uc3m | Universidad Carlos III de Madrid

Degree in Biomedical Engineering

2018-2019

Bachelor Thesis

"Characterization of postnatal microglia as a model for in vitro microglial studies"

Clara Gómez Cruz

Manuel Desco Menéndez

Eva Cano López

Leganés, 2019

Abstract

Microglia are the resident immune cells of the central nervous system. They have been found to play a major role in the development of different neurodegenerative diseases. In the case of Alzheimer's disease, understanding microglial interactions with Aβ peptides, a major hallmark of the disease, could unveil potential therapeutic targets. In order to study this interaction, it is important to be able to replicate it *in vitro*. However, there is no current model for microglial cells *in vitro*.

Primary cultures of rodent postnatal microglia are currently the most used *in vitro* model for microglial cells, as they are the simpler method to obtain large numbers of primary cells. Nevertheless, this study shows that postnatal microglia grown in vitro with granulocyte-macrophage colony-stimulating factor (GM-CSF) do not present the levels of expression of microglial signature markers (TMEM119, FCRLS, P2RY12, TREM2) found in adult microglia *in vivo*. This microglial signature was also downregulated in postnatal microglia compared to adult microglia. From immunostaining of tissue slices at postnatal day 3 (P3) it was shown that at least one of this characteristic microglial markers (P2RY12) was present in most of the neonatal microglial cells, although it had lower levels of expression than in adult cells.

Together, these results show that postnatal microglia have an immature phenotype and that current culture conditions are not able to promote differentiation of these immature cells into an adult phenotype. For this reason, neonatal microglia are currently not a good model for microglial studies concerning diseases that affect the adult brain.

Table of contents

List of figures

List of tables

1. Motivation and objectives

Motivation

Microglial cells are the central nervous system (CNS) resident parenchymal immune cells, accounting for around 5-12% of the cells in the adult brain, with major roles during disease, development and homeostasis [1], [2]. They were first characterized by Pio Del Río Hortega in a series of paper published in 1919 [3], but it was not until the late 1980s when studies of microglia began to emerge in neuroscience journals [4], [5]. This interest in microglial cells appeared as neuroinflammation was discovered to be a key element in the progression of neurodegenerative diseases including but not limited to Alzheimer's disease [6]–[8], Parkinson's disease [9], and amyotrophic lateral sclerosis [10].

In the case of Alzheimer's disease, neuroinflammation caused by microglial cells has been found to be a key component in the development of the disease, although the exact role these cells play has not been fully elucidated yet. An important step in unveiling this role would be to understand the mechanisms by which microglial cells interact with Aβ peptides, one of the main hallmarks in Alzheimer's disease. Understanding the pathways involved in this interaction could reveal new targets for potential therapies.

In order to further this line of research, the first step would be to have a reliable *in vitro* microglial model in which to perform the preliminary studies before moving on to *in vivo* studies.

Historically, the attainment of this reliable model has faced one major challenge: microglial characterization, and particularly, the differentiation between microglia and related cells, such as circulating macrophages. Until recently, microglia was distinguished from infiltrating macrophages based on their ramified morphology and flow cytometry surface expression markers, where macrophages are $CD11b^{+}CD45^{high}$ and microglia are $CD11b^{+}CD45^{med/low}$ [11]. However, activated microglia also present the amoeboid morphology typical of macrophages and CD45 expression can be compromised in the disease state. Other markers typically used to identify microglial cells (Iba-1, CD68, F4/80) are expressed by macrophages too [12], [13].

In the last decade, the development of new techniques such as single cell RNA-sequencing has allowed for the discovery of a new set of microglial signature genes (P2RY12, SALL1, FCRLS or TMEM119) that are differentially expressed in microglia, but not in other myeloid cells or other brain cells [14]–[18]. However, although the expression of these genes characterizes adult microglia in homeostasis, it is worth noting that the microglial phenotype is highly variable, and changes during development and in the disease state [19], so the expression of these genes may be regulated at different developmental stages or in the context of inflammation or neurodegeneration [20], [21].

Possible approaches to the development of a reliable microglial model would be the use of cell lines, freshly isolated adult cells, primary cultures of postnatal microglia or induced pluripotent stem cells (iPSCs). These different approaches will be analyzed in more detail when describing in vitro microglial models in the Background section.

In the case of cell lines, although they are able to yield high numbers of cells, the accuracy with which they model adult microglial cells is low [15] and their potential for improvement in this regard is limited. The opposite occurs with the use of freshly isolated microglial adult cells. This model allows for perfect replication of microglial adult cells and thus it is currently used for studies that require high accuracy; however, the protocol is complex and the number of cells obtained from each animal is limited, so it is not a viable long term solution. In the case of both iPSCs and primary cultures of postnatal microglia, these methods present potential to be used as models for adult microglia, as they combine the ability to yield high number of cells with the potential to develop a microglial adult phenotype.

This project will focus on primary cultures of microglial cells as they are currently the most used microglial model and therefore it is critical to characterize whether this method is suitable for modeling microglial cells [22]–[24]. This model allows for rapid attainment of large quantities of primary cells [25], and has been used to study signal transduction pathways as well as to gain an understanding of the functional consequences of gene expression manipulation. Nevertheless, due to the discovery of the aforementioned new microglial signature the suitability of this model might need to be revisited, as it is unclear whether cells from these primary cultures express the microglial signature required for them to be a proper model to study adult microglia.

Therefore, the motivation of this project will be to characterize whether postnatal microglia is a good model for adult microglia, in order to understand if it could be used in further studies concerning the interactions with Aβ peptides in the context of Alzheimer's disease.

Objectives

The number of microglial cells obtained from mouse brains is very low, so cell culture protocols are needed in order to ensure that enough cells are obtained for biochemical and drug testing studies.

Postnatal microglia are often used as a model for adult microglial studies (i.e.: testing the response to Aβ peptides). Nonetheless, after the discovery of a characteristic microglial signature, it is important to understand whether the primary cultures of postnatal microglial cells currently used as models present this characteristic phenotype.

Based on this general focus, the objectives of this project will be the following:

1. To characterize the microglial signature expression in cells cultured following the protocol described by Marshall et al [22], as it was considered for a long time the standard protocol for microglial culture. Some studies have already been done for other culture protocols [15], where they found that the primary cells did not present this characteristic phenotype.

The mRNAs analyzed will be P2RY12, TMEM119 and FCRLS as they are characteristic microglial markers and TREM2, as it is a risk factor for Alzheimer's disease typically expressed by microglia in the brain [26].

2. To characterize whether the original microglia extracted from postnatal day 3 (P3) mice expresses this microglial signature (P2RY12, TMEM119, FCRLS, and TREM2). With this objective, it will be possible to know whether the original microglia extracted from postnatal day 3 (P3) mice was expressing these markers and had lost them in culture, as occurs when culturing adult microglia [27] or whether postnatal microglia never expressed those markers to begin with.

3. Characterization of the microglial population in the P3 brain by immunohistochemistry. The project will study both the spatial distribution and morphology of these cells and their expression of the microglial marker P2RY12.

The legal framework and the socioeconomic impact of this project, including a detailed budget, can be found in section 5.

2. Background

Microglia: brain resident macrophages

Microglia are the innate immune cells of the central nervous system (CNS), and constitute a unique kind of tissue-resident macrophage due to their embryonic origin. They belong to the myeloid lineage, and they derive from myeloid progenitors in the extra-embryonic yolk sac, which arise before embryonic day 8 (E8) and colonize the brain by E9 in mice, before the formation of the blood brain barrier that will block the entrance of other immune cells formed in later stages of development [28], [29]. Brain border macrophages also derive from the same yolk sac hematopoietic progenitors, but microglia begins to express a differential phenotype when it enters the brain parenchyma and receives the environmental signals that are necessary for its differentiation [19]. The combination of their embryonic origin and the environmental cues received in the CNS are both necessary in order for microglial cells to maintain their identity [27].

Unlike other tissue resident macrophages, microglia are self-maintained due to their ability to self-renew [29] and remain yolk sac derived through the entire development and adulthood, without contributions from the circulating hematopoietic precursors. However, whether this microglial self-maintenance during adulthood is due to a small set of progenitors or to the self-replication of the entire microglial population is still obscure [20] .

Exceptionally, peripheral hematopoietic cells contribute to the microglial pool during pathology [30]. Studies have shown that circulating blood borne macrophages do not acquire the microglial phenotype, as macrophages engrafted in the CNS do not present the characteristic microglial signature [27], although they do adopt other microglial characteristics, like a more ramified morphology or longer lifespans [31].

Microglia in the adult brain are highly motile in that they are constantly remodeling their branches, but with no soma translation (non-migratory) [32]. This branch remodeling occurs as microglia sense their environment, which changed the paradigm to describe inactive cells as “surveying” rather than “resting” [33].

In general, microglia are able to change their phenotype rapidly in response to different developmental or contextual cues [34].

Microglia during development

Different studies have shown that the adult microglial population is the result of proliferation of microglial precursors [35] in the developing brain up to postnatal day 14 [16], [36]. The peak expression of proliferating microglia is seen between P6 and P9 in rats, and coincides with a period of high brain architecture formation, including dendritic growth, synapse formation and myelination [35]. This high levels of proliferation during the first stages of development may be driven by myeloid precursor cells (CD11b+ cells expressing the myeloid precursor marker ER-MP58) [36].

Microglia have been found to follow a step wise developmental program that comprises three different stages. The first stage could be described as "early microglia" and it is characterized by highly mobile precursors with a primitive amoeboid shape that overexpress cell cycle genes, important for the colonization of the brain parenchyma in the first stages of embryonic development [37]. After embryonic day 14 (E14.0) and until postnatal day 9 (P9) microglia mature to a so called "pre-microglia", with expression of genes regarding synaptic pruning and other structural functions[28], [35]. The late stage, corresponding to adult microglia would be characterized by their immune function and in this stage cells present a highly ramified morphology, with small soma and long, thin branching (Fig 1).

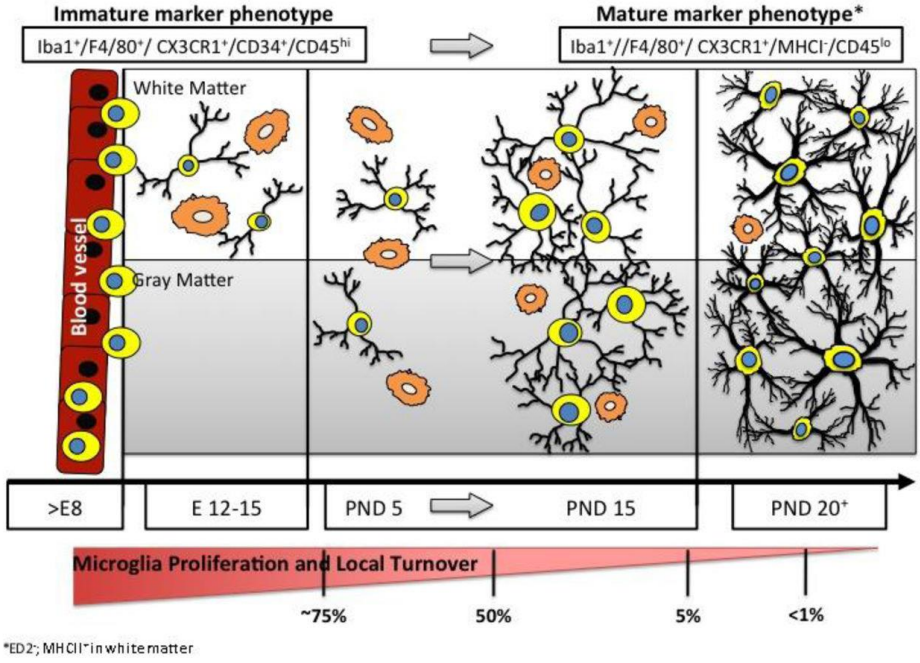

Fig 1. Different stages of microglial development [38]. Microglial differentiation is characterized by an increase in the number of cells and a tendency towards a more ramified

morphology, as their functions evolve from more structural roles during brain development to immune surveillance and responses during adulthood. Although microglia have been found to play a key role in the developmental biology of the brain, no studies currently exist to describe their motility and migration during this period [32].

Microglia have very different roles throughout the lifespan. In the developing brain, microglial cells have structural functions, and are essential for synaptic pruning, myelinogenesis, axon dynamics, cell genesis and survival, stimulation of vascularization, as well as cellular phagocytosis, as during the first postnatal week microglia are often found close to or engulfing apoptotic bodies [30], [32]. Due to this variety of functions, microglia is most diverse during early development, and becomes less heterogeneous in the homeostatic adult brain [19].

A characteristic population of microglial cells has been found in the neurogenic subventricular zone (SVZ) during early postnatal stages. Microglia had been shown to be a component in the adult neurogenic niches, controlling neural stem cells proliferation, but they are already present in this region at early stages. This population is characterized by a more amoeboid phenotype, with lower differentiation rates than other regions and higher cell numbers than other regions [39].

Microglia have been proved to be heterogeneous along a temporal axis, with different functions at different life stages; however, the level of heterogeneity of microglia along a spatial axis, especially during development, remains unanswered [20]. It is clear that environmental cues drive the phenotypic expression in microglia, but what are these cues and how microglial cells are driven to change between the different functions is still unsolved.

In vitro microglial models: iPSC, adult/neonatal primary cells and cell lines

Biological models are systems used to increase the scientific understanding of a particular biologic phenomenon. For the case of microglial models, as most diseases develop during the adult age, they should aim to reproduce the adult phenotype under physiological state. In vitro models could be distinguished in four main groups: cell lines, primary microglia derived from mixed glial cultures, *ex vivo* adult microglia and stem cell-derived microglia cultures, including the use of induced pluripotent stem cells (iPSC).

Immortalized cell lines for microglia include HMC3 or HMO6 for human, BV2 and N9 retroviral immortalized murine microglia, and spontaneously immortalized rodent microglial lines like EOC lines and HAPI cells [33]–[35]. Among these cell lines the most commonly used are the BV2 and N9 lines, due to the ease of use. The main advantage, common to all cell lines, is the ability to obtain high numbers of cells in short time spans without employing animals. However, although these cell lines present similar responses to different stimuli like LPS and Aβ, the magnitude of the responses is different [40], and recent studies have shown that they present differences in both genetic expression and function compared to ex vivo adult microglia [15], [41]. Besides, both BV2 and N9 are derived from neonatal microglia, meaning that any differences that were present between neonatal and adult primary microglia will also affect these cell lines.

In the case of primary cells, the gold standard for microglia is currently freshly isolated adult microglia. These *ex vivo* cells present the advantage that they were differentiated in the CNS, and thus present the characteristic genotype and phenotype of microglia *in vivo* [25]. The reason why these cells are only used for characterization, but not for studies regarding biological responses or biochemistry testing is because the number of cells yielded by each experiment is extremely low, and any experimentation performed in this model would require the sacrifice of huge amount of animals.

For this reason, primary cultures are used to optimize the amount of cells obtained. Cells are first grown in mixed glial cultures and then microglia is purified by density gradients, magnetic-activated cell sorting (MACS), fluorescence-activated cell sorting (FACS) or by specific shaking procedures [16], [22], [36], [42]. The main drawbacks of this technique are the evolutionary differences between rodents and humans, that can hamper the translation of basic research results to the clinic, and also the possible differences between the neonatal microglia being cultured and adult microglia.

To reduce the amount of animals employed while maintaining the biological relevance of primary cultures, the ideal model would be the use of stem-cell derived microglia, particularly iPSCs. However, to obtain any kind of cell from iPSCs it is vital to understand the key factors driving the developmental process and until 2010, even the origin of microglial cells was under debate [28]. In the last decade, different protocols have been described for the differentiation of iPSCs into microglial cells [43]–[45], including different factors like M-CSF, IL-34, TGF-β1, or cholesterol, that have

been found to be relevant in microglial development [30]. However, these methods are able to obtain microglia-like cells presenting an immature phenotype resembling that of in vivo embryonic microglia, so conclusions reached using these cells should not be extrapolated to adult studies.

As a summary, there is no current ideal model for studying microglia *in vitro*, as there is a lack of understanding of the environmental cues that are necessary for the differentiation and maintenance of the microglial phenotype. For this reason, in order to develop a useful model it is of the maximum importance to understand the biology of microglial development *in vivo*, in order to replicate the process *in vitro*.

Microglial visualization in tissue samples

The first drawings of the morphology of neuroglial cells in the early 20th century were performed based on unstained tissue by histologists such as Camillo Golgi, Santiago Ramón y Cajal, and Pio Del Rio Hortega [46]. Throughout the next decades, different methods were developed in order to selectively stain only particular types of cells, including Cajal's gold-chloride-sublimate staining, which allowed for the visualization of neuroglia. Later on, Rio Hortega was able to selectively stain microglia by using a modification of the ammoniacal silver staining method developed by Achúcarro [47].

Nonetheless, these methods only allowed for morphological staining. It was in the late 1980s when immunohistochemical methods appeared and it was possible to stain cells based on their functionality, for example by staining brain samples with anti-F4/80 antibodies, a macrophage specific antibody that was able to stain microglial cells, as well as other macrophages present in the CNS like those associated with the choroid plexus, ventricles and meninges [48].

Current techniques for visualization of microglia in tissue samples still rely mainly on immunostaining, particularly of markers such as Iba1, CD45 or CD68. Other approaches include the use of transgenic animals, which express eGFP following the promoter for another gene. For the case of microglial cells, the most used transgenic animals are the CX3CR1-eGFP and the CSF1R-eGFP transgenic models.

In order to facilitate the study of microglial cells in the brain, CSF1R-eGFP transgenic mice will be used in this project. CSF1R (Colony Stimulating Factor 1 Receptor) is a receptor for CSF-1, also known as M-CSF, and interleukin-34 (IL-34)

and it plays an important role in regulating the proliferation, differentiation and function of macrophages [49], [50]. CSF1R is expressed by all the mononuclear phagocyte system from the myeloid lineage [51]. In the brain, it is expressed not only by microglia, but also by perivascular macrophages, and macrophages from the meninges and the choroidal plexus. In order to overcome this issue, some recent studies [16] have relied on the use of immunostaining with antibodies binding characteristic microglial markers like TMEM119 in order to specifically stain only microglial cells and no other myeloid cells. However, the expression of these markers is not guaranteed on different stages of development and thus it will not be used in this project as it deals with postnatal microglia. Nevertheless, as the rest of CSF1R-expressing cells are localized in very specific regions of the brain, they can be easily distinguished from $CSF1R^+$ microglia found in the brain parenchyma. For this reason, microglia will be characterized as $eGFP^+$ cells found in the parenchyma of the brain.

All the images of tissue sections acquired for this work were obtained using confocal microscopy. The section below details the reasons why this technique was used over standard fluorescence microscopy.

Imaging: confocal microscopy vs standard fluorescence microscopy

Both immunostaining and the use of transgenic animals require fluorescence microscopy in order to visualize cells. This section will describe why confocal microscopy was used for imaging brain tissue samples instead of standard fluorescence microscopy.

Fluorescence is a form of energy emitted by some molecules (fluorochromes) when they are excited by light photons. Part of that excitation energy is lost in the form of heat, so the emitted fluorescence light always has less energy –and higher wavelength– than the original one. This physical property has been used for years in biological research with traditional fluorescence microscopes (Fig 2A). However, standard fluorescence microscopes present several disadvantages, including the overlapping between the excitation curves of different fluorochromes. As a practical example of this issue, eGFP is excited with blue light and emits in green, but it is also partially excited by green light and can emit in red, overlapping with the Cy3 emission in red. The other main disadvantage is that the light received at the detector not only comes from the focus plane, but also from all the other planes in the section. This can be particularly challenging in the case of imaging brain slices, as the brain has high autofluorescence

properties, particularly due to the accumulation of lipofuscins in the neural cells [52]. If the light captured comes from all the possible planes in a section, the unspecific fluorescence can mask the true signal from the eGFP protein of the transgenic model (Fig 2B).

These disadvantages can be overcome using confocal microscopy. The main characteristic that differentiates confocal microscopy from other kinds of fluorescence imaging is that it detects only the light coming from a focal plane in the three dimensional space. The thickness of this focus plane can be controlled by changing the pinhole, an opening diaphragm that controls the amount of light that gets to the detector. Opening the pinhole allows for more fluorescence to come in, as it takes the light coming from more planes, increasing the signal; while closing the pinhole improves the z-resolution of the images and reduces the amount of nonspecific light that gets to the detector.

The other problem solved by confocal microscopy is the overlap between the emissions of two fluorochromes. Confocal microscopy overcomes this challenge with two different solutions. For one, the excitation lights are lasers, which emit in only one wavelength, reducing the possibility of exciting two fluorochromes with the same one. Besides, confocal microscopy allows for sequential acquisition of the images. This means that the specimen is first excited with one laser, the light emitted is captured and then the next laser is switched on. With these two techniques, the overlapping between fluorescence is greatly reduced and becomes almost nonexistent.

For these reasons, confocal microscopy will be used along this project in order to visualize microglial cells in tissue slices.

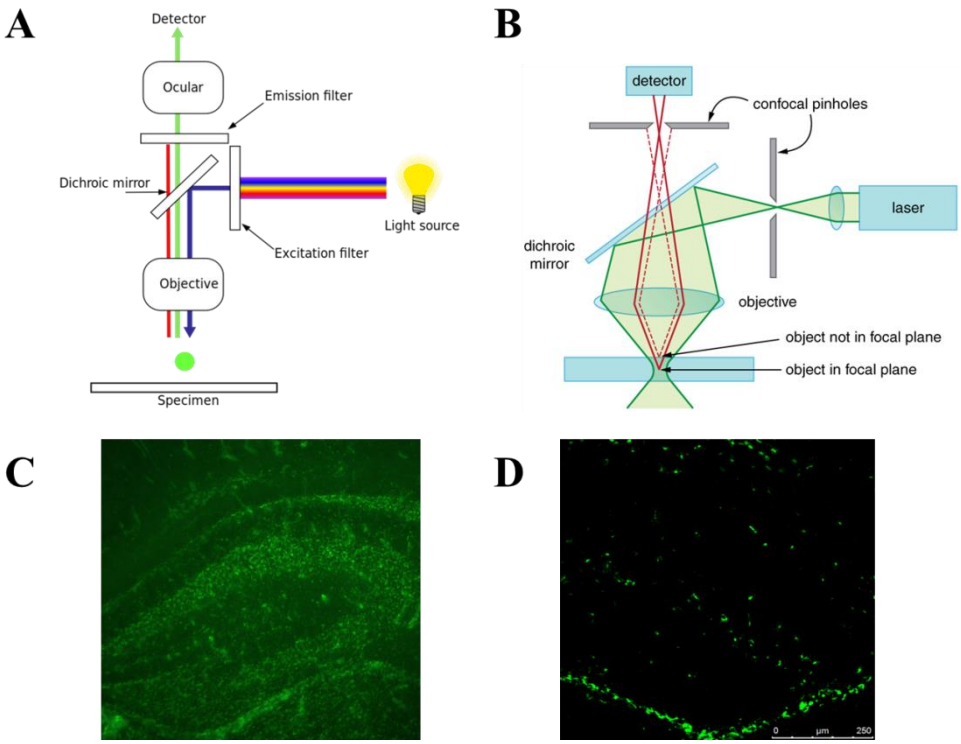

Fig 2. Comparative between standard fluorescence and confocal microscopy. (**A**) Schematics of a standard fluorescence microscope (Wikimedia Commons, modified) (**B**) Schematics of a confocal microscope. Light emitted by fluorochromes outside the focal plane (dotted line) gets blocked by the pinhole, and only light coming from the focal plane (block line) gets to the detector [4]. (**C**) Hippocampus of a neonatal (P3) mice imaged using standard fluorescence microscopy with a 10x objective (Leica DM IL LED). All the tissue shows fluorescence, the specific fluorescence coming from the transgenic CSF1r-eGFP cells cannot be distinguished. (**D**) Hippocampus of a neonatal (P3) mice imaged using confocal microscopy (20×/0.7 Plan Apo objective lens, Leica SP5 MP). Confocal microscopy greatly reduces the intensity of nonspecific fluorescence.

3. Materials and methods

Animals

Primary cultures of mixed glial cells were obtained from *Mus musculus* C57BL/6 neonatal (P2-P3) males and females. Animals employed included C57BL/6 wild type mice and mice from the FMS strain (B6.Cg-Tg(Csf1r-EGFP)1Hume/J), which was first generated and characterized by Sasmono el at in 2003 [54], [55] (Fig 3). Animals were provided by Instituto de Salud Carlos III (ISCIII). Pregnant females were allowed to give birth naturally, and the day of birth was considered P0. Animals were kept housed with sterile food and water ad libitum in "specific-pathogen free" (SPF) conditions.

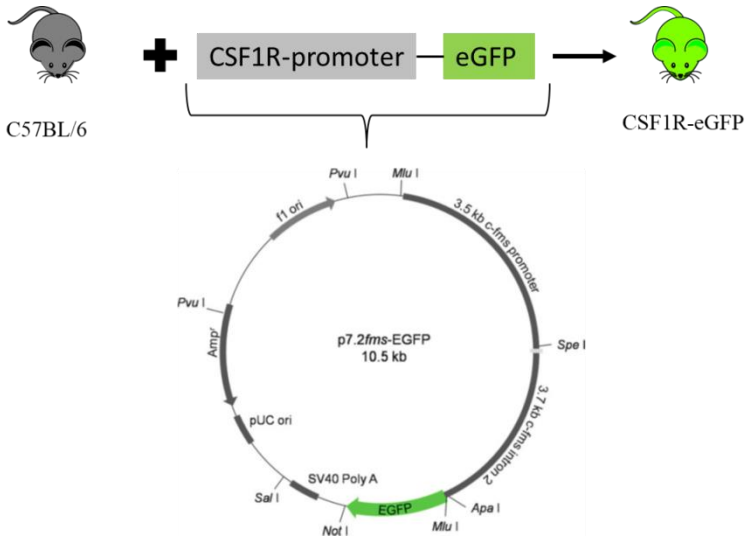

Fig 3. CSF1R-eGFP transgenic. Schematic of the transgenic mouse used and map of the p7.2 fms -EGFP plasmid construct used for the transfection [55].

Microglial extraction

Animals were sacrificed by decapitation in a horizontal flow cabin to maintain sterility. For immunostaining, brains of pups were maintained in the skull to preserve their structural integrity. When brains were going to be used for cell culture, flow cytometry or RNA analysis, they were extracted from the skull and the olfactory bulbs, the cerebellum and the meninges and choroid plexus were removed to reduce contamination by external myeloid cells.

Tissue disaggregation for cell culture and flow cytometry was performed following the protocol described by Marshall et al [22]. Brains were dissected and mechanically disaggregated with a 1000 µL pipette. The resulting mixture was filtered through a 70

µm cell strainer and pelleted by centrifugation at 1200 rpm for 5 min. Cell pellet was then retrieved for use in cell culture, flow cytometry or RNA analysis.

When extracted cells were employed for the analysis of microglial markers by real time quantitative PCR (RT-qPCR), CD45+ cells were retrieved by magnetic cell isolation using a MACS cell separation kit (MACS Miltenyi Biotec). Isolated cells were stored at -20ºC until use.

Cell culture

Cell culture was performed following the protocol described by Marshall et al. [22] (Fig 4). Briefly, cells were extracted as described in the section above, and then seeded into 25 cm^2 culture flasks in a neural growth medium (NGM) composed of Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12) with L-Glutamine and Penicillin/Streptomycin, supplemented with 5% FBS, recombinant human epidermal growth factor (EGF, 20ng/mL, PrepoTech), fibroblast growth factor (FGF, 10ng/mL, PrepoTech) and supplement N2 (Gibco). Cells were allowed to grow at 37ºC and 5% CO_2 until a confluent monolayer was formed. Cell were then transferred to a 75 cm^2 flask with the same culture media composition and again allowed to reach confluency. Confluent cells were seeded again at a 1:3 ratio to three 75 cm^2 flasks with microglial proliferation medium (MPM), which consisted of DMEM/F-12 supplemented with 10% FBS, N2 and granulocyte macrophage-colony stimulating factor (GM-CSF, 20ng/mL, PrepoTech).

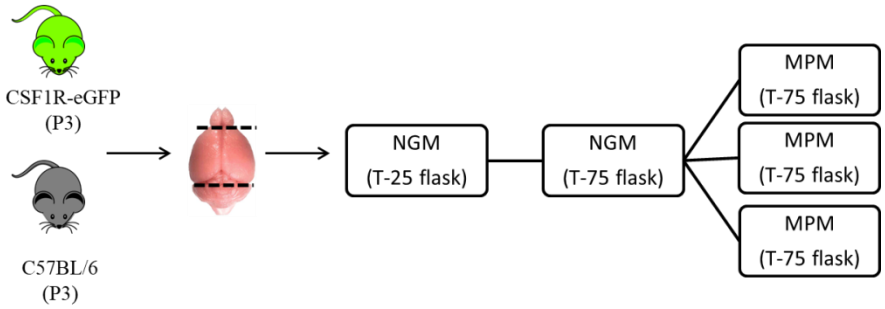

Fig 4. Culture protocol described by Marshall et al. [22]. From extracted brains, olfactory bulbs, cerebellum, meninges and choroidal plexus were removed. The tissue was mechanically disaggregated, filtered through a 70 µm cell strainer and seeded into cell culture flasks. Culture media used were neural growth medium (NGM): DMEM/F12 + 5%FBS + N2 supplement + EGF (20ng/mL) + FGF (10ng/mL) and microglial proliferation medium (MPM): DMEM/F12 +

10% FBS +N2 supplement + GM-CSF. Cells were passaged when cell monolayers reached confluency.

When cells reached confluency, they were retrieved for flow cytometry and RNA analysis. Microglial retrieval from mixed cell cultures was performed by agitation of the flasks at 150 rpm overnight.

Characterization of cell surface marker expression: flow cytometry

Flow cytometry was used to characterize cell populations both after extraction and after 14 days of culture. In flow cytometry, cells are stained using antibodies attached to different fluorophores. Each cell passes individually through the laser light and the light scatter and fluorescence emitted are electronically detected [56]. For this reason, this technique allows for very specific characterization of cell populations.

A minimum of 50,000 cells were used for each tube. All incubations described below were performed in dark and cold conditions. Cells were first incubated with an anti-CD16/CD32 (BD Pharmingen^{TM}) for 30 minutes to block the Fc region prior to incubation with antibodies for 45 minutes. The antibodies used were PE/Cy7 anti-mouse CD45 (BD Pharmingen^{TM}) and APC/Cy7 anti-mouse CD11b (BD Pharmingen^{TM}).

After incubation, 3 mL of PBS with 2% FBS were used to stop the reaction. Cell suspensions were centrifuged at 1200 rpm for 10 minutes and left in 500 µL of PBS 2% FBS. Cells were then passed through the cytometer (BD LSRFortessa^{TM} X-20) and the results analyzed using the FlowJo V10.1 software (Fig 5).

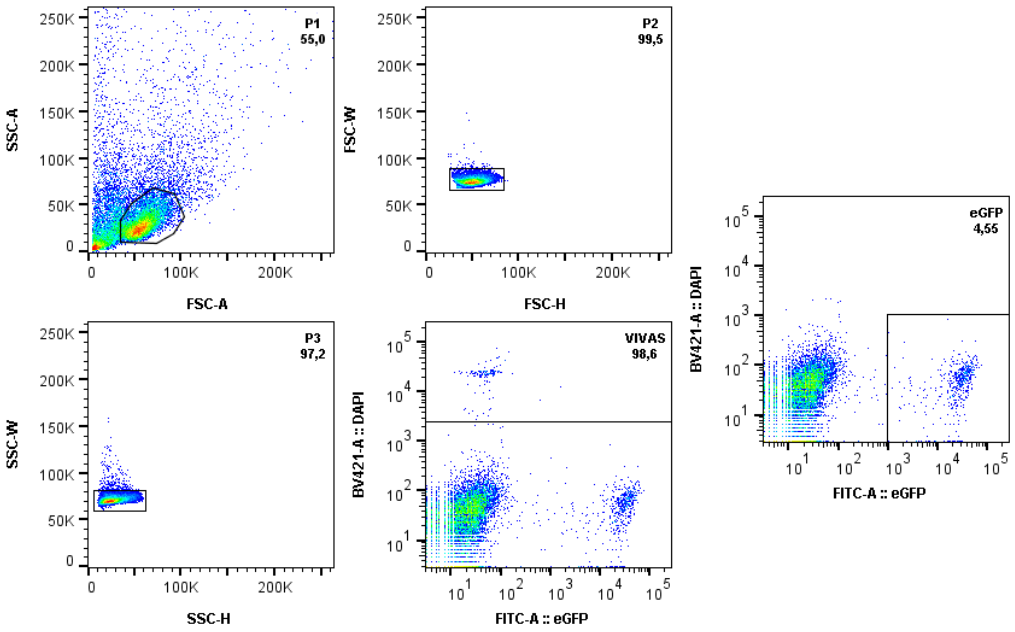

Fig 5. Representative figure of the selection criteria used during flow cytometry analysis. SSC-A/FSC-A was used to select the original population based on size and granularity. FSC-W/FSC-H and SSC-W/SSC-H were used to remove duplets and purify the population. DAPI staining was used to select only the living cells, as dead cells could have a compromised membrane and thus present non-reliable staining.

Characterization of mRNA expression: qRT-PCR

To study the expression of different microglial markers at RNA level, a quantitative RT-PCR was performed.

First, RNA was extracted with TriPure Isolation Reagent from frozen cell pellet samples following the phenol-chloroform standard protocol [57]. 1 mL of TriPure Isolation Reagent was added to pelleted cells in order to break them and denaturalize endogenous nucleases, preserving the integrity of nucleic acids. Samples were lysed using the embolus of a 5 mL syringe. Then the lysate was collected and incubated in a 1.5 mL Eppendorf for 5 min at room temperature.

After incubation, 200 µL of chloroform were added (1:5 proportion to TriPure) and mixed by vortex for 15 s to ensure proper mixture. After addition of chloroform, lysates were centrifuged (12,000 g, 15 min, 4ºC) to separate the phases. RNA is in the transparent aqueous phase, DNA in the interphase and proteins and other molecules in the organic phase below. Aqueous phase was extracted and transferred to a new Eppendorf with 500 µL of isopropyl alcohol (1:2 proportion to TriPure) to precipitate

isolated RNA. Tubes were inverted and incubated for 10 min at room temperature. Samples were centrifuged (12000g, 10 min, 4ºC), and then the pellet was resuspended in 200 µL of ethanol 75% to wash RNA samples. Samples were centrifuged again (7500 g, 5 min, 4ºC), and RNA pellet was left to dry at room temperature.

Once RNA was isolated, samples were resuspended in 12 µL of nuclease-free water (Gibco®). Samples were incubated for 7 min at 60ºC and RNA concentration measured using a Nanodrop (Nanophotometre Pearl, IMPLEN, bioNova cientifica), which quantifies RNA concentration using spectrophotometry principles.

When the number of cells in a sample was below 250.000 the RNeasy Micro Kit (QIAGEN) was used, according to the prescribed protocol.

As qRT-PCR is used to quantify relative amounts of DNA in a sample, to quantify the amount of RNA retrotranscription was used to obtain the complementary DNA (cDNA). Quantifying cDNA is homologous to quantifying RNA as during retrotranscription only one cDNA is obtained for each RNA molecule.

cDNA was synthesized from isolated RNA samples. RNA samples were diluted to 2 or 1 µg/µL in nuclease-free water, depending on the amount of RNA present in each sample, for a final volume of 10 µL. Diluted samples were converted to cDNA by retrotranscription. To each 10 µL sample, the following mixtures were added according to RT-PCR cycles:

-Mixture 1 (2 µL/10 µL sample) contained 0.2 µL of random primers (Invitrogen, 3 µg/µL 58875), 1 µL of DNTPs and 0.8 µL of nuclease-free water.

-Mixture 2 (7 µL/10 µL sample) contained 4 µL of Strand buffer (Invitrogen, y02321), 2 µL of DTT, 0.1 µL of the recombinant ribonuclease inhibitor RNAsa OutTM (40U/µl, Invitrogen, 100000840) and 0.9 µL of nuclease-free water.

-1 µL of MMLV enzyme.

Samples of cDNA obtained were diluted to 2 ng/µL in nuclease-free water. RT-qPCR (QuantStudio 3, Applied Biosystem) was performed following the SYBR Green protocol, with two technical replica of each biological replica. mRNAs analyzed were 36B4, as a housekeeping gene for control, and the characteristic microglial genes TMEM119, FCRLS, TREM2 and P2RY12 (Table I).

Table I. Primer sequences used for real time quantitative PCR (RT-qPCR).

Gene	Forward sequence	Reverse sequence
36B4	5'-AGATGCAGCAGATCCGCAT	5'-GTTCTTGCCCATCAGCACC
TMEM119	5'-GCATGAAGAAGGCCTGGAC	5'-CTGGGTAGCAGCCAGAATGT
FCRLS	5'-GCCTTTGATTGTGGACATGA	5'-GATCTTCAGAAAGTGCTGGTAA
TREM2	5'-TGGGACCTCTCCAACCAGTT	5'-GTGGTGTTGAGGGCTTGG
P2RY12	5'-CCCGGAGACACTCATATCCTT	5'-GTCCCAGGGGAGAAGGTG

Brain collection and sucrose cryoprotected tissue freezing

Isolated brains used for immunohistochemistry were fixed overnight in 4% PFA. The skulls were conserved to preserve tissue integrity. Brains were washed with PB1x 6 times for 10 minutes each in agitation and placed in 15% sucrose in PBS until they sank (12-24h) and then in 30% sucrose in PBS overnight or until they sank. This sucrose gradient was performed to avoid the formation of crystal due to the freezing of water inside the tissues. Brains were embedded in O.C.T. (Tissue-Tek O.C.T™, Sakura) and PB1x sucrose 30% solutions, at 1:3, 1:1 and 3:1 ratios successively, leaving them on each solution overnight.

For tissue freezing, each brain was introduced in the bulb of a 3 mL Pasteur pipette filled with O.C.T., vertically oriented, with the olfactory bulbs facing downwards. Bulbs were progressively introduced in an Iso-Pentane (VWR Chemicals) beaker at -80ºC, until every part but a small circle of O.C.T. was frozen. Bulbs were transferred to dry iced and allowed to fully freeze. Frozen brains were stored at -80ºC until sectioning was performed.

Cryostat sectioning and immunostaining

Once brains were embedded in O.C.T, they were coronally sectioned using a cryostat CM1950 (Leica Microsystems) following the protocol described by Diaz-Moreno in 2013 [58] generating 30 µm sections that were collected in Superfrost Ultra Plus® microscope slides (Thermo Fisher). Slides were kept at -20ºC until use.

As brains came from FMS mice, they inherently expressed eGFP in myeloid cells (when CSF1-R is expressed). Immunofluorescent staining was performed using indirect standard staining: samples were first incubated with a primary antibody, followed by

incubation with a secondary fluorescent antibody. All the incubations were performed in darkness to preserve fluorescence.

Samples were allowed to dry for two hours to avoid detachment from the slides prior to incubation in a blocking solution (0.1M PB, 10%FBS (Hyclone), 0.25% Triton-X100 (Sigma-Aldrich), 150mM glycine (Merck)) for 1 hour. Samples were subsequently incubated overnight at 4ºC with the primary antibodies made in the blocking solution. Rabbit anti-P2RY12 (Anaspec) and rabbit anti-GFAP (Z033429, Dako) antibodies were used.

The antibody solution was decanted and samples washed 3 times with PB. Then they were incubated with secondary antibodies for one hour. The secondary antibodies used were Alexa Fluor 647 donkey anti-rabbit (Dako) and Cy3 goat anti-rabbit (Dako). All incubations were performed in a wet chamber at room temperature in the dark, unless otherwise stated.

To better visualize the anatomy of the sections in the confocal microscope, samples were stained with DAPI (4′,6-diamidino-2-phenylindole). DAPI is a fluorescent stain that binds adenine-thymine regions of the DNA, effectively staining the nucleus of eukaryotic cells. At normal concentrations, it can only pass through the membrane of compromised cells and that is why it is used to discriminate between living and dead cells in flow cytometry, for example. However, as cells in tissue slides are fixed and thus their membranes compromised, DAPI can stain all nuclei and be used to see a general anatomical structure of the tissue samples. After washing the secondary antibody, sections were incubated for 10 minutes with DAPI. DAPI was rinsed with PB, and then slides mounted using Mowiol/DABCO 2.5% (v/v) mounting solution.

Imaging of tissue sections with confocal microscopy

Fluorescence images were captures using a Leica SP5 MP confocal imaging system. For confocal imaging of samples, the following laser lines were used: DAPI (405 nm Diode), eGFP (Argon 488 nm), Cy3 (HeNe 543 nm) and Alexa Fluor 647 (HeNe 633 nm). A sequential mode of acquisition was used to capture images to avoid cross excitation of the fluorophores. The confocal pinhole was set to one Airy disc unit to improve the z-resolution of the images and reduce the nonspecific fluorescence. To capture large fields of view (775 µm x 775 µm) images were collected using a 20×/0.7 Plan Apo objective lens, with an x-y resolution of 1.3 pixels/µm and a typical z-

resolution of 2.7 µm. For morphological imaging of single cells, a 63x/1.4-0.6 Plan Apo objective lens was used, giving an x-y resolution of 4.2 pixels/µm. Z-resolution was typically set between 0.3 and 0.7 µm.

Image stacks obtained were processed using the Leica LAS AF software or ImageJ (Wayne Rasband, NIH). A median filter in 3D was applied to image stacks to reduce impulse noise and then image stacks were projected with a maximum projection to obtain a two-dimensional visualization of the region.

Statistical Analysis

Results are expressed as mean ± SD, n values reported for each experiment. Raw data was analyzed using Prism (GraphPad Software, Inc.). Data sets were tested for normality with a Saphiro-Wilk test. For normal distributions of data, significant differences were tested using one-way ANOVA with Brown-Forsythe correction. Kruskal-Wallis test was used for non-normal distributions of data. Unpaired t-test with Welch's correction was used for comparison between two datasets. P values below 0.05 were considered significant.

4. Results

Microglial tracing using the CSF1R-eGFP transgenic mice

The use of transgenic mice as the CSF1R-eGFP is of great help to easily localize cells of interest, both in tissue preparations and in mixed cell cultures, where cells are growing alongside other cell types (as astrocytes or oligodendrocytes, in the case of mixed glial cultures). To ensure that eGFP expressing cells were indeed the population of interest, freshly isolated cells from postnatal brains (P3) were analyzed by flow cytometry. The markers analyzed were CD45 and CD11b, as microglial cells were classically characterized as being $CD11b^{+}$ $CD45^{me}$ [11]. $eGFP^{+}$ cells were $CD45^{+}$ and $CD11b^{+}$ (Fig 6) ensuring that the population of interest is being correctly followed using this transgenic.

The fact that $eGFP^{+}$ cells are $CD45^{+}$ and $CD11b^{+}$ does not imply that they are necessarily microglial cells, as other cell types also express these markers. However, as cell cultures were only made with the brain parenchyma and removing the olfactory bulbs, the meninges and the choroid plexuses (which could be the main source of macrophages and other myeloid cell types) it was considered for the purpose of this work that the majority of myeloid cells present in the cell mixture will be microglial cells.

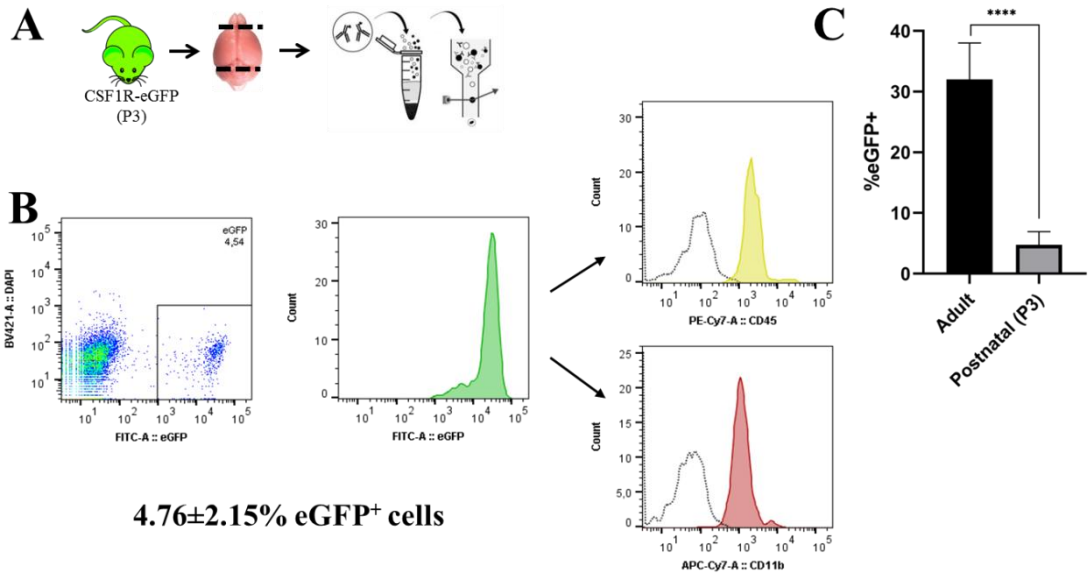

Fig 6. Percentage of eGFP+ cells obtained from whole brain extractions of neonatal CSF1r-eGFP mice. (A) Cytometry protocol followed. (B) Flow cytometry analysis of the eGFP+ cells among living cells. Population was selected as described in Fig 5. All eGFP+ cells

were CD45+ and CD11b+. (C) Percentage of eGFP+ cells obtained from neonatal brains compared to the same percentage obtained from 3-4 months adult mice (n=6). **** P<0.0001.

It is also important to notice that, as found in the literature [35], [36], the number of microglial cells is significantly lower in the neonatal brain compared to the brain of adult mice of 3 to 4 months, as microglial expansion occurs around day 14 of postnatal life in the murine brain [36]. This is one of the main reasons why cell cultures are a required step in any study that employs primary neonatal microglia, as obtaining the amount of cells needed for any experimental set up would require the sacrifice of big numbers of animals, violating the ethical guidelines that advocate for an optimization of the amount of animals employed for experimental research.

Microglial signature expression in cultures with GM-CSF

As stated in the previous section, a necessary step for microglial models is cell culture to increase the amount of cells available. In the culture protocol followed, the first step includes culture in neural growth medium (NGM) that enhances the growth of an astrocytic layer, and then cells are cultured in a microglial proliferation medium (MPM) that includes GM-CSF, a cytokine that stimulates macrophage proliferation [59]. MPM medium was successful in increasing the number of $eGFP^+$ cell in the cultures (Fig 7B and 7C). After culture, $eGFP^+$ cells were collected by agitation, taking advantage of the fact that they grow in a semi adherent fashion on top of the astrocytic layer.

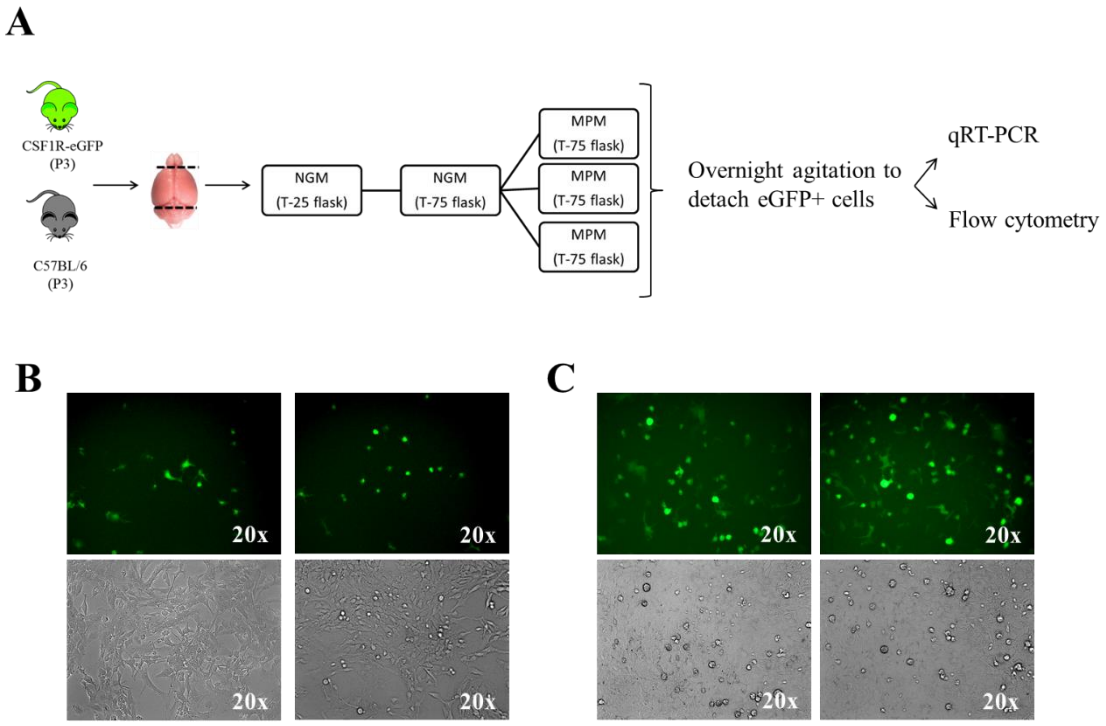

Fig 7. Mixed glial cultures following the Marshall et al protocol [22]. (**A**) Schematics of the culture protocol. (**B**) Representative fluorescence fields and the corresponding bright fields of cell cultures after 4 days in NGM. $eGFP^+$ cells grow over a basal layer of astrocytic cells. (**C**) Representative bright field and fluorescence fields of cell cultures after 14 days in culture (7 days in NGM and 7 days in MPM).

By flow cytometry, it was shown that 98.95±0.9% of cells detached by agitation were $eGFP^+$, and maintained the $CD11b^+$ $CD45^+$ expression (Fig 8A). However, qRT-PCR analysis revealed that these cells had very low expression of the microglial signature genes (P2RY12, FCRLS, TMEM119, TREM2) compared to the levels expressed by freshly isolated adult microglial cells (Fig 8B). These results match what has been reported in the literature for other cell culture protocols [15], where levels of expression of the microglial signature decay after culture, as microglial cells have been shown to require the environmental cues present in the central nervous system in order to maintain their characteristic phenotype, and rapidly loose it when extracted from it [27].

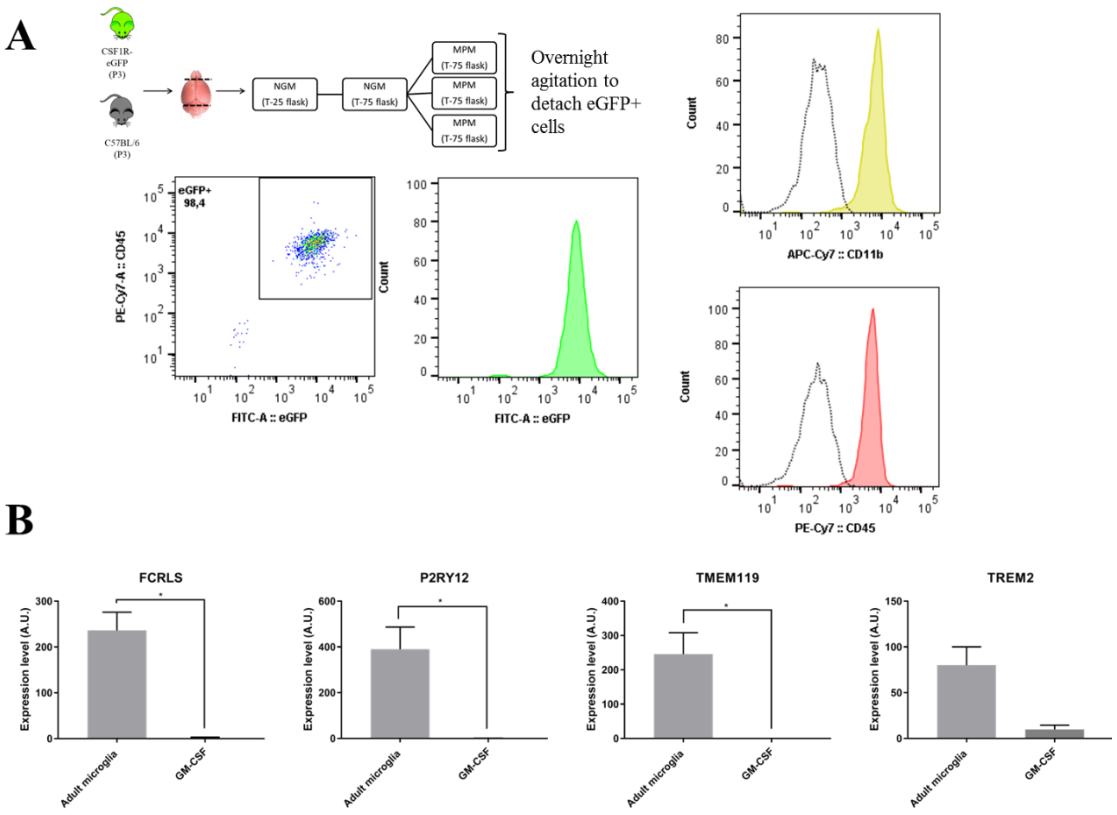

Fig 8. Characterization of the microglial population obtained by agitation after 14 days of culture. (**A**) Characterization by flow cytometry. 98.95±0.9% of detached cells are $eGFP^+$, meaning that agitation is a good method to obtain purified myeloid cells from mixed brain cultures. Detached cells maintain their myeloid characterization ($CD45^+$, $CD11b^+$). (**B**) Microglial signature expression in neonatal microglial cells after 14 days in culture (n=3) compared with the expression levels of microglia from adult mice of 3-4 months freshly isolated from the brain (n=1). * P<0.05.

Microglial signature expression in neonatal (P3) microglial cells

As microglial cells obtained from neonatal primary cultures do not express the characteristic microglial expression markers (P2RY12, TMEM119, FCRLS, TREM2), a question arises: did microglial cells lose their signature due to being in a cell culture environment different from their *in vivo* environment, or are these levels of expression normal for neonatal microglia?

To answer this question, expression of the same set of microglial markers was analyzed in freshly isolated microglial cells from neonatal (P3) brains. $CD45^+$ cells were isolated by MACS sorting and analyzed by qRT-PCR. Analysis of mRNA expression of these genes revealed low levels of expression of these markers in the

neonatal brain compared to the levels observed in the adult brain (Fig 9). Levels of expression of P2RY12 are expected to be low in the brains of P3 mice, with a steady growth up to a peak of expression at 4 months [15], [60]. In the case of Tmem119, although expression is expected to appear between P3 and P6, adult levels are not reached until postnatal day 14 [16]. FCRLS expression at P3 has been reported to be higher than for other markers [15], without reaching adult levels of expression.

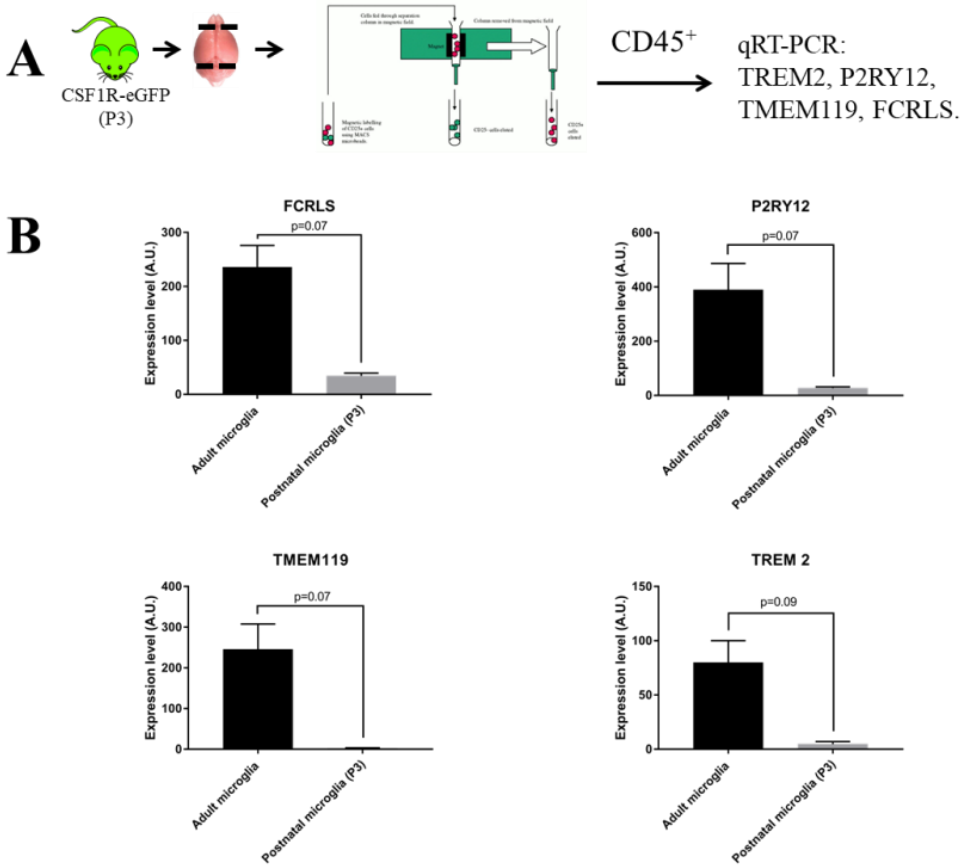

Fig 9. Microglial signature expression in P3 microglial cells. (**A**) Schematics of the neonatal microglial extraction protocol. $CD45^+$ cells were isolated from P3 brains (n=3) and analyzed by qRT-PCR. (**B**) Levels of expression are shown in comparison with expression levels of microglia from adult mice of 3-4 months freshly isolated from the brain (n=1). P-values are shown for each graph. No significance levels were obtained due to the low number of adult samples analyzed.

Overall, these results confirm what has been reported in the literature about microglial marker expression in postnatal brains using RNA studies. However, in order to fully characterize the microglial population at any stage, RNA expression is not a complete enough descriptor, as it misses the possible heterogeneity between microglial

populations of different brain regions. This heterogeneity in the population could mask the possibility that some cells have high levels of expression of these microglial markers, while others have no expression of it.

To unveil these possible differences, the next step of the study will be to analyze the spatial distribution of $eGFP^+$ cells in the microglial postnatal brain, and whether these cells are expressing the microglial marker P2RY12.

CSFR1-R+ cells in the neonatal murine brain: spatial distribution and P2RY12 expression

Microglial cells constitute between 5-12% of the cells in the adult rodent brain [2]. They are present in all the brain structures and have a highly ramified morphology, presenting a small cell soma with numerous long prolongations, which are vital for surveillance of their environment (FIG 10C). In the case of the postnatal brain, the distribution is highly heterogeneous (Fig 10B), and cells present a primitive ramified morphology (Fig 10D).

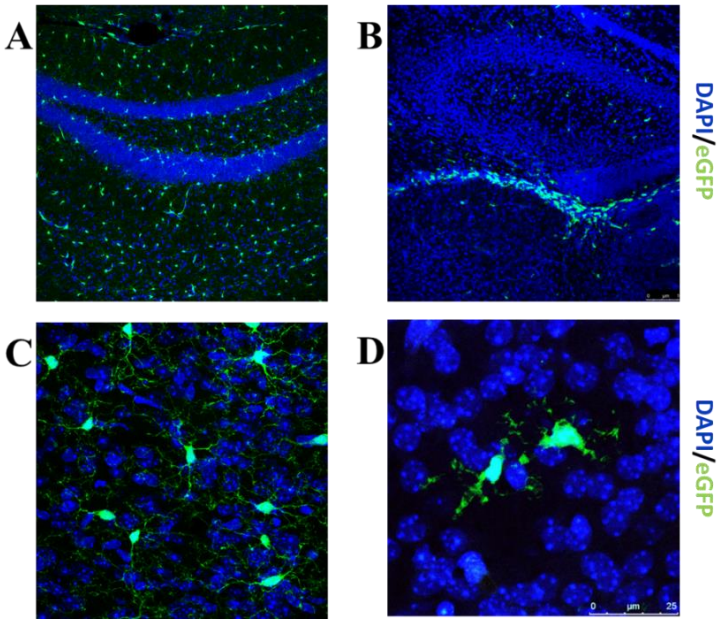

Fig 10. Comparison between microglia in the adult and postnatal brain. Comparison between the hippocampus of an adult (**A**) and a P3 (**B**) CSF1R-eGFP transgenic mice. The differences in the number of cells between both stages are evident. These differences are not only apparent in the number of cells but also in the phenotype of the typical cells in the adult brain (**C**) and in the postnatal brain (**D**), where adult cells present smaller somas and longer, thinner ramifications. Images from adult brains belong to the doctoral thesis of Elena Quintana from the Neuroinflammation unit of Instituto de Salud Carlos III [61].

The use of CSF1R-eGFP transgenic mice allows for a simple observation of microglial cells in the brain. There are also non-microglial cells expressing CSF1R in the brain, as meningeal macrophages and macrophages in the choroidal plexus, however, they are characteristically localized so they will not pose a problem for differentiation with the microglial population.

Four main regions will be analyzed in this study, the hippocampus, the cortex, the striatum and the region surrounding the lateral ventricles which includes the neurogenic subventricular zone (SVZ). In these regions, the number of eGFP+ cells will be analyzed, as well as the number of cells expressing P2RY12, to be able to characterize the microglial population at P3, as a starting point to understand its suitability as a model for adult microglial studies.

Description of the spatial distribution of microglial cells in the postnatal murine brain following the CSF1R-eGFP transgenic model

Regarding spatial distribution, microglial cells are much more heterogeneously distributed in the neonatal brain compared to the adult brain. The concentration of cells is lower in all regions, particularly in the cortex (Fig 12A). Cells in the striatum (Fig 13), in the cortex (Fig 12B) and in the hippocampus (Fig 11B) present a more ramified morphology, while cells close to the ventricles present a more amoeboid morphology (Fig 14). These results match results published in the literature observed for rat models [35], [62] and partial observations for murine models using the CX3CR1-eGFP transgenic model [16], [63]. It is worth noting the existence of a region with a very high number of $eGFP^+$ cells below the hippocampus (Fig 11C). These cells are disposed in what will be called “hippocampal stream” for simplicity, although the term is imprecise as there are no observations of mobility or migration along that axis. Cells in this region present a highly amoeboid morphology, similar to that in the ventricular region.

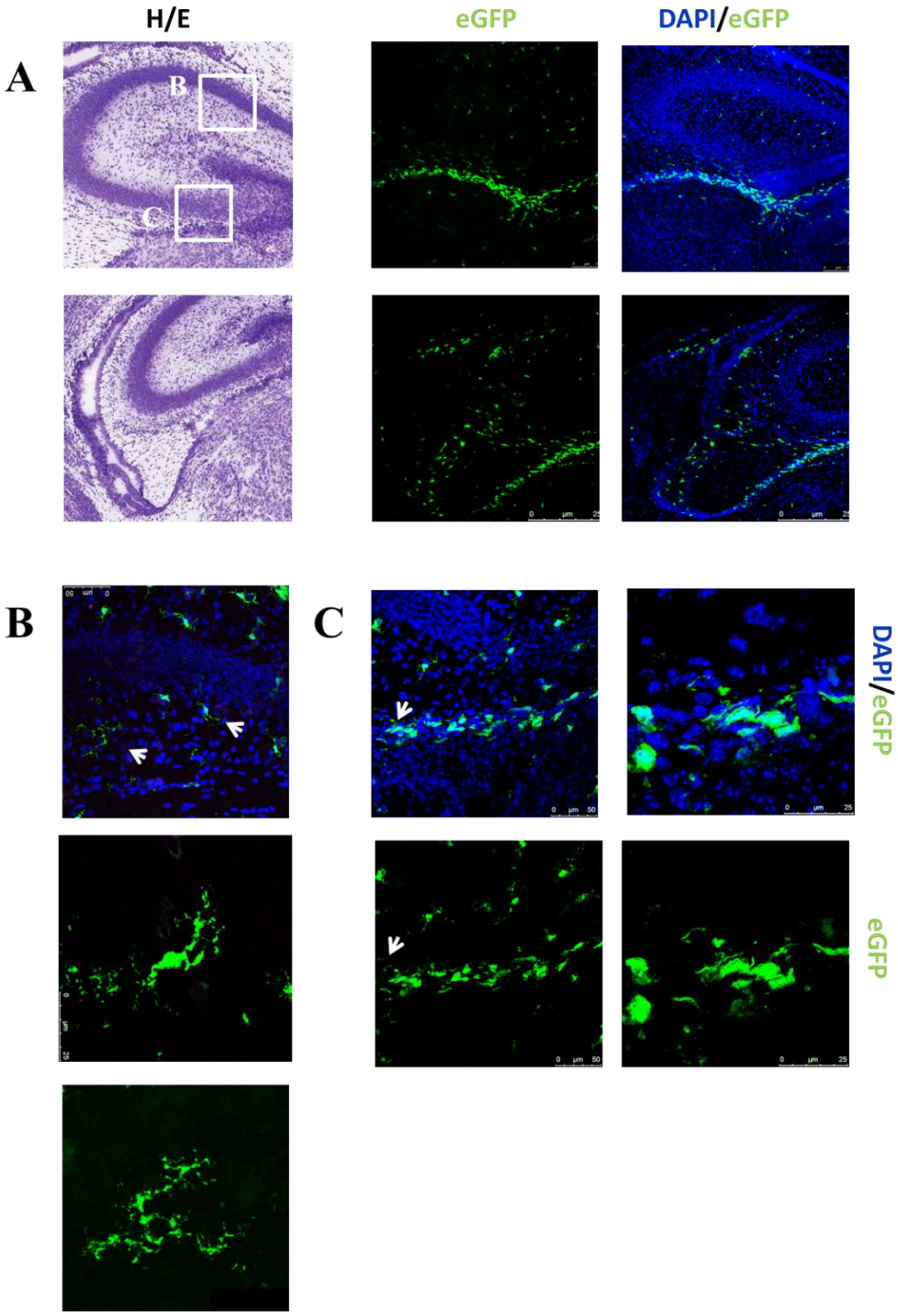

Fig 11. Microglial cells in the postnatal hippocampus. (**A**) 30 µm sections of the neonatal hippocampus, showing the location of $CSF1R^{+}$ cells (green), showing an unevenly distribution. Hematoxylin/Eosin staining from the Allen Brain Atlas (P4 NISSL coronal, positions 303 and 297) [64]. (**B**) Morphology of the cells inside the hippocampus. Cells show primitive ramified morphology, an intermediate morphology characteristic of developing microglial cells [62]. (**C**) Characteristic morphology of cells in the "hippocampal stream", more amoeboid than cells in the surrounding regions.

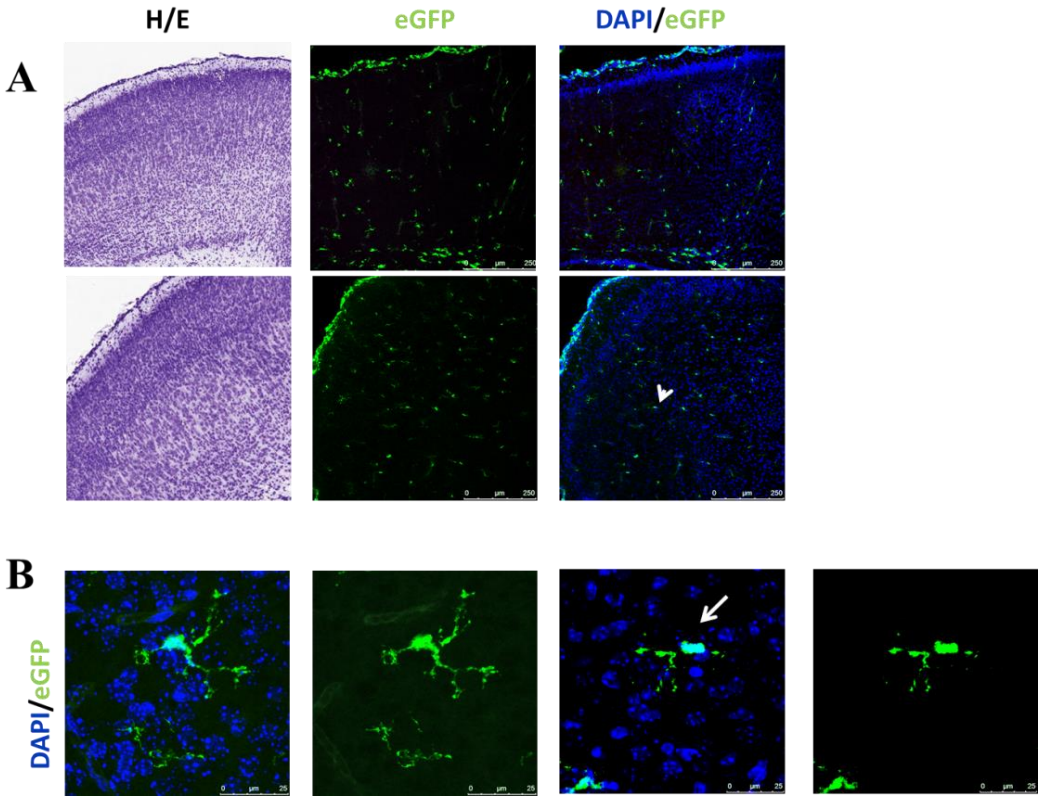

Fig 12. Microglial cells in the postnatal cortex. (**A**) 30 µm sections of the neonatal cortex, showing the location of $CSF1R^{+}$ cells (green). Cells are evenly distributed through the cortex, with a high concentration of eGFP expressing cells in the outer meninges, corresponding to the meningeal macrophages. Hematoxylin/Eosin staining from the Allen Brain Atlas (P4 NISSL coronal, position 342) [64]. (**B**) Morphology of the cells in the cortex. Cells present a higher ramification degree than cells present in other structures, which could indicate later stages of development.

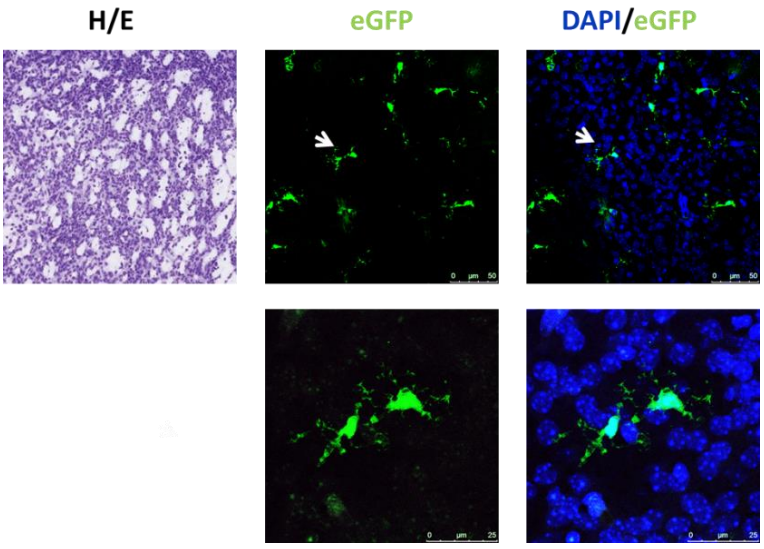

Fig 13. Microglial cells in the postnatal striatum. 30 µm sections of the neonatal striatum, showing the location of CSF1R$^+$ cells (green). Hematoxylin/Eosin staining from the Allen Brain Atlas (P4 NISSL coronal, position 342) [64]. Representative morphology of the cells in the striatum.

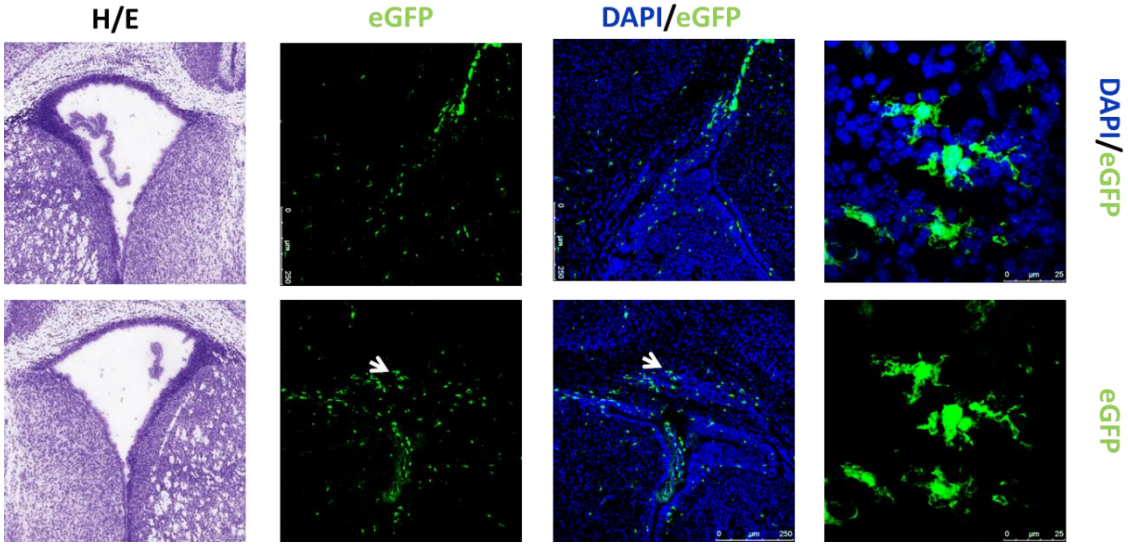

Fig 14. Microglial cells surrounding the lateral ventricles. 30 µm sections of the neonatal ventricles, showing the location of CSF1R$^+$ cells (green). Some of the CSF1R+ are inside the ventricles, as the choroid plexuses are the interface between the blood and the cerebrospinal fluid and thus contain blood immune cells [65]. Hematoxylin/Eosin staining from the Allen Brain Atlas (P4 NISSL coronal, position 342) [64]. Representative morphology of the cells in the regions surrounding the ventricles.

P2RY12 differential expression in the postnatal murine brain

Once microglial spatial distribution had been characterized for the CSF1R-eGFP transgenic brain, the next objective was to study whether these eGFP$^+$ cells expressed the microglial marker P2RY12.

Contrary to what could have been expected by the low levels of mRNA expression found in these cells (Fig 9), the majority of cells expressed this surface marker in all the regions observed (Fig 15, Fig 16, Fig 17). It is worth noting that although not all the eGFP$^+$ cells were P2RY12, all P2RY12$^+$ cells were eGFP$^+$, as could be expected. However, in some cases the level of fluorescence of the eGFP$^+$ cells was very low, especially in the cells expressing P2RY12 (Fig 15B). Also, as the eGFP protein is expressed in the cytoplasm of the cell, while the P2RY12 is a marker found in the surface of the cell, the intensity of the eGFP signal was higher in the soma of the cell, as

the majority of the cytoplasm volume is in that region, while the P2RY12 signal was distributed along the surface, which enhanced the visualization of the microglial processes (Fig 17B). It is worth noting that indeed P2RY12 was only expressed in the microglial cells and not in the meningeal macrophages (Fig 17A) or in the cells inside the choroid plexus (Fig 19).

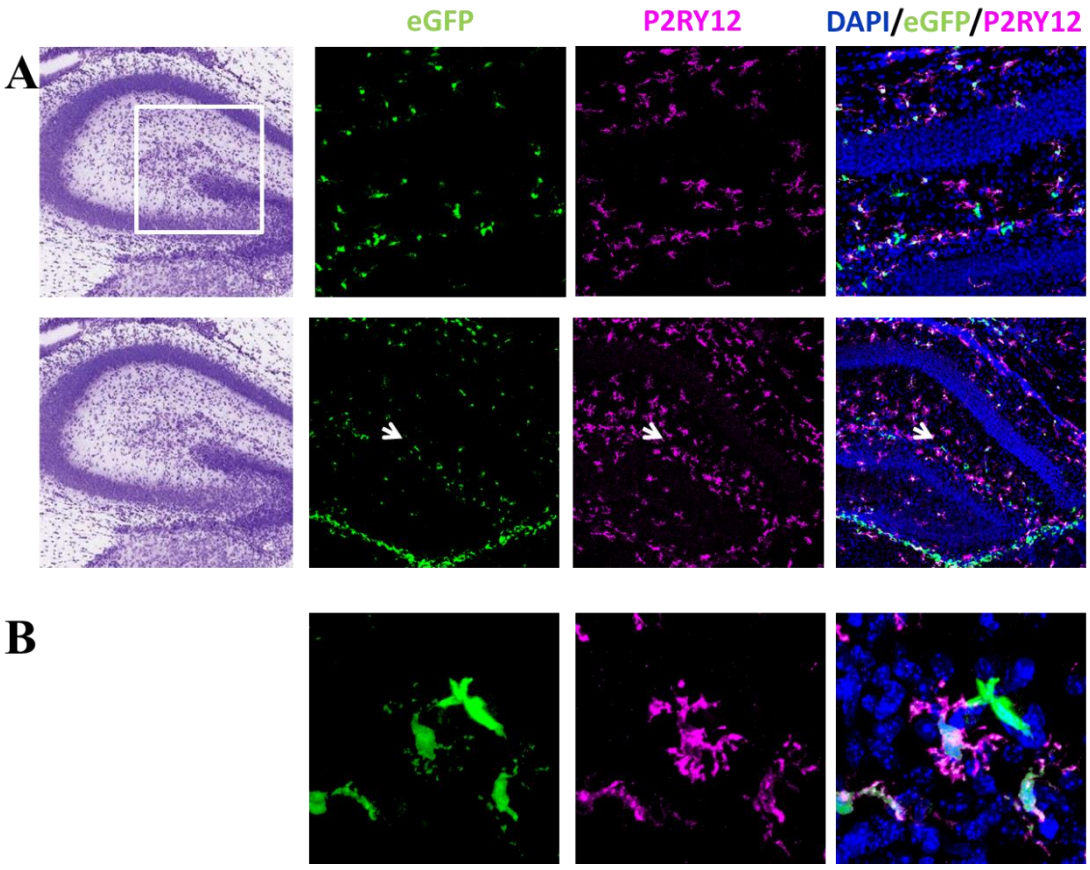

Fig 15. P2RY12 expression in the postnatal hippocampus. (**A**) eGFP+ cells in the hippocampus mostly expressed P2RY12 in their surface membrane, with the main exception of the cells found in the "hippocampal stream". Hematoxylin/Eosin staining from the Allen Brain Atlas (P4 NISSL coronal, positions 303) [60]. (**B**) Representative eGFP+ cells in the hippocampal parenchyma. Cells expressing the P2RY12 marker show a more ramified morphology (left) than cells that do not express this marker (right).

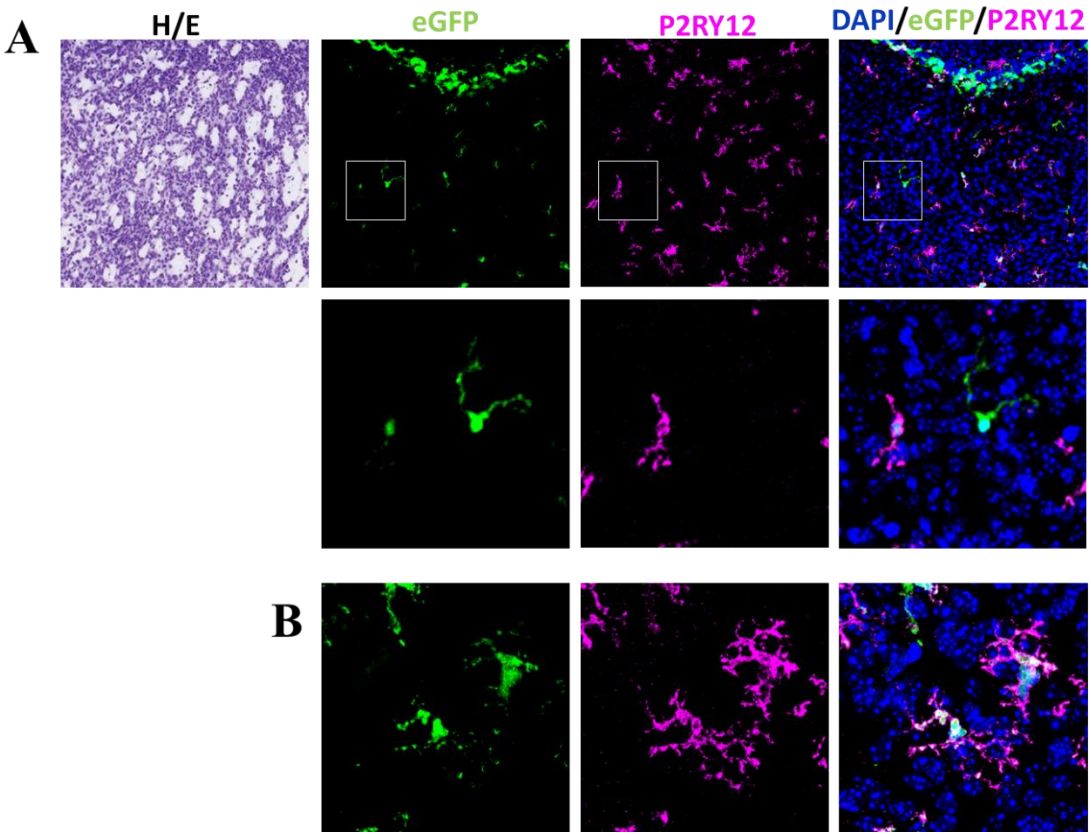

Fig 16. P2RY12 expression in the postnatal striatum. (**A**) $eGFP^{+}$ cells in the striatum mostly express P2RY12 in their surface membrane, with some exceptions. As occurred in the hippocampus, it appears as if the cells that do not express P2RY12 (right) have a higher intensity in the eGFP signal than the cells that do express P2RY12 (right). Hematoxylin/Eosin staining from the Allen Brain Atlas (P4 NISSL coronal, positions 342) [60]. (**B**) Representative $eGFP^{+}$ $P2RY12^{+}$ cells in the striatum.

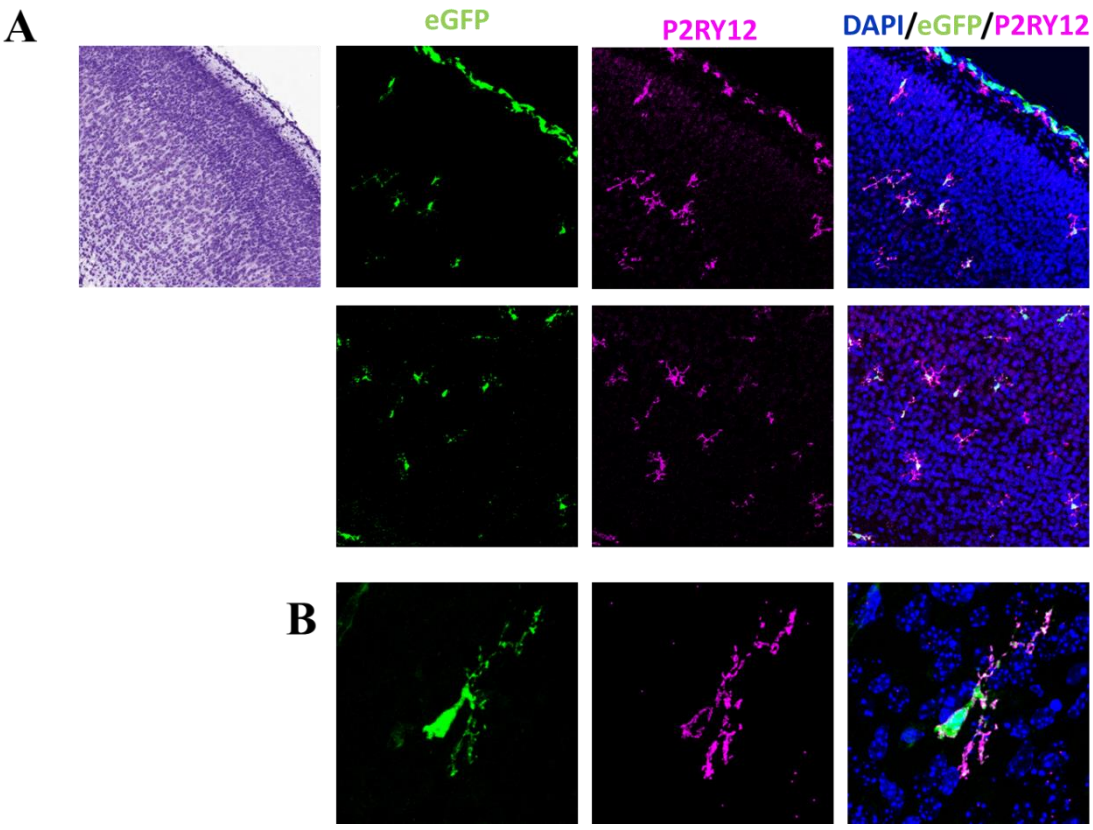

Fig 17. P2RY12 expression in the postnatal cortex. (**A**) All eGFP^{+} cells in the cortex express P2RY12 in their surface membrane and present a primitive ramified morphology. Hematoxylin/Eosin staining from the Allen Brain Atlas (P4 NISSL coronal, positions 342) [60]. (**B**) Representative eGFP^{+} P2RY12^{+} cell in the cortex. It can be appreciated how eGFP expression is mostly found in the soma of the cells, while P2RY12 is found in the surface of the cell.

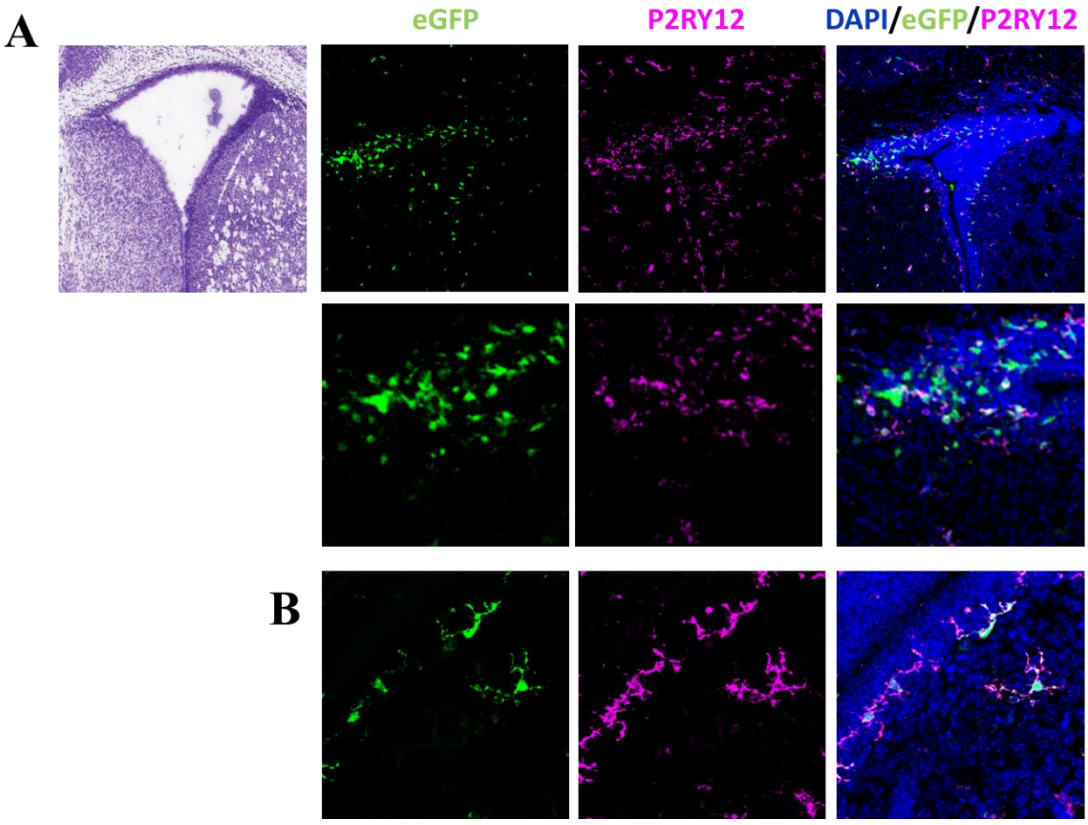

Fig 18. P2RY12 in the area surrounding the lateral ventricles. (**A**) general view and close up of the eGFP^{+} cells in the lateral ventricles region. Hematoxylin/Eosin staining from the Allen Brain Atlas (P4 NISSL coronal, positions 342) [60]. (**B**) Cells in the lining of the ventricles show a primitive ramified morphology and P2RY12 expression.

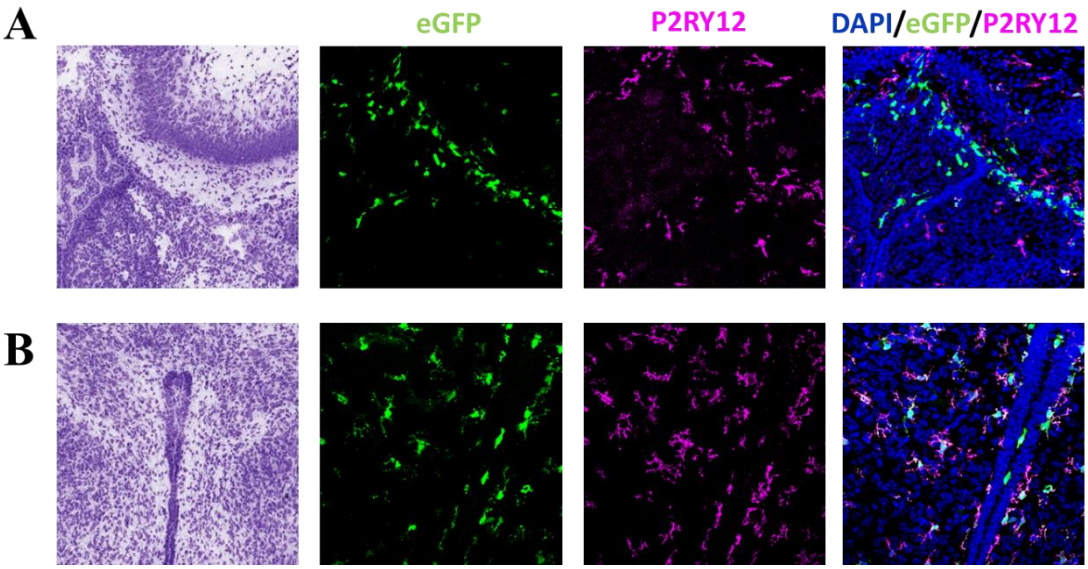

Fig 19. P2RY12 expression in the interior of the ventricles. (**A**) eGFP+ cells in the interior of the third ventricle below the hippocampus are negative for P2RY12 staining, while microglia

in the brain parenchyma are P2RY12+. The same applies for other ventricular regions (**B**). Hematoxylin/Eosin staining from the Allen Brain Atlas (P4 NISSL coronal, positions 280) [60].

Considering the "stream" found below the hippocampus, the cells had been found to present a more amoeboid morphology than other cells in the surrounding regions. These cells also lack the P2RY12 staining that was present in other cells of the hippocampus (Fig 20), which may lead to believe that they could be a different microglial population.

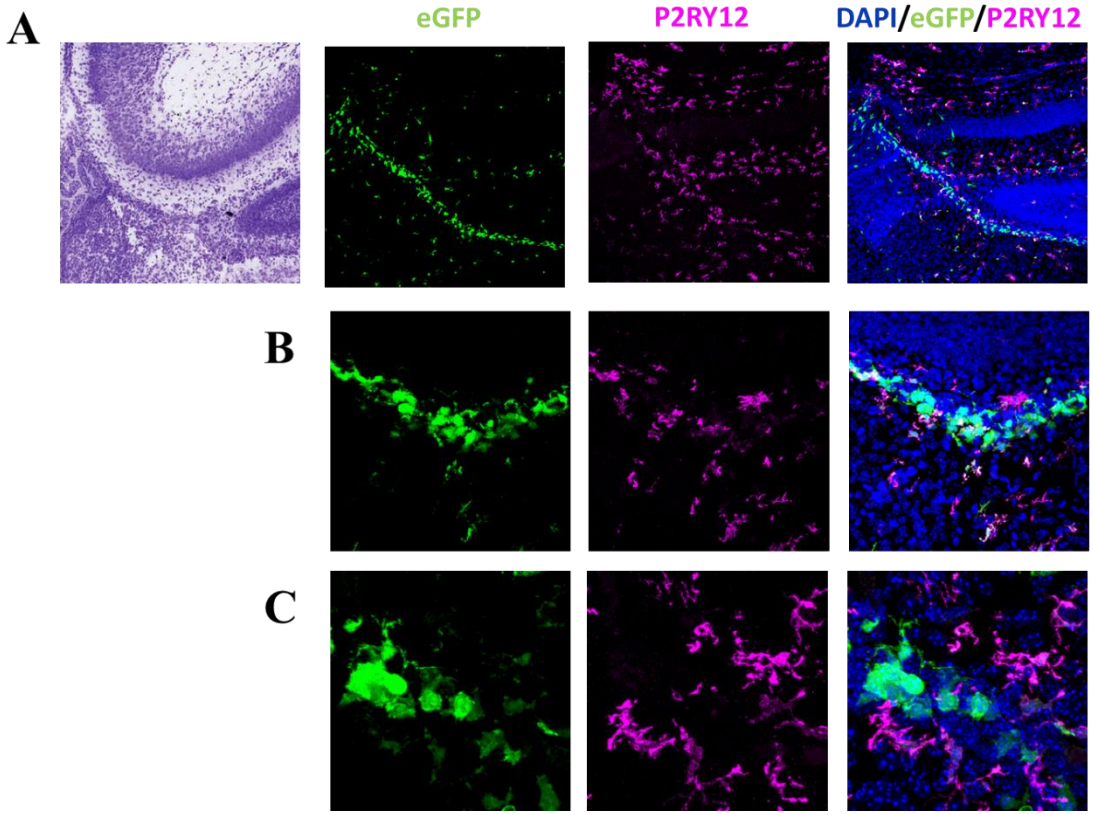

Fig 20. P2RY12 expression in the "hippocampal stream". $eGFP^+$ cells in the stream present a more amoeboid morphology, and are also $P2RY12^-$. As occurred with $P2RY12^-$ in other regions, these cells present a higher eGFP fluorescence level than the surrounding $eGFP^+$ $P2RY12^+$ cells. Hematoxylin/Eosin staining from the Allen Brain Atlas (P4 NISSL coronal, positions 280) [60].

5. Discussion

The first objective of this project was to characterize the expression of microglial markers in the cells obtained following the Marshall et al protocol [22]. Several studies had found that primary cultures of microglial cells express lower levels of the recently characterized microglial signature [15], [16] compared to adult microglial cells. This first goal was achieved, and the study showed that the lack of microglial signature in primary cultures remains true for the Marshall et al protocol [22], so cells obtained using this procedure should not be considered models of adult microglial cells, particularly for studies that are testing the response to neurodegenerative signals such as the Aβ peptides found in Alzheimer's disease, as the cells are phenotypically different from adult microglial cells. Although studies using these cell cultures can still provide useful insights, it is important to report that the conclusions reached may not hold true for adult microglial cells, as they present different phenotypes and are likely to respond differently to the same stimuli.

Nevertheless, the fact that cells obtained from these mixed glial cultures do not show adult levels of expression of the microglial signature genes does not imply that the cells are not microglial cells, but only that they are not phenotypically adult microglial cells. In fact, in consonance with the second objective of this project, postnatal microglial cells were also found to present lower values of the microglial signature expression when compared to the adult microglia (Fig 9).

For this reason, it is not possible to conclude whether the cells obtained following the aforementioned protocol are immature microglial or blood-derived monocytes and macrophages. The fact that they present low levels of the microglial markers could be a consequence of the immaturity of the cells also found in the freshly extracted postnatal microglia, combined with the fact that the culture protocol was unable to induce maturation in the cells in the same way the innate CNS environment would, probably because GM-CSF is not the most suitable cytokine for this purpose. Indeed, although some authors use this cytokine in their protocols [43], [66],other papers have tried using other cytokines as M-CSF or IL34 that are also ligands for the CSF1R (necessary for the formation and maturation of microglial cells [50], [67], [68]), but that are naturally present in the CNS.

In order to determine if these cells are indeed postnatal microglial cells and could be used as models for postnatal microglia, it would be necessary to find a set of genes that are characteristically expressed in postnatal microglia and study their expression in the different microglial culture protocols. In any case, what becomes apparent from these results is that postnatal microglial cultures are not reliable models for adult microglia.

Overall, the first and second objectives of this project were achieved, as both primary cultures and neonatal microglia have been characterized.

From these results, and knowing that the best options for microglial models in vitro would be postnatal microglial cultures or, ideally, the differentiation of iPSCs into microglial cells, understanding the microglial developmental process *in vivo* appears as a necessary step in order to replicate this process *in vitro*.

As a first approximation for any attempt to reproduce this developmental process the first step would be to characterize the original population. For this reason, the third objective of this work was to describe the microglial population at postnatal day 3 (P3), both their spatial distribution and the expression of the microglial signature marker P2RY12.

Considering the spatial distribution, it is remarkable that some $eGFP^+$ cells were concentrated in a "hippocampal stream" below the hippocampus, and that these cells showed a more amoeboid morphology than cells from other regions. Immunostaining also showed that these cells were mainly $P2RY12^-$, as opposed to other cells in the surrounding regions.

These cells present a similar morphology and expression to the $eGFP^+$ cells found in the subventricular zone (SVZ) (Fig 14). In a 2015 study, Xavier et al [39] characterized postnatal microglia in the neurogenic SVZ as being a copious population that outnumbered other region microglia during early postnatal stages. They also found that these cells were less proliferative and showed a delayed maturation compared to cortical microglia. The high number of cells and the fact that they present a more immature morphology compared to cells in the surrounding regions could lead to hypothesize that cells in the "hippocampal stream" are a similar population to the cells found in the SVZ. In that case, the "hippocampal stream" could be close to the subgranular layer of the hippocampus dentate gyrus, another neurogenic region. In order

to test this hypothesis, it would be interesting to analyze whether cells in the surroundings express Nestin, Sox2 or any other marker of neural progenitors [69].

Another hypothesis for the origin of this "hippocampal stream" can be found in a study by Mercier et al. in 2012. In this study, the authors describe a meningeal projection below the hippocampus, continuous with the choroid plexus stroma [70], that would match what this study was describing as "hippocampal stream". The authors of this paper characterized an N-sulfated HS neurogenic niche comprised as a single anatomical system that was formed by the olfactory bulb, the rostral migratory stream, the subventricular zone, the sub-callosum and subcapsule zones and that appeared to continue to sub-cortical meninges that connected with the hippocampal neurogenic zone. A follow up study found that these meninges found below the hippocampus were associated with $Nestin^{+}$ neural progenitors [71]. The main limitation to describing these cells as belonging to a meningeal projection would be that this "hippocampal stream", to the best of our knowledge, is not found in the adult brain, and meninges are anatomical structures that are present throughout the lifespan without major changes.

Nevertheless, the results obtained in this project are not sufficient to adventure a hypothesis about the origin and nature of these cells, and further research into their ontogeny and phenotype would be needed in order to determine whether these cells are microglial cells part of a stem cell niche, meningeal macrophages belonging to a meningeal projection or if they are involved in a completely different function that is yet to be discovered.

Broadening the focus to microglial cells found in the other regions of the brain, it was found that microglial cells at P3 showed the phenotype that had been previously described as primitive ramified microglia [35], where they show a bigger cell soma and a limited number of processes that are shorter than the ramifications found in the adult brain. Besides, the number of cells is lower than in the adult brain, with cells distributed non-homogeneously among the different regions.

Regarding the expression of P2RY12, it would have been expected that most cells showed little to none expression of this marker, according to the results found in the RNA studies. However, the majority of cells in all brain regions analyzed expressed the P2RY12 marker in their surface.

This discordance between the results found by RNA analysis and by immunohistochemistry may have two main possible causes. On the one hand, it is possible that cells expressing this marker do so at the levels of adult cells, but that the populations of cells that do not express this marker are a sufficiently large percentage of the total population of $eGFP^+$ cells that they produce this mismatch. However, from the imaging, this explanation does not seem to hold on its own, as the number of cells that are $P2RY12^-$ is not large enough to induce these differences. What appears to be more likely is that although these postnatal cells are expressing the P2RY12 marker, they are doing so at levels that are lower than the levels of expression found in adult microglia.

P2RY12 is a G protein-coupled purinergic receptor [72] which has been found to regulate microglial translocation [73], microglial closure of the injured blood–brain barrier [74] and microglial activation by extracellular nucleotides [75]. As all of these functions are more related to the role microglial cells have in the adult brain (sensing, immune cells), it may seem reasonable that this marker is more expressed in mature cells than it is during development, when microglial cells have more structural roles.

From the results of the immunohistochemistry analysis, the microglial population at P3 can be described as being less numerous than at later stages, with a primitive ramified morphology and mostly expressing the microglial marker P2RY12.

To sum up, the three objectives for this project were attained, and overall the results of this project provide a good general characterization of the microglial population at P3. The main conclusions and future lines of work can be found in section 6.

Legal framework

This work involves animal research, and as such, it is subjected to the corresponding ethical and legal regulations. All animal procedures were approved by the local ethics committee. Animals were handled according to Real Decreto 53/2013 of Spanish law, which obliges with the EU Directive 2010/63/UE concerning the protection of animals used for scientific purposes. This research was done according to the last modification of this law presented on the Spanish BOE on the 20th of November, 2018.

Besides, animal handling for experimental or other scientific purposes requires specific permits. In this regard, all animal handling performed during this project was done by certified personnel according to Order ECC/566/2015 from Spanish law.

Socioeconomic impact

24 million people worldwide are affected by Alzheimer and other dementias according to the World Health Organization [76], and this number is expected to grow as the population ages. There is currently no treatment for Alzheimer's disease and other neurodegenerative diseases, in many cases, because the cause is unknown. Understanding the role microglial cells play in these diseases may unveil potential targets for treatment.

This project will have no direct socioeconomic impact in this issue, as it has no direct application for any potential therapy or treatment for any of the diseases where microglial cells are involved. Nevertheless, this project helped support the thesis that current ways of modeling microglial cells should be revisited, and that gaining an understanding of microglial development should be a focus for the research field. If new protocols for obtaining microglial implement this approach, the models for microglial testing will be more significant, and thus the results more reliable. In the end, an easy, reliable model for microglial biochemical and drug testing would greatly enhance the research into diseases that affect the CNS, and provide great value to society.

Budget

The approximate costs of this project were calculated accounting for three different types of expenses: human resources, technical equipment and expendable laboratory materials, including reagents.

Human resources

The human resources costs account for the salaries of all the people working in this project and are detailed in Table II.

Table II. Human resources costs

Concept	Hours	Cost/hour	Total cost
Project supervisor	100	35	3500€
Student	580	20	11600€

Confocal scientist	25	35	875€
Lab technicians	200	25	5000€
		Total	**21000€**

Technical equipment

Technical equipment costs and software licenses costs were approximated based on the total cost of each equipment, its expected life and the months of use. The details of the technical costs are shown in Table III.

Table III. Technical equipment costs

Concept	Expected life	Cost	Months used	Total cost
FlowJo License (with academic discount)	5 years	2695	8	360€
GraphPad Prism 7 license	1 year subscription	150	8	100€
ImageJ		free	8	0€
Leica DM IL LED	5 years	2750	8	367€
Other laboratory devices (centrifuge, shaker)	5 years	5472+447	8	789€
Personal computer	5 years	800	8	107€
			Total	**1723€**

Although the use of the confocal microscope is free of charge for ISCIII users, the estimate cost per use is 20€/hour, accounting to a total of 800€.

Laboratory materials

For general laboratory reagents (PBS, sucrose, ethanol, chloroform…) that are bought in bulk an estimate of the monthly budget is provided. For the cytokines, antibodies, cell culture reagents and qRT-PCR reagents, price was calculated based on the prices on the providers' websites on the 27th of May, 2019. Expendable laboratory material (pipette tips, falcon tubes, cell culture flasks, etc.) was provided by Insituto de

Salud Carlos III. The estimate price for each order was around 30€, and they were done approximately monthly.

Table IV. Laboratory materials costs

Concept	Units	Cost per unit	Total
Cell culture reagents	2	200	400 €
Cell culture cytokines	1	1140	1140€
qRT-PCR reagents	2	960	1920€
General laboratory reagents	8	50	400€
FC antibodies	2	200	400€
Laboratory reagents			
IHC Primary antibodies	2	400	800€
IHC Secondary antibodies	2	200	400€
Expendable laboratory material	8	30	240€
		Total costs	**5700€**

Total costs

The final total cost of the project including all expenses was 27948 as shown in Table V.

Table V. Total costs

Concept	Total cost
Human resources	19725
Technical equipment	1723
Confocal microscope use	800
Laboratory materials	5700
Total	**29198€**

6. Conclusions and future work

Conclusions

The main conclusions reached after ending this project are the following:

1. Primary cultures of postnatal microglial (P3) cells stimulated with GM-CSF are not a good model for adult microglial studies, as they lack the expression profile found on adult microglial cells (FCRLS, P2RY12, TMEM119, TREM2).

2. Neonatal microglia from P3 mice also show lower RNA expression levels than adult microglia for these markers due to their immature phenotype.

3. Since neonatal microglia does not present the adult levels of microglia markers either, it cannot be said that cells obtained from postnatal primary cultures are not microglia, but rather that they come from immature microglia and are not differentiating in the *in vitro* cultures.

4. This immature phenotype of microglial cells is also found in immunohistochemistry analysis of postnatal brains. As had been shown for other microglial models (using CX3CR1-eGFP transgenic mice, for example), microglia in postnatal day 3 present a primitive ramified morphology and are less numerous than at later stages.

5. Most microglial cells at P3 express the purinergic receptor P2RY12, one of the main microglial markers.

Future work

From the conclusions reached above, the main takeaway moving forward is that in order to obtain primary postnatal microglial cultures that are reliable models for microglial function in the adult brain, the purpose of the cell cultures should not only be to amplify the number of cells, but also to guide their differentiation towards a more mature phenotype. In order to do so, the culture environment should include all the environmental cues that drive this development *in vivo*. This approach has proven successful in differentiating iPSC to microglia that resemble fetal microglia [43], [44], [66].

Next steps in this work would be to culture postnatal microglial cells with cytokines that are involved in the development from neonatal to adult microglia, like M-CSF, IL-34 and TGF-β1 and analyze whether they drive the immature cells towards a

more mature phenotype. A first approach towards this end, changing GM-CSF by M-CSF in the Marshall et al. protocol tested in this project, seemed to yield promising results.

However, the knowledge of the developmental process is uncomplete, and until there is a clear understanding of the *in vivo* process, attempts to replicate it *in vitro* will yield only partial results. So the main focus of any research in this area should be to characterize the microglial development, for example, by using transgenic models that are KO for different factors or receptors that are suspected of being involved.

References

[1] A. Aguzzi, B. A. Barres, and M. L. Bennett, “Microglia: scapegoat, saboteur, or something else?,” *Science*, vol. 339, no. 6116, pp. 156–161, Jan. 2013.

[2] M. Mittelbronn, K. Dietz, H. J. Schluesener, and R. Meyermann, “Local distribution of microglia in the normal adult human central nervous system differs by up to one order of magnitude,” *Acta Neuropathol. (Berl.)*, vol. 101, no. 3, pp. 249–255, Mar. 2001.

[3] A. Sierra, F. de Castro, J. del Río-Hortega, J. R. Iglesias-Rozas, M. Garrosa, and H. Kettenmann, “The ‘Big-Bang’ for modern glial biology: Translation and comments on Pío del Río-Hortega 1919 series of papers on microglia,” *Glia*, vol. 64, no. 11, pp. 1801–1840, Nov. 2016.

[4] “Rise of the Microglia - Scientific American.” [Online]. Available: https://www.scientificamerican.com/article/rise-of-the-microglia/?redirect=1. [Accessed: 26-May-2019].

[5] A. J. Svahn, T. S. Becker, and M. B. Graeber, “Emergent Properties of Microglia: Microglia Interactions and Interactome Entities,” *Brain Pathol.*, vol. 24, no. 6, pp. 665–670, Nov. 2014.

[6] S. Itagaki, P. L. McGeer, H. Akiyama, S. Zhu, and D. Selkoe, “Relationship of microglia and astrocytes to amyloid deposits of Alzheimer disease,” *J. Neuroimmunol.*, vol. 24, no. 3, pp. 173–182, Oct. 1989.

[7] R. Guerreiro *et al.*, “TREM2 Variants in Alzheimer’s Disease,” *N. Engl. J. Med.*, vol. 368, no. 2, pp. 117–127, Jan. 2013.

[8] B. Cameron and G. E. Landreth, “Inflammation, microglia, and alzheimer’s disease,” *Neurobiol. Dis.*, vol. 37, no. 3, pp. 503–509, Mar. 2010.

[9] P. L. McGeer, S. Itagaki, B. E. Boyes, and E. G. McGeer, “Reactive microglia are positive for HLA-DR in the: Substantia nigra of Parkinson’s and Alzheimer’s disease brains,” *Neurology*, vol. 38, no. 8, pp. 1285–1291, 1988.

[10] S. Boillée *et al.*, “Onset and Progression in Inherited ALS Determined by Motor Neurons and Microglia,” *Science*, vol. 312, no. 5778, pp. 1389–1392, Jun. 2006.

[11] A. L. Ford, A. L. Goodsall, W. F. Hickey, and J. D. Sedgwick, “Normal adult ramified microglia separated from other central nervous system macrophages by flow cytometric sorting. Phenotypic differences defined and direct ex vivo antigen presentation to myelin basic protein-reactive CD4+ T cells compared,” *J. Immunol. Baltim. Md 1950*, vol. 154, no. 9, pp. 4309–4321, May 1995.

[12] Y. Imai, I. Ibata, D. Ito, K. Ohsawa, and S. Kohsaka, “A novel gene iba1 in the major histocompatibility complex class III region encoding an EF hand protein expressed in a monocytic lineage,” *Biochem. Biophys. Res. Commun.*, vol. 224, no. 3, pp. 855–862, Jul. 1996.

[13] D. E. Korzhevskii and O. V. Kirik, “Brain Microglia and Microglial Markers,” *Neurosci. Behav. Physiol.*, vol. 46, no. 3, pp. 284–290, Mar. 2016.

[14] C. Beutner *et al.*, “Unique transcriptome signature of mouse microglia: Transcriptome Analysis of Microglia,” *Glia*, vol. 61, no. 9, pp. 1429–1442, Sep. 2013.

[15] O. Butovsky *et al.*, “Identification of a Unique TGF-β Dependent Molecular and Functional Signature in Microglia,” *Nat. Neurosci.*, vol. 17, no. 1, pp. 131–143, Jan. 2014.

[16] M. L. Bennett *et al.*, “New tools for studying microglia in the mouse and human CNS,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 113, no. 12, pp. E1738–E1746, Mar. 2016.

[17] S. E. Hickman *et al.*, “The microglial sensome revealed by direct RNA sequencing,” *Nat. Neurosci.*, vol. 16, no. 12, pp. 1896–1905, Dec. 2013.

[18] V. Haage *et al.*, “Comprehensive gene expression meta-analysis identifies signature genes that distinguish microglia from peripheral monocytes/macrophages in health and glioma,” *Acta Neuropathol. Commun.*, vol. 7, no. 1, p. 20, Feb. 2019.

[19] T. R. Hammond *et al.*, “Single-Cell RNA Sequencing of Microglia throughout the Mouse Lifespan and in the Injured Brain Reveals Complex Cell-State Changes,” *Immunity*, vol. 50, no. 1, pp. 253-271.e6, Jan. 2019.
[20] A. Silvin and F. Ginhoux, “Microglia heterogeneity along a spatio-temporal axis: More questions than answers,” *Glia*, vol. 66, no. 10, pp. 2045–2057, Oct. 2018.
[21] O. Butovsky and H. L. Weiner, “Microglial signatures and their role in health and disease,” *Nat. Rev. Neurosci.*, vol. 19, no. 10, pp. 622–635, Oct. 2018.
[22] G. P. Marshall, M. Demir, D. A. Steindler, and E. D. Laywell, “Subventricular Zone Microglia Possess a Unique Capacity for Massive In Vitro Expansion,” *Glia*, vol. 56, no. 16, pp. 1799–1808, Dec. 2008.
[23] J. Roy, “Primary microglia isolation from mixed cell cultures of neonatal mouse brain tissue,” *Brain Res.*, vol. 1689, pp. 21–29, 15 2018.
[24] H. Lian, E. Roy, and H. Zheng, “Protocol for Primary Microglial Culture Preparation,” *BIO-Protoc.*, vol. 6, no. 21, 2016.
[25] M. J. Carson, J. Crane, and A. X. Xie, “Modeling CNS microglia: the quest to identify predictive models,” *Drug Discov. Today Dis. Models*, vol. 5, no. 1, pp. 19–25, 2008.
[26] T. K. Ulland and M. Colonna, “TREM2 — a key player in microglial biology and Alzheimer disease,” *Nat. Rev. Neurol.*, vol. 14, no. 11, pp. 667–675, Nov. 2018.
[27] F. C. Bennett *et al.*, “A Combination of Ontogeny and CNS Environment Establishes Microglial Identity,” *Neuron*, vol. 98, no. 6, pp. 1170-1183.e8, Jun. 2018.
[28] F. Ginhoux *et al.*, “Fate mapping analysis reveals that adult microglia derive from primitive macrophages,” *Science*, vol. 330, no. 6005, pp. 841–845, Nov. 2010.
[29] F. Ginhoux, S. Lim, G. Hoeffel, D. Low, and T. Huber, “Origin and differentiation of microglia,” *Front. Cell. Neurosci.*, vol. 7, p. 45, 2013.
[30] K. M. Lenz and L. H. Nelson, “Microglia and Beyond: Innate Immune Cells As Regulators of Brain Development and Behavioral Function,” *Front. Immunol.*, vol. 9, Apr. 2018.
[31] A. Shemer *et al.*, “Engrafted parenchymal brain macrophages differ from microglia in transcriptome, chromatin landscape and response to challenge,” *Nat. Commun.*, vol. 9, no. 1, p. 5206, Dec. 2018.
[32] U. B. Eyo, S. A. Miner, J. A. Weiner, and M. E. Dailey, “Developmental changes in microglial mobilization are independent of apoptosis in the neonatal mouse hippocampus,” *Brain. Behav. Immun.*, vol. 55, pp. 49–59, Jul. 2016.
[33] U.-K. Hanisch and H. Kettenmann, “Microglia: active sensor and versatile effector cells in the normal and pathologic brain,” *Nat. Neurosci.*, vol. 10, no. 11, pp. 1387–1394, Nov. 2007.
[34] T. Masuda *et al.*, “Spatial and temporal heterogeneity of mouse and human microglia at single-cell resolution,” *Nature*, vol. 566, no. 7744, pp. 388–392, Feb. 2019.
[35] I. Dalmau, J. M. Vela, B. González, B. Finsen, and B. Castellano, “Dynamics of microglia in the developing rat brain: Proliferation and Death of Microglia in Immature Brain,” *J. Comp. Neurol.*, vol. 458, no. 2, pp. 144–157, Mar. 2003.
[36] M. Nikodemova, R. S. Kimyon, I. De, A. L. Small, L. S. Collier, and J. J. Watters, “Microglial numbers attain adult levels after undergoing a rapid decrease in cell number in the third postnatal week,” *J. Neuroimmunol.*, vol. 278, pp. 280–288, Jan. 2015.
[37] O. Matcovitch-Natan *et al.*, “Microglia development follows a stepwise program to regulate brain homeostasis,” *Science*, vol. 353, no. 6301, p. aad8670, Aug. 2016.
[38] G. J. Harry, “Microglia during development and aging,” *Pharmacol. Ther.*, vol. 139, no. 3, pp. 313–326, Sep. 2013.
[39] A. L. Xavier, F. R. S. Lima, M. Nedergaard, and J. R. L. Menezes, “Ontogeny of CX3CR1-EGFP expressing cells unveil microglia as an integral component of the postnatal subventricular zone,” *Front. Cell. Neurosci.*, vol. 9, Feb. 2015.

[40] B. Stansley, J. Post, and K. Hensley, “A comparative review of cell culture systems for the study of microglial biology in Alzheimer’s disease,” *J. Neuroinflammation*, vol. 9, p. 115, May 2012.
[41] A. Das *et al.*, “Transcriptome sequencing reveals that LPS-triggered transcriptional responses in established microglia BV2 cell lines are poorly representative of primary microglia,” *J. Neuroinflammation*, vol. 13, no. 1, p. 182, 11 2016.
[42] T. T. Tamashiro, C. L. Dalgard, and K. R. Byrnes, “Primary Microglia Isolation from Mixed Glial Cell Cultures of Neonatal Rat Brain Tissue,” *J. Vis. Exp.*, no. 66, Aug. 2012.
[43] H. Pandya *et al.*, “Differentiation of human and murine induced pluripotent stem cells to microglia-like cells,” *Nat. Neurosci.*, vol. 20, no. 5, pp. 753–759, May 2017.
[44] E. M. Abud *et al.*, “iPSC-Derived Human Microglia-like Cells to Study Neurological Diseases,” *Neuron*, vol. 94, no. 2, pp. 278-293.e9, Apr. 2017.
[45] W. Haenseler *et al.*, “A Highly Efficient Human Pluripotent Stem Cell Microglia Model Displays a Neuronal-Co-culture-Specific Expression Profile and Inflammatory Response,” *Stem Cell Rep.*, vol. 8, no. 6, pp. 1727–1742, 06 2017.
[46] C. Sousa, K. Biber, and A. Michelucci, “Cellular and Molecular Characterization of Microglia: A Unique Immune Cell Population,” *Front. Immunol.*, vol. 8, Mar. 2017.
[47] M.-È. Tremblay, C. Lecours, L. Samson, V. Sánchez-Zafra, and A. Sierra, “From the Cajal alumni Achúcarro and Río-Hortega to the rediscovery of never-resting microglia,” *Front. Neuroanat.*, vol. 9, p. 45, 2015.
[48] V. H. Perry, D. A. Hume, and S. Gordon, “Immunohistochemical localization of macrophages and microglia in the adult and developing mouse brain,” *Neuroscience*, vol. 15, no. 2, pp. 313–326, Jun. 1985.
[49] E. R. Stanley and V. Chitu, “CSF-1 Receptor Signaling in Myeloid Cells,” *Cold Spring Harb. Perspect. Biol.*, vol. 6, no. 6, Jun. 2014.
[50] S. Nandi *et al.*, “The CSF-1 receptor ligands IL-34 and CSF-1 exhibit distinct developmental brain expression patterns and regulate neural progenitor cell maintenance and maturation,” *Dev. Biol.*, vol. 367, no. 2, pp. 100–113, Jul. 2012.
[51] P. V. Byrne, L. J. Guilbert, and E. R. Stanley, “Distribution of cells bearing receptors for a colony-stimulating factor (CSF-1) in murine tissues,” *J. Cell Biol.*, vol. 91, no. 3 Pt 1, pp. 848–853, Dec. 1981.
[52] “Challenges of Autofluorescence in Neuroscience,” *Bitesize Bio*, 30-Nov-2016. .
[53] “neuroscience - Voltage sensitive dyes technique: 2-photons microscopy vs confocal microscopy,” *Psychology & Neuroscience Stack Exchange*. [Online]. Available: https://psychology.stackexchange.com/questions/13829/voltage-sensitive-dyes-technique-2-photons-microscopy-vs-confocal-microscopy. [Accessed: 26-May-2019].
[54] R. T. Sasmono, “A macrophage colony-stimulating factor receptor-green fluorescent protein transgene is expressed throughout the mononuclear phagocyte system of the mouse,” *Blood*, vol. 101, no. 3, pp. 1155–1163, Feb. 2003.
[55] R. T. Sasmono and E. Williams, “Generation and Characterization of MacGreen Mice, the Cfs1r-EGFP Transgenic Mice,” in *Leucocytes*, vol. 844, R. B. Ashman, Ed. Totowa, NJ: Humana Press, 2012, pp. 157–176.
[56] “Flow Cytometry Fundamental Principle, How FACS Works | Boster.” [Online]. Available: https://www.bosterbio.com/protocol-and-troubleshooting/flow-cytometry-principle. [Accessed: 18-May-2019].
[57] P. Chomczynski, “A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples,” *BioTechniques*, vol. 15, no. 3, pp. 532–534, 536–537, Sep. 1993.
[58] M. Díaz-Moreno *et al.*, “Aβ increases neural stem cell activity in senescence-accelerated SAMP8 mice,” *Neurobiol. Aging*, vol. 34, no. 11, pp. 2623–2638, Nov. 2013.

[59] I. Ushach and A. Zlotnik, “Biological role of granulocyte macrophage colony-stimulating factor (GM-CSF) and macrophage colony-stimulating factor (M-CSF) on cells of the myeloid lineage,” *J. Leukoc. Biol.*, vol. 100, no. 3, pp. 481–489, 2016.
[60] J. M. Crain, M. Nikodemova, and J. J. Watters, “Expression of P2 nucleotide receptors varies with age and sex in murine brain microglia,” *J. Neuroinflammation*, vol. 6, no. 1, p. 24, Aug. 2009.
[61] E. Q. Menéndez, “Caracterización de células mieloides cerebrales y sus alteraciones en un modelo murino de envejecimiento,” p. 188.
[62] I. Dalmau, B. Finsen, J. Zimmer, B. González, and B. Castellano, “Development of microglia in the postnatal rat hippocampus,” *Hippocampus*, vol. 8, no. 5, pp. 458–474, 1998.
[63] A. L. Xavier, J. R. L. Menezes, S. A. Goldman, and M. Nedergaard, “Fine-tuning the central nervous system: microglial modelling of cells and synapses,” *Philos. Trans. R. Soc. B Biol. Sci.*, vol. 369, no. 1654, pp. 20130593–20130593, Sep. 2014.
[64] “Image Thumbnails :: Allen Brain Atlas: Developing Mouse Brain.” [Online]. Available: http://developingmouse.brain-map.org/experiment/thumbnails/100034998?image_type=nissl. [Accessed: 25-May-2019].
[65] R. B. Meeker, K. Williams, D. A. Killebrew, and L. C. Hudson, “Cell trafficking through the choroid plexus,” *Cell Adhes. Migr.*, vol. 6, no. 5, pp. 390–396, Sep. 2012.
[66] P. Douvaras *et al.*, “Directed Differentiation of Human Pluripotent Stem Cells to Microglia,” *Stem Cell Rep.*, vol. 8, no. 6, pp. 1516–1524, 06 2017.
[67] Y. Wang *et al.*, “IL-34 is a tissue-restricted ligand of CSF1R required for the development of Langerhans cells and microglia,” *Nat. Immunol.*, vol. 13, no. 8, pp. 753–760, Aug. 2012.
[68] S. Wu *et al.*, “Il34-Csf1r Pathway Regulates the Migration and Colonization of Microglial Precursors,” *Dev. Cell*, vol. 46, no. 5, pp. 552-563.e4, Sep. 2018.
[69] L. Vinci *et al.*, “Immunohistochemical Markers of Neural Progenitor Cells in the Early Embryonic Human Cerebral Cortex,” *Eur. J. Histochem. EJH*, vol. 60, no. 1, Feb. 2016.
[70] F. Mercier and E. Arikawa-Hirasawa, “Heparan sulfate niche for cell proliferation in the adult brain,” *Neurosci. Lett.*, vol. 510, no. 2, pp. 67–72, Feb. 2012.
[71] I. Decimo, G. Fumagalli, V. Berton, M. Krampera, and F. Bifari, “Meninges: from protective membrane to stem cell niche,” *Am. J. Stem Cells*, vol. 1, no. 2, pp. 92–105, May 2012.
[72] G. H. Reference, “P2RY12 gene,” *Genetics Home Reference*. [Online]. Available: https://ghr.nlm.nih.gov/gene/P2RY12. [Accessed: 31-May-2019].
[73] U. B. Eyo *et al.*, “P2Y12R-Dependent Translocation Mechanisms Gate the Changing Microglial Landscape,” *Cell Rep.*, vol. 23, no. 4, pp. 959–966, Apr. 2018.
[74] N. Lou, T. Takano, Y. Pei, A. L. Xavier, S. A. Goldman, and M. Nedergaard, “Purinergic receptor P2RY12-dependent microglial closure of the injured blood–brain barrier,” *Proc. Natl. Acad. Sci.*, vol. 113, no. 4, pp. 1074–1079, Jan. 2016.
[75] S. E. Haynes *et al.*, “The P2Y 12 receptor regulates microglial activation by extracellular nucleotides,” *Nat. Neurosci.*, vol. 9, no. 12, p. 1512, Dec. 2006.
[76] “WHO | Neurological disorders affect millions globally: WHO report,” *WHO*. [Online]. Available: https://www.who.int/mediacentre/news/releases/2007/pr04/en/. [Accessed: 31-May-2019].