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Assessing the Influence of Neuroinflammation on Neurogenesis: *In Vitro* Models Using Neural Stem Cells and Microglia as Valuable Research Tools

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1. Introduction

1.1 Neural stem cells

Neural stem cells are localized in two limited regions of the adult mammalian brain: the subgranular zone of the dentate gyrus (DG) of the hippocampus, a cell layer located between the granule cell layer and the hilus (Eriksson *et al.*, 1998; Limke and Rao, 2002), and the subventricular zone (SVZ), located next to the ependyma of the lateral walls of the lateral ventricles (Doetsch and Scharff, 2001; Curtis *et al.*, 2007). These regions are thought to provide a specific microenvironment, the stem cell niche, characterized by the presence of several agents involved in the maintenance of self-renewal and/or multipotency of neural stem cells (Alvarez-Buylla and Lim, 2004).

Although neurogenesis has been intensively studied over the past decades, only recently it has been established that newly formed neurons in the adult mammalian brain are functional and integrate into the existing neuronal network (Carlen *et al.*, 2002). The several stages of adult neurogenesis include proliferation of adult neural stem cells, fate determination, migration, integration and maturation of the newborn neurons. Using specific cell markers it is possible to independently investigate the different phases of development. Hippocampal neurogenesis plays an important role in normal hippocampal function, learning and memory (Gould *et al.*, 1999a; Shors *et al.*, 2001; Drapeau *et al.*, 2007). Newborn cells emerging from the SVZ migrate through the rostral migratory stream and integrate into the neuronal network of the olfactory bulb, establish functional synaptic connections and develop electrophysiological properties of mature neurons (Carlen *et al.*, 2002; Petreanu and Alvarez-Buylla, 2002; Belluzzi *et al.*, 2003). Furthermore, neurogenesis in the olfactory bulb is involved in important functions such as odor memory and discrimination (Gheusi *et al.*, 2000; Rochefort *et al.*, 2002; Shingo *et al.*, 2003). Under

physiological conditions, neural stem cells are tightly controlled contributing for the maintenance of brain homeostasis (Morshead *et al.*, 1994; Morshead *et al.*, 1998), however they seem to be also involved in neuronal replacement in response to pathophysiological conditions, particularly in conditions associated with neuroinflammation. Although little is known about the molecular mechanisms involved in the regulation of neural stem cells, several factors, both intrinsic and extrinsic, have been described to modulate the neurogenic process, such as hormones, trophic factors, neurotransmitters, neuromodulators and glial cells (for review see Ming and Song, 2005).

The existence of neurogenesis in areas beyond the SVZ and the DG of the adult mammalian brain have also been reported, namely in the neocortex (Gould *et al.*, 1999b; Dayer *et al.*, 2005), striatum, amygdala (Bernier *et al.*, 2002), hypothalamus (Gould *et al.*, 2001; Xu *et al.*, 2005), mesencephalon (Zhao *et al.*, 2003) and spinal cord (Yamamoto *et al.*, 2001). However, these findings need further experimental support, thus more studies need to be conducted.

1.2 Neuroinflammation

The central nervous system (CNS) was considered an immunologically privileged site, not susceptible to immune activation, due to its protection by the blood-brain barrier, which selectively allows certain inflammatory agents to enter and/or exit (Lucas *et al.*, 2006). Nowadays it is well established that immune surveillance takes place in the CNS due to the selective permeability of the blood-brain barrier to immune cells such as T cells, macrophages and dendritic cells (Hickey, 1999). Following injury or exposure to pathogens, an inflammatory response is driven by the activation of two types of immune cells: CNS resident cells, such as microglial cells and astrocytes, and CNS infiltrating cells, such as lymphocytes, monocytes and macrophages from the hematopoietic system (Stoll and Jander, 1999; Streit *et al.*, 1999). The activation of immune cells leads to the production and release of a plethora of regulatory substances, like cytokines, chemokines, neurotransmitters, reactive oxygen species and reactive nitrogen species (reviewed by Whitney *et al.*, 2009). These inflammatory mediators are essential for the recruitment of immune cells, particularly microglial cells, but also for changing the permeability of the blood-brain barrier and recruitment of monocytes and lymphocytes from the hematopoietic system to the compromised area (Hickey, 1999; Lossinsky and Shivers, 2004; Taupin, 2008), which creates a positive feedback loop to the inflammatory response.

Microglia, frequently referred to as the resident macrophages of the brain parenchyma, play a central role in the inflammatory response. Unlike astrocytes, oligodendrocytes and ependymal cells, microglial cells derive from the mesodermal germ layer. During adult life, the microglial cell pool is renewed by division of CNS resident cells. Moreover, microglia are distributed throughout the CNS with distinct densities (Lawson *et al.*, 1990). In the healthy brain, microglia are present in a resting state assuming a typical and dynamic morphology, whose function has been clarified by different studies (Davalos *et al.*, 2005; Nimmerjahn *et al.*, 2005; Davalos *et al.*, 2008). This resting state consists of a constant surveillance activity of the brain parenchyma, which enables microglial cells to screen different brain regions without disturbing the neuronal network (Hanisch and Kettenmann, 2007). Therefore, microglial cells can rapidly react to subtle homeostatic variations by changing morphology and acquiring an array of functions that allow the targeted migration

into a site of injury and release of inflammatory mediators (Gehrmann, 1996; Kreutzberg, 1996; Haynes *et al.*, 2006). Reactive microglia have the ability to rapidly upregulate a large number of receptor types, like cytokine receptors, toll-like receptors or cell adhesion molecules, but also to release a plethora of inflammatory agents (for review see Block and Hong, 2005). In fact, chemokines released by reactive microglial cells attract more microglia that, following activation, contribute to further propagate the neuroinflammatory event (Whitney *et al.*, 2009).

Astrocytes constitute the majority of glial cells in the CNS, and play an important structural function, providing support for neurons, playing also regulatory functions, including maintenance of extracellular ion balance, signaling to neurons, repair and scarring process of the CNS (Svendsen, 2002). During inflammation, astrocytes also become activated and release inflammatory factors, growth factors and excitatory amino acids, such as glutamate, which are involved in the regulation of the inflammatory response (Song *et al.*, 2002).

1.3 Neuroinflammation and neurogenesis

Neuroinflammation is a complex event with different outcomes in the neurogenic process, which can therefore enhance or suppress neurogenesis. The secreted products during inflammation have been shown to act as pro- or anti-neurogenic agents, contributing to beneficial or detrimental outcomes of neuroinflammation on the different steps of neurogenesis. Moreover, these effects seem to be particularly dependent on how and for how long microglial cells are activated. Inflammation and microglia activation were initially thought to inhibit adult neurogenesis (Ekdahl *et al.*, 2003; Monje *et al.*, 2003), while recent evidence indicates that microglia under certain circumstances can support neurogenic events (reviewed by Hanisch and Kettenmann, 2007). It has been suggested that mediators released by reactive microglia, such as cytokines and nitric oxide (NO), can inhibit adult neurogenesis in inflammatory conditions (Vallieres *et al.*, 2002; Monje *et al.*, 2003; Liu *et al.*, 2006). On the other hand, neurogenesis seems to be induced by microglial cells activated by IL-4 or low level of IFN-gamma, which has been associated with increased neuroprotection (Wong *et al.*, 2004; Song *et al.*, 2005; Baron *et al.*, 2008). Moreover, some inflammatory mediators like NO seem to have opposite roles in regulating neurogenesis in inflammatory conditions (Carreira *et al.*, 2010). Apparently, microglial cells and the factors they release play a dual role in neurogenesis acting as antiproliferative or proliferative agents. Indeed, self-renewal, proliferation, migration, differentiation, integration and, more importantly, survival of newborn neurons is modulated by the local microenvironment characterizing the neuroinflammatory response. Neural stem cells become "activated" following brain injury and migrate into the lesioned areas, which suggests that mediators present in the inflammatory microenvironment can guide the migration of newborn cells (Arvidsson *et al.*, 2002; Nakatomi *et al.*, 2002).

The role of neuroinflammation in regulating neurogenesis and neuroprotection is not clear yet, and is the subject of numerous studies (for comprehensive review see Whitney *et al.*, 2009; and Gonzalez-Perez *et al.*, 2010). There is, however, evidence for some of the most important mediators of the inflammatory response in their role in the regulation of neurogenesis and neuroprotection (Table 1).

Inflammatory factor	Neurogenesis	Neuroprotection	References
IFN-gamma	Pro-neurogenic	Decreased	(Ben-Hur *et al.*, 2003; Wong *et al.*, 2004; Butovsky *et al.*, 2006; Johansson *et al.*, 2008)
Interleukin-6	Anti-neurogenic	Decreased	(Ekdahl *et al.*, 2003; Liu *et al.*, 2005; Nakanishi *et al.*, 2007; Koo and Duman, 2008; Bauer, 2009; Islam *et al.*, 2009)
Interleukin-18			
Nitric oxide	Anti-neurogenic (nNOS) Pro-astrogliogenic (iNOS)	Decreased	(Contestabile *et al.*, 2003; Moreno-Lopez *et al.*, 2004; Matarredona *et al.*, 2005; Ciani *et al.*, 2006; Covacu *et al.*, 2006; Fritzen *et al.*, 2007; Luo *et al.*, 2007; Carreira *et al.*, 2010)
TNF-alpha	Anti-neurogenic (TNF-R1) Pro-neurogenic (TNF-R2)	Decreased or Increased	(Ben-Hur *et al.*, 2003; Wong *et al.*, 2004; Cacci *et al.*, 2005; Heldmann *et al.*, 2005; Liu *et al.*, 2005; Iosif *et al.*, 2006; Bernardino *et al.*, 2008)

Table 1. Effect of some inflammatory factors on neurogenesis and their neuroprotective role.

We are only beginning to understand how inflammatory factors and microglial cells influence neurogenesis in an inflammatory scenario, and the mechanisms, function and modulation of neurogenesis during inflammation require further investigation. This field of work is of particular interest for a better understanding of the mechanisms underlying the effects of neuroinflammation on neurogenesis, and further studies need to be conducted to increase the potential therapeutic value of regulating neuroinflammation in cellular regeneration in the diseased brain.

1.4 Brain repair and stem cell based therapies

Repair of damaged tissues is essential for the survival of living organisms. Each tissue or organ has an intrinsic, albeit limited ability for the replacement of dead cells, and correct integration of the newborn cells that, ideally, should restore the original structure. Cell replacement and correct integration of the newborn cells in the CNS is not so efficient as in other tissues such as skin or bone, which present a higher cell turnover. The CNS, on the other hand, has weak capabilities for both endogenous cell replacement and pattern repair. Some approaches have been used to attempt to develop therapeutic strategies for brain repair, namely transplantation of neural stem cells, stimulation of endogenous neurogenesis, neuroprotective strategies and anti-inflammatory approaches.

Transplantation of neural stem cells is one of the promising methods in study to be used in the reconstruction of neuronal circuits. However, the cells to be transplanted should be phenotypically plastic and able to proliferate *ex vivo* in response to external stimulus (Wang *et al.*, 1998; Sheen *et al.*, 1999). Intracerebral transplantation of SVZ-derived neural stem cells

has been successfully used in experimental models of Parkinson's disease (Zigova *et al.*, 1998; Richardson *et al.*, 2005), Huntington's disease (Vazey *et al.*, 2006), and in Multiple Sclerosis (Cayre *et al.*, 2006). Cell replacement could also be achieved by inducing endogenous neural stem cells to differentiate into neurons in the adult CNS, which consists in a less invasive strategy when compared to cell transplantation.

Indeed, *in situ* stimulation of endogenous adult neural stem cells and modulation of injury-induced neurogenesis is a therapeutic strategy, developed to upregulate endogenous neurogenesis, for instance through the control of the inflammatory response in a safe and efficient way. This approach seems to be a more advantageous strategy for multifocal diseases such as Alzheimer's disease, when compared to grafting strategies. Therefore, increased neurogenesis has been achieved by different strategies, such as administration of mitotic agents or trophic factors (Craig *et al.*, 1996; Kuhn *et al.*, 1997; Zigova *et al.*, 1998), treatment with neuroleptics like olanzepine (Green *et al.*, 2006), administration of NO donors or 5-phosphodiesterase inhibitors (Zhang *et al.*, 2003; Imitola *et al.*, 2004; Sun *et al.*, 2004; Sun *et al.*, 2006).

Other strategies designed to improve brain repair are being investigated, such as neuroprotective approaches consisting in the administration of radical scavengers, apoptosis inhibitors, neurotrophic agents, metal ions chelators and gene therapy, which seem to be useful to limit injury-induced lesion, but also for the enhancement of the survival of newborn cells (Polazzi and Monti, 2010). The use of anti-inflammatory drugs as a strategy to promote neurogenesis has also been explored and, although the chronic use of nonsteroidal anti-inflammatory drugs is detrimental for the gastrointestinal tract, it has also been associated with a decreased risk for neurodegenerative diseases (McGeer and McGeer, 1995; Lim *et al.*, 2000; Chen *et al.*, 2003). In fact, control of the inflammatory response seems to be an important strategy to increase proliferation of neural stem cells and/or differentiation of newborn neurons.

Strategies to promote regeneration of lesioned areas or cell replacement therapies will have to take into account the effects of inflammation on the formation and survival of newly generated neurons, either from the brain's own pool of neural stem cells, or from transplanted neural stem cells. Thus, the understanding of the mechanisms underlying the effect of neuroinflammation in proliferation, fate determination, migration and differentiation of neural stem cells is the first step in the development of specific strategies that could target the deleterious effect of inflammation in neurogenesis. Since the neuroinflammatory event is mostly characterized by the activation of resident microglial cells, the use of *in vitro* models that allow the study of the effects of microglia activation in the modulation of neural stem cells proliferation, fate determination, migration and differentiation into neurons is of high importance for the development of therapeutic strategies.

2. *In vitro* models to assess the crosstalk of neurogenesis and neuroinflammation

In vitro culture systems are critical tools for the study of various aspects related to the mechanisms that regulate biological functions. The removal of cells from their native microenvironment allows the study in a more focused way without the restrictions or

control of other cell types. When using *in vitro* systems it is essential to recognize that some of the isolated cells must be studied within a short period of time following isolation, or instead, the experimental model must reproduce the microenvironment of the CNS from where cells were isolated. These limitations can, however, be useful to investigate the factors that regulate the phenotype of isolated cells. Different *in vitro* models using neural stem cells and microglial cells may be used, to better understand how inflammation affects the formation of new neurons from neural stem cells.

2.1 Neural stem cell cultures

Reynolds and collaborators performed the first adult neural stem cell culture in the 90's (Reynolds *et al.*, 1992; Reynolds and Weiss, 1992), as free floating cell clusters, commonly referred to as neurospheres. These adult neural stem cells found *in vivo* were dissociated *in vitro* and kept their main properties: self-renewal capacity and multipotency, when in presence of mitogens such as basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF). This cell culture system is extensively used by researchers in neural stem cell biology, and models based on adherent adult neural stem cells cultured in a monolayer on matrix are also widely used (Pollard *et al.*, 2006).

The neural stem cell cultures can be obtained from different regions from the neuroaxis of the adult mammalian CNS, from the olfactory bulb to the spinal cord, and kept in uncoated dishes under serum-free conditions plus mitogens and other essential supplements (Golmohammadi *et al.*, 2008). These adult neural stem cells can be identified based on the expression of specific protein markers such as the transcription factor Sox2, nestin, musashi-1 and the EGF receptor, among others (Kaneko *et al.*, 2000; Ming and Song, 2005). After removal of mitogens these cells can give rise to three different cell types, namely neurons, astrocytes and oligodendrocytes (Levison and Goldman, 1997; Luskin *et al.*, 1997; Palmer *et al.*, 2001; Sanai *et al.*, 2004). Thus, in cultures we can find cells expressing the referred markers but also cells expressing other specific markers, such as glial fibrillary acidic protein (GFAP), polysialylated-neural cell adhesion molecule (PSA-NCAM) and beta-IIII tubulin (Suslov *et al.*, 2002; Ming and Song, 2011).

It is believed that the neurosphere culture may closer resemble the *in vivo* architecture than adherent cultures since it is believed that the stem cell niche is created by clustered cells. On the other hand, the sphere size can be a limitation of this culture in comparison to adherent neural stem cell cultures since the cells that are in the sphere core can have lower access to the nutrients and oxygen, thus undergoing cell death (Ostenfeld *et al.*, 2002; Bez *et al.*, 2003).

Adult neural stem cell culturing systems have been a relevant tool in the study of biological processes within the mammalian nervous system such as neurogenesis and their distinct phases. Cultures are good platforms for expansion of adult neural stem cells, being easily manipulated without loss of function. Additionally, they can be used as experimental models for the study of differentiation and intrinsic specification, and also for screening of drugs with the potential to enhance neurogenesis. However, further investigation should be performed for characterization of stem cells in these models, since a specific marker for neural stem cells is still lacking.

On the other hand, adult stem cell cultures have some limitations, as described next. Cells are sensitive to the culturing protocols, namely the overall number of passages, mitogen

concentration and also to the methodology adopted to dissociate spheres – mechanically or by enzymatic digestion (Caldwell, 2001; Caldwell *et al.*, 2001; Morshead *et al.*, 2002; Irvin *et al.*, 2003). The overall size of spheres has been linked to the heterogeneity of sphere composition, since it increases with sphere size, the artificiality of the cell cultures, since cells propagate without instructions of their niche, and the fact that all dividing cells propagate resulting in a mixture of different cell types, are all limitations of the neurosphere culture (Reynolds and Weiss, 1996; Suslov *et al.*, 2002; Parmar *et al.*, 2003). Moreover, the non-limited expansion of cultures could be a disadvantage once the proliferative capacity could be lost by fast dividing cells over multipotent cells or by loss of stem cell capacity over the number of passages. This situation may occur at the expense of differentiation. Moreover, long-term culturing emphasizes the tendency for neural stem cells to adopt an astrocytic phenotype, with reduced capacity to generate oligodendrocytes and neurons (Chang *et al.*, 2004; Vukicevic *et al.*, 2010). Despite these limitations, free floating neural stem cell culturing systems have several advantages and are by far the most used tool concerning the study of neural stem cell biology. The use of neural stem cell cultures allows the easy access to different stages of adult neurogenesis, including proliferation of neural stem cells or progenitors, differentiation and fate determination of progenitor cells, migration of newborn cells and cell survival. By choosing the right tools and correct techniques, these different stages can be independently studied *in vitro*.

Adult neurogenesis was initially reported *in vivo* using autoradiography to track tritiated ([^{3}H])-thymidine. [^{3}H]-thymidine is incorporated in the DNA of dividing cells, thus proving evidence for the existence of newborn cells in the hippocampus (Altman and Das, 1965) and later, in the olfactory bulb (Altman, 1969). Proliferation of neural stem cells, the first stage of neurogenesis, can be also detected *in vitro*. Different methods have been developed since, such as the evaluation of 5-bromo-2′deoxyuridine (BrdU) incorporation, a thymidine analogue that can be incorporated by S-phase cells during DNA synthesis, to detect cell proliferation instead of [^{3}H]-thymidine (Gratzner, 1982; Nowakowski *et al.*, 1989). BrdU has been the golden standard in the detection of cell proliferation for the last 20 years both *in vivo* and *in vitro*. Detection of BrdU can be easily performed with antibodies, either by immunocytochemistry, microplate assay or by flow cytometry. However, BrdU detection requires aggressive treatment for DNA denaturation, in order to allow exposure of the incorporated BrdU to antibodies. Such harsh treatment can be a major drawback in the technique, as head or acid treatment can destroy several epitopes, thus precluding multiplex labeling with other antibodies, and DNA denaturation causes the loss of binding sites for cell cycle dyes.

The use of 5-ethynyl-2′-deoxyuridine (EdU) has recently been proposed as an alternative to BrdU, since EdU detection does not require DNA denaturation, thus improving DNA structural preservation (Salic and Mitchison, 2008). EdU is also a thymidine analog that is incorporated into DNA by dividing cells during active DNA synthesis, and can be used *in vitro* as well as *in vivo* (Rostovtsev *et al.*, 2002). EdU detection is based on click chemistry, via the copper-mediated covalent coupling of the ethynyl group of EdU to a fluorescent dye-conjugated azide (Rostovtsev *et al.*, 2002). Detection can be performed by microscopy, high-throughput analysis equipment or flow cytometry. Particularly, flow cytometry is extremely useful for fast cell cycle analysis together with detection of EdU incorporation, while at same time it is possible to co-label the proliferative cells with other cell-type specific markers. The use of cell cycle markers (described next) complement detection of proliferation by ^{3}H-thymidine, BrdU or EdU, allowing for a more accurate timing of the birth of newborn cells

(Eisch and Mandyam, 2007). Other thymidine analogues that can be detected with antibodies are also available, such as iododeoxyuridine (IdU) and chlorodeoxyuridine (CldU).

Proteins related to the cell cycle have different expression patterns in the neurogenic regions accordingly to the phases of the cell cycle: retinoblastoma protein (Rb), a nuclear protein involved in the control of cell cycle progression, has a functional domain that binds to transcription factors and is expressed mostly in late G1 phase (Yoshikawa, 2000). Proliferating cell nuclear antigen (PCNA), a catalytic nuclear protein associated with DNA polymerase δ, is detected throughout all four phases of the cell cycle, however it is most abundant at late G1 and early S and scarce during G2 and M (Kawabe *et al.*, 2002). Ki-67, a nonhistone nuclear protein, is present during G1, S, G2 and M phase (Gerdes *et al.*, 1984). Cyclin-dependent kinase 1 (CDK1) or Cdc2 (the p34cdc2) is one of the mitosis-promoting factors and has an important role in the initiation of mitosis (Draetta *et al.*, 1988; Okano *et al.*, 1993).

Multi-labeling cells with specific cell markers and proliferation makers could easily identify newly generated neurons and glial cells, such as astrocytes and oligodendrocytes, which allows the distinction between these cell types. Proteins such as RNA-binding protein Hu and musashi-1 are exclusively expressed in mitotic active neural precursor cells, and they are absent in fully differentiated neuronal cells (Sakakibara *et al.*, 1996; Akamatsu *et al.*, 1999). The expression pattern of these markers can be detected by immunolabeling or quantitative real-time PCR (qRT-PCR). Mature neurons can be identified by assessing the presence of markers such as beta-III-tubulin, which contributes to microtubule stability in neuronal cell bodies and axons (Lee *et al.*, 1990; Memberg and Hall, 1995), or by evaluating the presence of neuronal nuclear antigen (NeuN) (Mullen *et al.*, 1992). Also the transcription factor NeuroD can be used since it is expressed throughout maturation until new neurons develop dendrites (Seki, 2002). Other markers that are commonly used can also be found in non-neuronal cells, namely PSA-NCAM (Seki and Arai, 1993; Kiss and Rougon, 1997); nestin, which is expressed in newly generated cells that still have the capacity to divide and differentiate into neurons or astrocytes (Reynolds and Weiss, 1992; Daniel *et al.*, 2008); Sox2, a transcription factor essential to maintain self-renewal of stem cells (Pevny and Placzek, 2005); and doublecortin (DCX) which has a transient expression in proliferating progenitor cells and newly generated neuroblasts or glial cells (Brown *et al.*, 2003; Kempermann *et al.*, 2003; Rao and Shetty, 2004). Oligodendrocytes are easily identified by imunolabeling against 2', 3'–cyclic nucleotide 3'-phosphodiesterase (CNPase), APC or O4 (Vernadakis *et al.*, 1984; Wu *et al.*, 2008; Girolamo *et al.*, 2010), while astrocytes can be identified by immunolabeling against GFAP, a specific protein for astrocytes (Bock *et al.*, 1977).

Concerning the migration of newly formed cells, it has been extensively studied *in vivo* (Kempermann *et al.*, 2003; Rao and Shetty, 2004), but also *in vitro*, by measuring DCX immunoreactivity (Francis *et al.*, 1999; Cohen *et al.*, 2008). DCX is a microtubule-associated protein having an important role in neuronal migration, by stabilizing microtubules and causing bundling (Sapir *et al.*, 2000). While immunolabeling is currently used, other assays have been developed in order to evaluate migration and simultaneously the mechanisms controlling cell migration, cell protrusion and cell polarization, such as the scratch-wound migration assay (Etienne-Manneville, 2006). Additionally, Durbec and collaborators compared three different assays to evaluate migration of neural stem cells *in vitro*: matrigel, a three-dimensional substrate mimicking the *in vivo* extracellular matrix, detection of soluble factors influencing radial migration and the chemotaxis chamber assay, where the researcher can evaluate whether the cells prefer or not a chemical factor (Durbec *et al.*, 2008).

When mature, not all neurons in culture are functional or survive. It is important to check their viability, namely identify functional synapses by morphological, electrophysiological and immunological characterization (Hartley *et al.*, 1999). Several methods have been used, including immunocytochemical assays, Western blotting and qRT-PCR which allow identification and quantification of proteins, neurotransmitters, neurotrophic factors, among others, involved in neuronal or glial neurotransmitter systems (Hartley *et al.*, 1999; Elmariah *et al.*, 2005; Goodfellow *et al.*, 2011). Using patch-clamp techniques *in vitro* the electrophysiological characterization of neural stem cell cultures can be performed by evaluating the formation of action potentials and activity patterns (Li *et al.*, 2008; Cheyne *et al.*, 2011). Also single-cell calcium currents may be evaluated to discriminate neuronal profile and viability in response to different stimuli, as reported by Bernardino and collaborators (Bernardino *et al.*, 2008).

2.2 Microglial cell cultures

Microglial cells may be obtained for culturing by several methods. One of the most used models for the study of microglial cell function consists in the isolation and expansion of microglia from the neonatal brain. However, there are several limitations and criticisms to this approach since it consists in the isolation of microglial cells from the neonatal brain, not the adult brain. One of the main problems associated with the use of microglial cells *in vitro* is related to the characterization of microglia phenotype. Since there are no truly, unique and specific microglial cell markers, microglia phenotype is defined through a combined analysis of morphology and presence or absence of certain antigens. Several works lack a proper evaluation of microglia phenotype that would allow to distinguish microglia from macrophages. In most studies, the presence of microglial cell markers is excluded from cells that are positive for astrocytic or neuronal markers, but do not distinguish between microglia or macrophages. One of the most used immunocytochemical marker of microglial cells that is the ionized calcium binding adapter molecule 1 (Iba1) (Ito *et al.*, 1998). Other markers that have been identified include the beta-integrin marker CD11b (Ling and Wong, 1993; Gonzalez-Scarano and Baltuch, 1999), the glucose transporter 5 (GLUT5) (Sasaki *et al.*, 2004), CD163 (Roberts *et al.*, 2004; Borda *et al.*, 2008), CCR2 (Albright *et al.*, 1999; Zhang *et al.*, 2007), CD34 (Asheuer *et al.*, 2004; Ladeby *et al.*, 2005) and C-type lectin CD209b (Park *et al.*, 2009). Toll-like receptor 2 (TLR2) and Toll-like receptor 4 (TLR4) have been also used as markers of microglial cells as they appear to be involved in determining the phenotype and function of microglia (Li *et al.*, 2009). A combination of several of these markers would allow for a better characterization of microglia phenotype, rather than the use of a single marker, which is the current standard. The use of multiplex detection systems would be the best approach for a full molecular characterization of microglia (Albright and Gonzalez-Scarano, 2004; Duke *et al.*, 2004; Gebicke-Haerter, 2005; Glanzer *et al.*, 2007; Moran *et al.*, 2007).

The most popular protocol to isolate microglial cells is the shaking method described by Guilian and Baker (Giulian and Baker, 1986) and Frei and colleagues (Frei *et al.*, 1986). In this method, microglial cells are separated from confluent primary mixed glial cultures, isolated from the rodent neonatal cortex, by agitation in an orbital shaker. Although this method allows the preparation of highly pure microglial cultures, the yield of this protocol is low. Saura and colleagues described a method to isolate microglial cells from primary mixed glial cultures of rodent brain by a mild trypsinization protocol, which allows the preparation of

high purity microglial cultures, with a higher yield when compared to the shaking method (Saura *et al.*, 2003). Similarly to the shaking method, several works describe the isolation of microglia from adult rodents, and the large majority of these studies take advantage from the astrocyte-microglia interaction for the success of cell cultures (Rosenstiel *et al.*, 2001; Ponomarev *et al.*, 2005). These studies showed that microglial cells, when grown on a monolayer of astrocytes, develop a highly branched morphology which seems to be associated with the downregulation of the nuclear factor kappa B (NF-kappaB) (Rosenstiel *et al.*, 2001). It has been shown that microglial cells isolated from the neonatal or adult brain are sensitive to the treatment with granulocyte macrophage colony-stimulating factor (GM-CSF), which induced a differentiation into a phenotype more similar to those of dendritic cells (Suzumura *et al.*, 1990; Aloisi, 2001). On the other hand, the isolation of adult microglial cells and subsequent culture with low concentrations of macrophage colony-stimulating factor (M-CSF) leads to increased proliferation and survival of cells that persists for several weeks (Suzumura *et al.*, 1990; Ponomarev *et al.*, 2005). M-CSF seems to be a key factor for the maintenance and survival of microglial cells *in vitro*, and has been used in several works (Wegiel *et al.*, 1998; Ponomarev *et al.*, 2005; Carreira *et al.*, 2010). Other methods are also described for the isolation of microglial cells, which include isolation from CNS tissue by Percoll gradient (Dick *et al.*, 1995; Ford *et al.*, 1995), isolation from primary cultures by nutritional deprivation (Hao *et al.*, 1991) or by collecting floating cells in mixed glial cultures (Ganter *et al.*, 1992), but the yield is generally very low.

The use of *in vitro* models allows for the understanding of many aspects of the dynamics associated with the biological functions of microglial cells in a quick and simple manner. However, one cannot overlook that the relevance of the observations obtained can only be extrapolated following *in vivo* studies. Several groups work with microglial cell lines, such as BV-2, HAPI or N9, however the use of microglial cell lines should be carefully considered since immortalization could significantly affect cell biology when compared to the use of primary microglial cultures (Corradin *et al.*, 1993; Lockhart *et al.*, 1998; Horvath *et al.*, 2008).

Concerning primary cultures of microglial cells it is always important to assess the purity of the cultures, this parameter being intrinsically linked to the method of isolation adopted. The isolation method described by Saura and collaborators is, therefore, one of the methods that seems to offer the best value yield/purity (Saura *et al.*, 2003). We favor the isolation of microglial cells by shaking from mixed glial cultures treated with low levels of M-CSF as an alternative to the method of Saura (Saura *et al.*, 2003), with a high purity of the microglia obtained (>90%) and, unlike previous methods, with a high yield (Carreira *et al.*, 2010).

When microglial cells become activated in response to immunologic stimuli or brain injury, activation is characterized by changes in microglia morphology (Streit *et al.*, 1988; Kreutzberg, 1996; Streit *et al.*, 1999; Liu and Hong, 2003), from resting ramified into activated amoeboid microglia (Kreutzberg, 1996). There is also a complex cellular response after activation of microglial cells, which is characterized by upregulation of surface molecules, such as complement receptors and major histocompatibility complex molecules (Oehmichen and Gencic, 1975; Graeber *et al.*, 1988). In addition, activated microglia release a large variety of soluble factors, with a pro- or anti- inflammatory nature and potentially cytotoxic (for review see Block and Hong, 2005). It is therefore important, when establishing primary

cultures of microglia, to assess whether microglial cells *in vitro* are also responsive to inflammatory stimuli similarly to what occurs *in vivo*. Microglial cells can be challenged with different stimuli *in vitro*, and by far the most widely used stimulus in primary cultures of microglia isolated from rodents is the bacterial endotoxin lipopolysaccharide (LPS) (Qin *et al.*, 2005a; Qin *et al.*, 2005b; Pei *et al.*, 2007). LPS mimics the infection by Gram-negative bacteria, which induces an increase in the synthesis of inflammatory mediators, namely cytokines, such as IL-1, IL-6 and tumor necrosis factor-alpha (TNF-alpha), chemokines, such as stromal derived factor-1 alpha (SDF-1alpha), free radicals and nitric oxide (Block and Hong, 2005). Other stimuli may consist in the use of ATP, interleukins, IFN-gamma or LPS plus IFN-gamma (Wollmer *et al.*, 2001; Saura *et al.*, 2003).

To characterize the activation of microglial cells after an inflammatory stimulus, we suggest to define at least three parameters to evaluate the activation of microglial cells following exposure to an inflammatory stimulus, including: change to an amoeboid morphology (Suzumura *et al.*, 1991; Wollmer *et al.*, 2001), the expression of NF-kappaB (Heyen *et al.*, 2000; Wollmer *et al.*, 2001), expression of the inducible nitric oxide synthase (iNOS) and subsequent evaluation of the production of NO (Boje and Arora, 1992; Chao *et al.*, 1992b), or the release of TNF-alpha (Sawada *et al.*, 1989; Chao *et al.*, 1992a). The various mechanisms by which microglial cells are activated and the identity of the inflammatory factors released by microglia have been studied and characterized, but there still is a great controversy whether these factors are neuroprotective or neurotoxic when released. The hypothesis that seems to be more acceptable is that, depending on the aggressiveness of the inflammatory response, the activation of microglial cells may shift from a beneficial to a harmful outcome for neurogenesis.

2.3 Combination of neural stem cells and microglial cell cultures

The study of the link between brain inflammation and neurogenesis, in particular the role of microglia in the modulation of the various steps of the neurogenic process, is of particular relevance. In order to operate at a therapeutic level there is an urgent need to understand the crosstalk between microglia and neural stem cells and the implications of the inflammatory response for the neurogenic outcome. Several studies *in vivo* have been developed in recent years, but the potential of *in vitro* studies becomes indisputable when the aim is to study the effect of a particular inflammatory factor or a very specific parameter related to the inflammatory response and its effect on neurogenesis. Whether the function of microglial cells is pro- or anti-neurogenic and whether it is possible to control microglial activation in order to reach a beneficial effect are important questions that need to be answered. Thus, the development of basic models for the *in vitro* study of these issues is an asset to the studies in this area. The use of combined primary neuronal and microglial cell cultures has been a very useful tool in studying the effect of the inflammatory response on neurons from different brain regions. In fact, there are numerous published studies where different approaches have been adopted for the study of the crosstalk between microglial cells and neurons *in vitro* (Boje and Arora, 1992; Lambertsen *et al.*, 2009). Here we describe the use of three different *in vitro* models, which address different aspects of the effects of inflammatory factors released by microglial cells in the neurogenic process.

2.3.1 Co-cultures of neural stem cells with microglia

The inflammatory response has been identified as responsible for the down-regulation of neurogenesis. This hypothesis has been supported by several studies *in vivo* (Ekdahl *et al.*, 2003; Monje *et al.*, 2003), but also by *in vitro* studies where the survival of new neurons is compromised when these are co-cultured with microglial cells activated by LPS (Monje *et al.*, 2003; Cacci *et al.*, 2005; Liu *et al.*, 2005; Cacci *et al.*, 2008). Co-cultures of neural stem cells with microglia, without physical contact between the two cell types, is an experimental model that allows the researcher to assess the role of soluble neuroinflammatory factors using co-cultures of microglial cells seeded in membrane inserts placed on top of multiwell plates containing neural stem cells. The use of techniques of immunodepletion, but also the use of genetically modified animals, allowed to correlate this anti-neurogenic inflammatory response to different interleukins produced during the activation of microglial cells, including IL-6 and IL-1beta (Vallieres *et al.*, 2002; Monje *et al.*, 2003; Nakanishi *et al.*, 2007; Goshen *et al.*, 2008; Koo and Duman, 2008; Spulber *et al.*, 2008). Other factors involved in the inflammatory response appear to contribute to the inhibition of neurogenesis. For example, the increased production of TNF-alpha by microglial cells appears to reduce the survival and differentiation of neural stem cells (Vezzani *et al.*, 2002; Monje *et al.*, 2003; Liu *et al.*, 2005; Iosif *et al.*, 2006).

Although some studies have described IFN-gamma as having a deleterious effect on neurogenesis, it has been demonstrated that microglia stimulated with low levels of IFN-gamma can support the neurogenic process, promoting neuronal differentiation *in vitro* (Butovsky *et al.*, 2006). In other studies it was observed that IFN-gamma is involved in the modulation of proliferation and differentiation of neural stem cells into neurons (Wong *et al.*, 2004; Song *et al.*, 2005; Baron *et al.*, 2008). Recent *in vitro* studies based on the establishment of co-cultures of microglia and neural stem cells, without physical contact between cells, reported that microglia might have a more complex role in neurogenesis contrarily to initial thoughts. Microglia seems to play a dual role in adult neurogenesis, being detrimental or beneficial and support the different steps in neurogenesis, such as stem cell proliferation, differentiation, migration and survival (reviewed in Ekdahl *et al.*, 2009). This dual effect becomes associated to different soluble factors produced by activated microglial cells, such as TNF-alpha or nitric oxide.

The establishment of experimental models such as co-cultures of microglia and neural stem cells allows to mimic the chemical microenvironment that surrounds the SVZ and/or the DG during inflammatory conditions when microglial cells are recruited and activated. On the other hand, the fact that both cell types share the same culture environment is important to determine the effect of factors produced by microglial cells on neural stem cells. The fact that this is a system without physical contact between the two cell types also allows determining more quickly, and using more economic approaches, the modulation of the multistep neurogenic process mediated by the inflammatory response. Thus, experimental approaches to determine cell proliferation and cell cycle, such as flow cytometry, cell migration, could be performed without the need for prior characterization to distinguish neural stem cells from microglial cells as in mixed cultures. Moreover, signaling pathways present in both cell types can be studied this way, as is the case of TLR4 that directly modulates self-renewal and the decision-cell-fate in neural stem cells (Rolls *et al.*, 2007) and in microglial cells is involved in its activation, particularly in the regulation of gene expression of iNOS (Graeber and Streit, 2010).

However, there are also some disadvantages associated with the use of this experimental methodology. Firstly, the fact that it does not allow an easy processing of microglia cells, which are placed in membrane inserts, after experimental treatment. In fact, simple experimental procedures such as protein, RNA or DNA extraction from microglial cells becomes difficult to perform. On the other hand, it is not possible to perform immunostaining techniques for subsequent microscopic analysis of microglial cells plated in inserts. In addition, this model does not answer a question that seems to be increasingly important which is the influence of cell-to-cell contact in the modulation of neurogenesis by the inflammatory response (Song *et al.*, 2002; Aarum *et al.*, 2003; Alvarez-Buylla and Lim, 2004). Despite these disadvantages, the use of co-cultures of neural stem cells with microglia, without physical contact between the two cell types, is a good approach for some studies.

2.3.2 Neural stem cell cultures exposed to microglia-conditioned medium

The production of cytokines and other molecules by activated microglial cells with implications in cellular processes has been demonstrated in many studies based on *in vitro* models (Banati *et al.*, 1993; Minghetti and Levi, 1998; Gebicke-Haerter *et al.*, 2001; Hanisch, 2002; Hausler *et al.*, 2002). However, there is still much to be learned about how cellular pathways in neural stem cells are regulated by these soluble factors from microglial origin. It is therefore important to assess how these diffusible factors influence phenomena as diverse as proliferation, differentiation, migration or cell survival.

Culturing neural stem cells with microglia conditioned medium, obtained from a separate microglia culture, allows the isolation of the unidirectional communication between activated microglia and neural stem cells, with further investigation of soluble inflammatory factors. According to studies using this experimental model, the conditioned medium of microglial cells acutely challenged with LPS reduced the survival of neural stem cells, preventing their differentiation into neurons (Monje *et al.*, 2003; Cacci *et al.*, 2008). One of the inflammatory agents reported to be responsible for this antineurogenic effect is the cytokine IL-6, as evidenced by the works of Monje and collaborators or Nakanishi and colleagues that by using a specific antibody against IL-6 rescued neurogenesis (Monje *et al.*, 2003; Nakanishi *et al.*, 2007). On the other hand, several *in vitro* studies described a pro-neurogenic effect of microglial cells and their conditioned medium, in which neural stem cells grow (Aarum *et al.*, 2003; Morgan *et al.*, 2004; Walton *et al.*, 2006; Nakanishi *et al.*, 2007).

Despite the advantages of this experimental model, namely the fact that it allows a study of the unidirectional effect of microglia on neural stem cells, there are also some disadvantages. This model does not allow inferring any conclusion about the influence of cell-to-cell contact between microglia and neural stem cells, an event that has been described to occur between glial cells and neural stem cells (Song *et al.*, 2002; Aarum *et al.*, 2003; Alvarez-Buylla and Lim, 2004). On the other hand, this model completely neglects the fact that some of the factors released by microglial cells have physical characteristics that do not allow their study in a conditioned medium transferred from a cell culture to another. Particularly nitric oxide, a gaseous molecule with a short half-life, cannot be studied because it is highly reactive in aqueous solution at 37 °C and physiological pH

(pH = 7.4). Thus, although stable end products of NO can be detected in conditioned medium from activated microglial cell cultures, the effect of NO in the neural stem cells cannot be analyzed. These are negative aspects that must be taken into account when a researcher decides to select this experimental model. Despite these aspects, the use of conditioned medium of microglia in cultures of neural stem cells is a good model to further study the influence of inflammation on neurogenesis. This model is useful to complement other *in vitro* approaches, including co-cultures of microglia and neural stem cells, with or without physical contact.

2.3.3 Mixed cultures of neural stem cells with microglia

The progression of the neurogenic process until the differentiation of neural stem cells into neurons appears to be regulated by the inflammatory microenvironment but also by cell-to-cell interactions involved (Arvidsson *et al.*, 2002; Nakatomi *et al.*, 2002; Ben-Hur *et al.*, 2003; Thored *et al.*, 2006; Thored *et al.*, 2009). Therefore, the optimization of an *in vitro* system that allows the study of physical interactions between microglia and neural stem cells is of great interest to understand how both cell types crosstalk in inflammatory conditions.

Mixed cultures are co-cultures of neural stem cells with microglia with physical contact between the two cell types. In this culture model, the role of physical contacts between microglia and neural stem cells can be studied. The mixed culture system is, probably, the *in vitro* approach that more closely mimics what happens *in vivo*, where microglial cells physically contact with the neural stem cells from neurogenic areas. Adopting this experimental model, the researcher can study the influence of the inflammatory response on the several steps of the neurogenic process, but also cell-cell interactions, which is an advantage compared to the *in vitro* models already described. An example of a mixed culture of neural stem cells cultured together with forebrain microglia is shown in Fig. 1. Enhanced green fluorescent protein (EGFP)-positive SVZ cells were isolated from the SVZ of postnatal day 1-3 actin-EGFP C57Bl6 mice, thus being readily distinguishable from microglia isolated from wild-type mice (Fig. 1A).

The mixed culture model allows simultaneous evaluation of microglia and neural stem cells. Thus, following stimulation of microglial cells, the researcher can evaluate the activation of these cells as well as several biological processes of neural stem cells, such as proliferation, differentiation and/or survival. Moreover, multi-labeling experiments of proliferation markers, such as BrdU or EdU (Fig. 1B), with microglia-specific (Iba-1 or CD11b), neuron-specific (NeuN or Tuj-1) or glia-specific (GFAP) proteins by confocal microscopy or flow cytometry are a good way to determine the phenotype of proliferating cells (Nixon and Crews, 2004). In addition, it is also possible to evaluate the effect of diffusible factors that are produced following activation of microglial cells. Separation of the two cell populations for posterior analysis (e.g. of protein or nucleic acids) is possible using a cell sorter. The researcher can confirm whether the effects observed in mixed cultures are caused by physical interactions or by diffusible factors released by microglial cells by combining such experiments with a comparative study using co-cultured cells without physical contact.

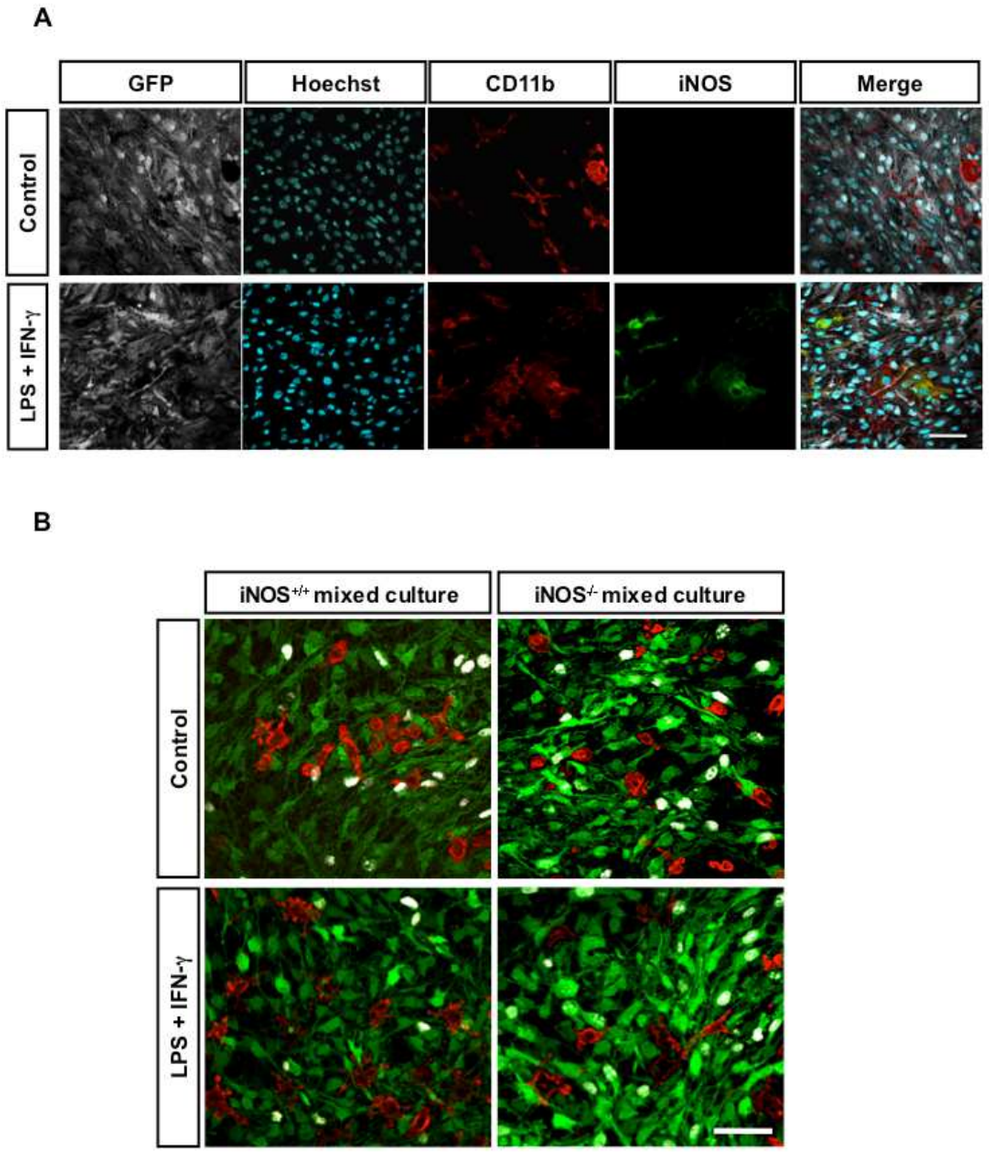

Fig. 1. Mixed cultures of primary microglial cells and subventricular zone (SVZ)-derived neural stem cells. SVZ cells (isolated from transgenic mice expressing green fluorescence protein (GFP) under the actin promoter (shown in white) are readily distinguishable from CD11b-positive microglia (red) (A). Microglia (red) cultured with GFP-positive SVZ cells (white) show immunoreactivity for inducible nitric oxide synthase (iNOS, green), following treatment with lipopolysaccharide (LPS; 100 ng/ml) plus interferon-gamma (IFN-gamma; 0.5 ng/ml), for 24 h. Nuclei are labeled with Hoechst 33342 (blue). Scale bar: 20 µm. B) Stimulation with LPS plus IFN-gamma decreases the proliferation of GFP-positive SVZ-derived neural stem cells (green), in mixed cultures of SVZ and microglia obtained from wild type mice ($iNOS^{+/+}$), which are CD11b-positive (red). Cell proliferation was assessed by 5-ethynyl-2'-deoxyuridine (EdU) incorporation (white). The antiproliferative effect of LPS plus IFN-gamma on EdU incorporation is abolished in mixed cultures in which the microglia was obtained from iNOS-knockout mice ($iNOS^{-/-}$). Scale bar: 20 µm.

3. Summary and future directions

Microglial cells may cause different effects on the neurogenic process, promoting or inhibiting it. Experimental evidence has been presented indicating that microglia, depending on their activation status and phenotype, could favor or hinder adult neurogenesis, in physiological or pathophysiological conditions. In fact, microglia can have a dual role in different steps of the neurogenic process, namely in the formation, maturation and integration of newly formed neurons. Therefore the need to explore in more detail how microglia regulate adult neurogenesis in physiological and pathophysiological conditions is of particular importance (Graeber and Streit, 2010).

Genetic mouse models in which the researcher can selectively ablate genes have already been described as useful strategies to study the involvement of particular effectors of the neuroinflammatory response on neural stem cells. Experimental models may have as an objective the determination of how modulation of microglial cell activation can be used as a therapeutic target to regulate neurogenesis in the adult brain (Ekdahl *et al.*, 2009; Whitney *et al.*, 2009; Polazzi and Monti, 2010). These models are suitable to evaluate the neurogenic potential of anti-inflammatory drugs or identify pro-neurogenic targets. Thus, these experimental approaches will allow the design of therapeutic strategies to enhance the formation, proper migration, differentiation, integration and survival of new neuronal cells in the injured nervous system. Moreover, all culture models are suitable for pharmacological or genetic manipulation, including obtaining the cells used in the cultures from wild-type or genetically modified animals, and can be adapted for high-throughput analysis and drug screening. The use of anti-inflammatory drugs with a selective mechanism of action at the level of microglial cells, or the use of anti-inflammatory drugs which may release molecules that may enhance the neurogenesis are strategies under investigation (Keeble and Moore, 2002; Napoli and Ignarro, 2003; Ajmone-Cat *et al.*, 2008; Koc and Kucukguzel, 2009). In order to develop more specific therapeutic interventions in the future, it is necessary to identify the mechanisms and factors that regulate the switch between the enhancing or detrimental effect of the inflammatory response on neurogenic events. The *in vitro* strategies discussed here are important as a first step in identifying and characterizing these events (Table 2).

Experimental model	**Parameters evaluated**			
	Diffusible/soluble factors	**Cell-to-cell interaction**	**Cellular characterization**	**Protein, RNA and DNA content**
Co-culture	Very Good	-	Very Good	Very Good
Conditioned medium	Good	-	Very Good	Very Good
Mixed culture	Very Good	Very Good	Good (requires multiplex analysis)	Good (requires cell sorting)

Table 2. Evaluation of experimental i*n vitro* models using neural stem cells and microglial cells as research tools to evaluate the effect of neuroinflammation in the neurogenesis.

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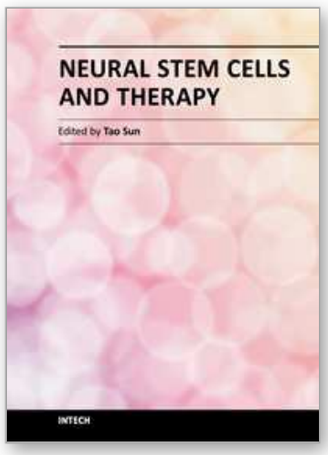

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This book is a collective work of international experts in the neural stem cell field. The book incorporates the characterization of embryonic and adult neural stem cells in both invertebrates and vertebrates. It highlights the history and the most advanced discoveries in neural stem cells, and summarizes the mechanisms of neural stem cell development. In particular, this book provides strategies and discusses the challenges of utilizing neural stem cells for therapy of neurological disorders and brain and spinal cord injuries. It is suitable for general readers, students, doctors and researchers who are interested in understanding the principles of and new discoveries in neural stem cells and therapy.

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