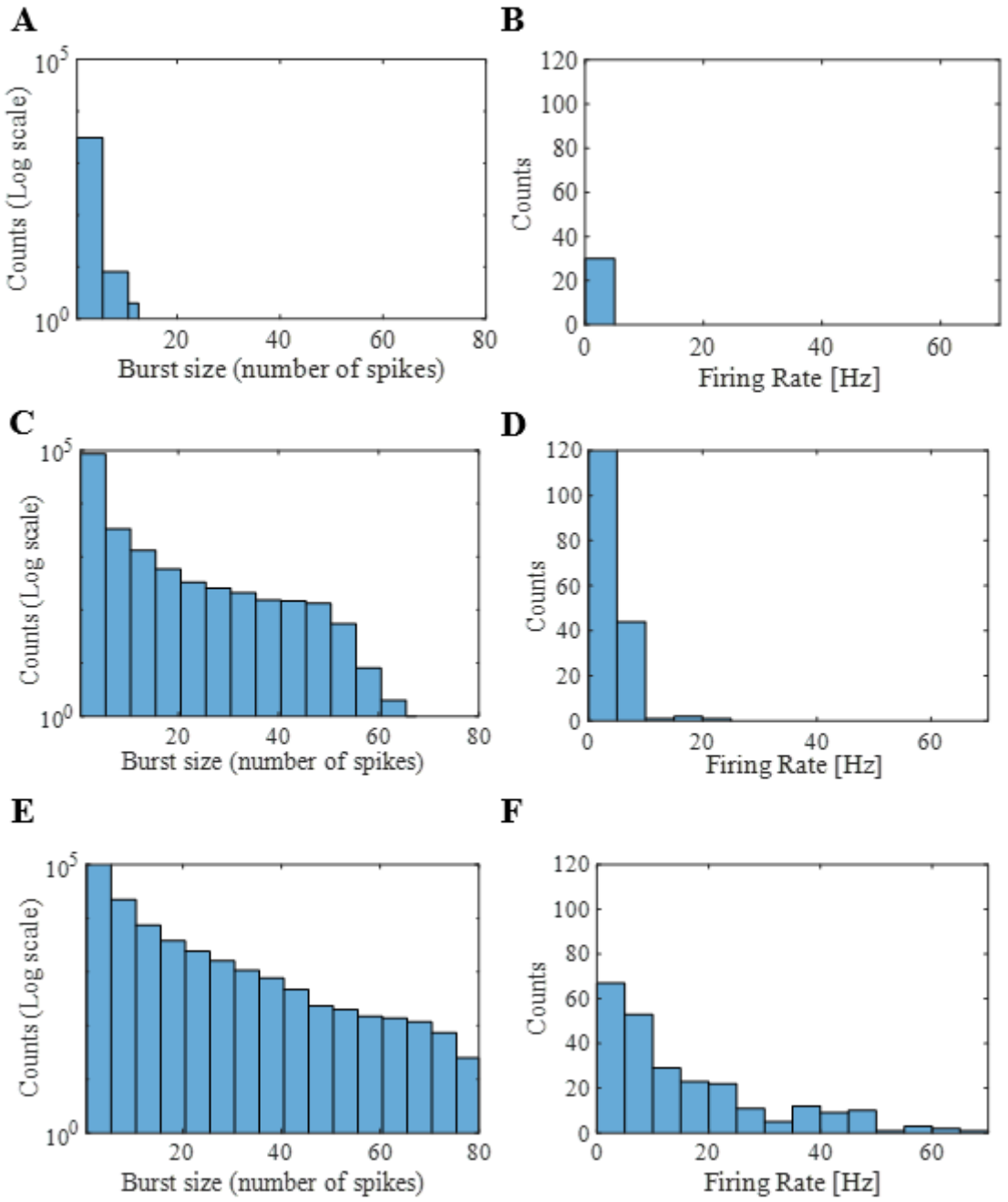

Figure 11. Characterization of the neuronal networks activity at different days in vitro (DIV). (A, C, E) Burst size histograms showing the number of spikes detected in neuronal networks cultured for 7, 14 and 21 DIV, respectively. (B, D, F) Histograms showing the mean firing rate of neuronal networks cultured for 7, 14 and 21 DIV, respectively. **Note**: Neuronal networks cultured for 4 DIV did not displayed detectable levels of spontaneous activity. Therefore, the activity of these cultures were not characterized.

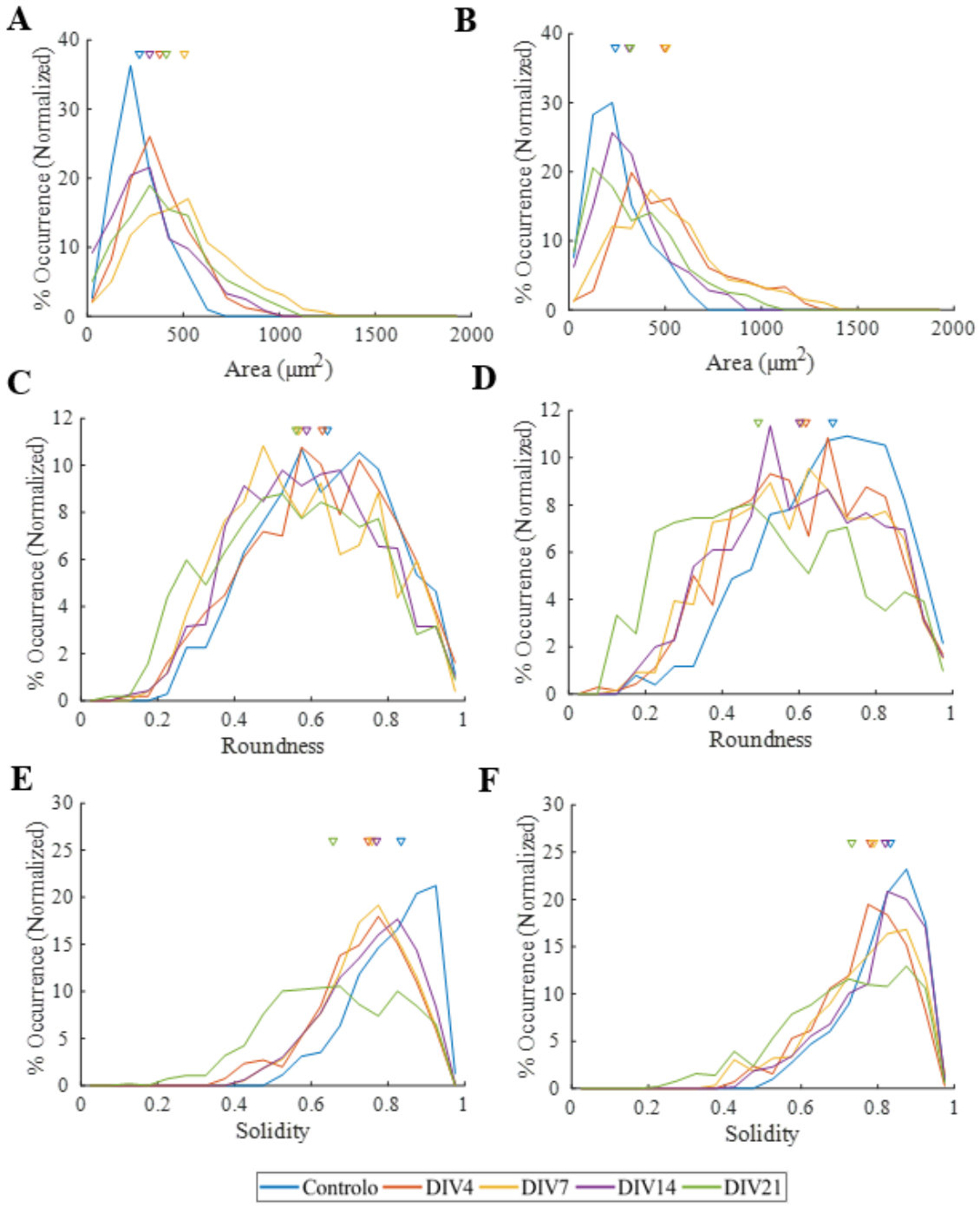

Figure 12. Frequency distribution of area, roundness and solidity, in microglial cells treated with neuron-conditioned medium (NCM) from cultures at 4, 7, 14 and 21 days in vitro (DIV). Graphs illustrating the morphologic parameter in microglia after 24 (A, C, E) and 48 h (B, D, F) in culture with each NCM. ▼ Color-matched median values (blue: Control group, no NCM; red: DIV4 NCM; yellow: DIV7 NCM; purple: DIV14 NCM; green: DIV21 NCM).

Table 4. Summary of the descriptive statistics for the microglial cells treatment with neuron-conditioned medium (NCM) from cultures at 4, 7, 14 and 21 days in vitro (DIV). The area, roundness and solidity parameters were quantified after 24 and 48 h in culture with each NCM. N, number of cells analyzed; Min, minimum value; Max, maximum value; SD, standard deviation; CI, confidence interval.

Parameters		N	Min	Max	Median	Mean ± SD	95% CI of mean
Area							
24h	CTRL	711	45.17	634.9	271.4	289.8 ± 120.7	[281.0:298.7]
	DIV 4	557	45.05	921.1	377.0	397.1 ± 168.2	[383.1:411.1]
	DIV 7	757	45.45	1229	505.6	527.3 ± 245.8	[509.7:544.8]
	DIV 14	1205	10.87	959.7	323.8	355.8 ± 202.6	[344.3:367.2]
	DIV 21	569	46.46	1069	410.9	431.0 ± 223.7	[412.6:449.5]
48h	CTRL	513	43.48	666.2	240.9	271.4 ± 141.4	[259.2:283.7]
	DIV 4	719	50.79	1249	497.0	539.9 ± 255.6	[521.2:558.7]
	DIV 7	660	55.81	1343	505.2	545.5 ± 276.2	[524.4:566.6]
	DIV 14	705	43.04	857.9	311.8	341.0 ± 177.4	[327.9:354.1]
	DIV 21	510	43.76	1075	318.8	363.0 ± 227.9	[343.2:382.8]
Roundness							
24h	CTRL	711	0.23	0.99	0.64	0.64 ± 0.17	[0.62:0.65]
	DIV 4	557	0.14	0.98	0.63	0.62 ± 0.18	[0.61:0.64]
	DIV 7	757	0.17	0.98	0.57	0.58 ± 0.18	[0.57:0.60]
	DIV 14	1205	0.14	0.99	0.59	0.59 ± 0.18	[0.58:0.60]
	DIV 21	569	0.08	0.98	0.56	0.56 ± 0.20	[0.54:0.58]
48h	CTRL	513	0.17	0.98	0.69	0.67 ± 0.17	[0.66:0.69]
	DIV 4	719	0.09	0.99	0.62	0.61 ± 0.18	[0.60:0.63]
	DIV 7	660	0.13	0.99	0.61	0.60 ± 0.19	[0.59:0.62]
	DIV 14	705	0.16	0.99	0.60	0.60 ± 0.19	[0.59:0.62]
	DIV 21	510	0.11	0.97	0.49	0.52 ± 0.22	[0.50:0.53]
Solidity							
24h	CTRL	711	0.50	0.96	0.83	0.81 ± 0.10	[0.80:0.82]
	DIV 4	557	0.39	0.95	0.75	0.73 ± 0.12	[0.72:0.74]
	DIV 7	757	0.44	0.97	0.76	0.75±0.11	[0.74:0.75]
	DIV 14	1205	0.43	0.96	0.77	0.76±0.11	[0.75:0.76]
	DIV 21	569	0.14	0.95	0.66	0.66±0.17	[0.64:0.67]
48h	CTRL	513	0.52	0.96	0.83	0.81 ± 0.10	[0.80:0.82]
	DIV 4	719	0.44	0.96	0.78	0.76 ± 0.11	[0.75:0.77]
	DIV 7	660	0.39	0.97	0.79	0.76±0.13	[0.75:0.77]
	DIV 14	705	0.45	0.96	0.82	0.80±0.11	[0.79:0.80]
	DIV 21	510	0.22	0.96	0.73	0.71±0.16	[0.67:0.73]

Table 5. Summary of the statistical tests performed to analysis the difference between the area, roundness and solidity medians, from microglial cells treatment with neuron-conditioned medium (NCM) from neuron cultures at 4, 7, 14 and 21 days in vitro (DIV), after 24 and 48 h in culture with each NCM.

		Area				Roundness				Solidity			
		DIV4	DIV7	DIV14	DIV21	DIV4	DIV7	DIV14	DIV21	DIV4	DIV7	DIV14	DIV21
24h	CTRL	###	###	###	###	ns	###	###	###	###	###	###	###
	DIV4		###	###	ns		###	###	###		ns	#	###
	DIV7			###	###			ns	ns			ns	###
	DIV14				##				ns				###
48h	CTRL	###	###	###	###	###	###	###	###	###	###	ns	###
	DIV4		ns	###	###		ns	ns	###		ns	###	###
	DIV7			###	###			ns	###			###	###
	DIV14				ns				###				###

$p<0.001$; Kruskal-Wallis Test followed by Dunns Multiple Comparison Test to compare every other treatment median

ns – statistically non-significant

Chapter 5

Discussion

Microglia-neuron interaction is an active and dynamic partaker for the proper CNS functioning. Several evidences show that the regulation of neuronal network development and maturation by microglia is highly coordinated with the functional status of the networks, suggesting the existence of a very close signaling crosstalk between microglia and neurons. But, little is known regarding the mechanisms by which microglia sense and understand the level and/or pattern of neuronal activity. While a series of potential chemical messengers have been postulated as substrates for this communication, including cytokines, purines and neurotransmitters, microglia-neuron communication in the electrophysiological domain remains elusive. Therefore, this thesis aimed to study microglia-neuron communication in the electrophysiological domain and, determine if microglia can directly readout electrical activity of neurons.

The complexity of microglia-neuron communication difficult the interpretation of results from in vivo experiments. Thus, to study this communication at the electrophysiological domain, one used a physiologically relevant in vitro model with full control of the electrophysiological microenvironment. In standard conditions, microglia in vitro do not entirely retain the in vivo signature, but this can be modulated by adapting culture conditions with CNS instructive cues (e.g. astrocyte-conditioned medium, or defined serum/glycine/ serine levels) [64, 180, 181]. In this work, one has tested three of the most used microglia culture media in the literature – DMEM medium supplemented with low and intermediate levels of serum (2 and 10%, respectively) and serum-free Neurobasal medium [181-183]. Although in vivo microglia are not exposed to serum, in this work the presence of 10% serum in culture medium showed to increase cell viability and resulted in more morphologically homogenous microglial cells population. This is in concordance with previous reports [181, 184]. Under these settings, these microglial cultures are a crucial resource to study the impact of different stimuli in these cells through morphometric analysis. Thus, this medium formulation was chosen as the control culture medium for the experiments developed in this thesis.

To determine how microglia sense neuronal activity, this work explored the microglia responsiveness to: i) direct electrical activity (i.e. 40 or 400 V/m EF). The impact of these

stimuli in microglia behavior was characterized, by imaging analysis, in terms of cell morphological changes; and, ii) NCM (secretome) from neuronal cultures, at different days in vitro, with different levels/patterns of neuronal activity.

Microglia phenotypes have been categorized into resting or activated based on cell morphology, but also taking into account its immunological state [185]. In terms of morphology, microglia in the "resting" state are normally described as round to oval in shape with small somas, filopodia extensions and relatively long processes [186]. Microglia activation, apart from immunological phenotypic changes, is characterized by morphological changes that include a progressive decrease in ramification up to an amoeboid shape and, with big somas [13, 187]. The correlation of microglia morphology to its function remains difficult essentially due to the heterogeneous phenotype of these cells. Still, subtle changes in cells morphology can be a good indicator of their sensitivity towards specific stimuli. Therefore, here one have applied few imaging analysis tools to adequately quantify microglia morphological changes towards the two different stimuli mentioned before. As morphometric parameters one has selected the area, roundness and solidity (all available in the ImageJ/Fiji free software). These are commonly used parameters that aid in detecting alterations in microglia soma size (area), soma shape (roundness) and ramification (solidity) [176, 178, 186, 188, 189].

EFs are intrinsic to many biological processes such as embryogenesis, wound healing and tissue regeneration [190]. Every cell has in its plasma membrane a number of charged proteins, which allow them to experience a net force when exposed to an applied EF [191]. This has been explored in vitro with various cell types in a tentative to understand the implicated changes of EF in cells migration, proliferation and morphology [6, 192-194]. The behavioral responses of a specific cell to an applied EF can be monitored by the use of galvanotaxis chambers. In respect to the nervous system, exogenous EF have been applied to neuronal cells, such as dorsal root ganglia neurons [193], neural crest cells [195], Schwann cells [171] and astrocytes [172, 196]. Regarding microglia cells, to the best of our knowledge, there is no study to date evaluating the impact of EF on primary microglia cells. Only few studies were reported for immortalized murine BV-2 microglia cell lines [6, 21], that are known to not fully reproduce the main biological features of primary microglial cells[8].

Taking into account the effects reported for microglia cell lines [6, 21], we hypothesize that primary microglial cells behavior will also be affected in some manner. Thus, for this

study we explored whether primary microglia are capable to sense EFs of 40 and 400 V/m, by evaluating microglia morphological changes. The morphometric analysis showed that microglial cells exposed to EFs responded differently according to the strength and time of EF application. The 400 V/m resulted in a decrease in the roundness and solidity values without changing cell area, suggesting that EF treatment induced the formation of new cytoplasmic protrusions. In contrast, EFs of 40 V/m induced an increase in microglia area with time and a decrease in cell roundness and membrane irregularity (i.e. decreased roundness and increased solidity values) with time. Apart from the unchanged area observed in microglia treated with 400 V/m, our results corroborate the findings from previous studies with microglia cell lines, demonstrating an inverse relation of microglial cells roundness to EFs strength [6]. Our results suggest that primary microglial cell scan sense an EF and respond to it by changing their morphological properties. Although, the parameters analyzed in our studies were statistically different, the differences in the microglia phenotype are very subtle. Meaning, that the detection of differences could be caused by the elevated number of cells analyzed in this work and not by significant morphologic changes in the microglia.

The effect of neuronal network secretome was also evaluated by collecting the NCM from neuronal cultures for different days in vitro thus, with different levels/ patterns of neuronal activity, and by exposing microglia to it. Our results showed that NCM from older and more active neuronal cultures induced microglial cells to be significantly more irregular in boundary as compared to microglia from control group, suggesting that microglia phenotype becomes nearest the “resting” state.

Previous studies suggest that microglia activation is efficiently controlled by healthy active neurons and that, conversely, neuronal activity blockade impairs this control and induce microglial cells to escape from the resting state [197, 198]. Here, the modulation of microglia behavior by the secretome of active neurons indicate that microglia can sense and respond to the activity of neuronal networks, in an electrically-independent way, through soluble mediators. Indeed, it is well-known that microglia express a diversity of surface receptors, like CX3CR1, CD200 and CD45, which corresponding ligands are known to be released by neurons [199]. Chemokines, such as monocyte chemoattractant protein-1 (MCP-1), can be also released by neurons and have been reported to be involved in the attraction/ migration of microglia/macrophage cells to neurodegenerative regions, in both adult and neonatal brain [200-204]. Microglia proliferation can be also promoted

by Csf1 released by neurons [198]. Moreover, the ATP released as a result of neuronal activity has been shown to influence both, microglia migration and activation [58]. Our findings, although lacking a molecular characterization of secretome and microglia activation state, seems to be in line with these previous demonstrations showing that factors released during neuronal activity, independently of electrical activity, are essential to modulate microglia behavior [12, 13, 109].

This study showed for the first time that primary microglial cells sense EFs and respond to it by changing their morphology, namely by increasing their membrane protrusions. This increase in membrane irregularities – present in "resting" microglia – might be a tentative of microglial cells to increase their contact with neighboring cells, as described for "resting" microglia during their microenvironment scanning state [187, 205]. Still, the mechanisms behind these changes remains unknown as well as the correlation of these changes with microglia function.

Chapter 6

Conclusion

Recent studies have been showing that microglia regulate neuronal networks development and maturation in a tight coordination with the functional status of the networks. These findings suggest the existence of a very close signaling crosstalk between microglia and neurons. Still, the mechanisms by which microglia sense and understand the status of neuronal activity is still far from being elucidated. Several chemical messengers have been put forward as substrates for this intimate communication. But, the microglia-neuron communication in the electrophysiological domain remains elusive.

This work demonstrates that microglia can discern the activity status of neuronal networks by two independent pathways. First, microglial cells can sense and react to EFs without any chemical cue usually involved in the microglia-neuron interaction. Using a custom-made galvanotaxis system, one has tested the effect of EFs on microglia behavior, namely through a morphometric evaluation. Microglia not only were capable to sense EFs but also responded in different ways to different EF strengths. In general, with time, the higher EF strength used resulted in an increase of microglia ramification, while the lower EF strength was associated with less ramified microglia cells, as compared with the control (unstimulated) cells.

Second, microglial cells can sense and react to the secretome of active neuronal cultures without any electrical signal. When exposed to NCM from neuronal network cultures with different levels of spontaneous electrical activity, microglia change their morphology as compared to the control cells, becoming more ramified and thus, more close to a “resting”-like status.

To the best of our knowledge, this is the first study reporting the ability of microglial cells to sense, directly and independently of chemical signals, electrical activity per se. Yet, the mechanisms by which microglia sense and react to the electrical activity remain unknown.

Chapter 7

Future Work

This work showed that microglia can sense and respond to EFs directly and independently of chemical cues. Our results suggest that primary microglial cells can sense an EF and respond to it by changing their morphological properties. But, in this study, the morphological differences observed between treatment conditions were not correlated with microglia functionality. Therefore, future studies should investigate other microglia properties that can aid in the understanding of the impact of electrical activity on microglia functionality. Examples are the microglia migration and phagocytic activity under EF exposure. Macrophages, that share the same lineage as microglia, migrate toward the anode when exposed to EFs [116]. Moreover, their phagocytic activity is up-regulated after EF stimulation. Being migration and phagocytosis key functions of microglial cells, it is crucial to evaluate if electrical activity can per se modulate these properties in microglia as well.

This study showed that exposure to EF of 400 V/m increases microglial cells ramification, as compared to unstimulated cells, suggesting an approximation to the "resting"-like status. Still, the morphological changes observed were subtle and are not strong enough to allow inferences regarding the activation status of these microglia cells. Therefore, future studies should also investigate in more detail the activation status of EF-treated microglia, through the molecular and genetic characterization of microglia activation markers. Examples of activation markers include IFN-γ, TNF-α, IL-1β, IL-6, nitric oxide, iNOS, among others.

Apart from these planned experiments, one aims to address, in future studies, a new approach to investigate the effect of electrical activity in microglia behavior, based on the use of the state of the art high-density microelectrode arrays (MEAs) technology. MEAs consist on a grid of microelectrodes embedded in a substrate that is used as a cell culture vessel. MEA systems are important tools that allow both electrical stimulation and recording of local field potentials and extracellular action potentials from a neuronal network in a non-invasive way. Therefore, using MEA technology, the spontaneous activity of neuronal networks in vitro (with different patterns and/or levels of activity) can be recorded and then, played back on microglia cultured in MEAs. In this way, MEA

systems can be used to modulate microglia populations and monitor their response towards the electrical stimulus applied.

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