K_{ATP} Channel blockade instructs microglia to foster brain repair and neurogenesis after stroke

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K_{ATP} CHANNEL BLOCKADE INSTRUCTS MICROGLIA TO FOSTER BRAIN REPAIR AND NEUROGENESIS AFTER STROKE

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Chapter 6.

RESULTS BLOCK III

Microglial K_{ATP}-channel blockade instructs activation of neural precursors from the subventricular zone

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6.1 Summary

Microglia, the macrophage population of the CNS, have been recognized as a component of the neurogenic niche, releasing soluble factors that influence positively the proliferation, migration and differentiation of neural precursor cells, as well as, fostering brain repair. In this regard, controlling the microglial activation and the neuroinflammatory mediators released following damage to the brain parenchyma for instance during stroke, may offer clinical therapeutics for inflammation-related brain pathologies. As such we set out to characterize the functional properties acquired by microglia upon exposure to K_{ATP} channel blocker, glibenclamide and using the neurosphere assay, we explored the possibility that microglia have the capacity to promote neurogenesis trough the blockade of the K_{ATP}. We showed that after inhibition of neural precursors (NPs) proliferation by LPS+IFNγ, the specific blockade of the microglial K_{ATP}-channel causes a release of soluble factor that enhance the activation of NPs. Moreover, progenitor cells cultured with non-challenged microglia and treated with Gbc gave rise to a higher number of beta-III tubulin-positive cells. The characterization of releasing cytokines determined that the blockage of the K_{ATP}-channel boost the production of MCP-1. Our results revealed that the microglial K_{ATP}-channel is a regulator of the NPs proliferation and differentiation from the SVZ. This represents a potential new drug target to stimulate microglia-mediated brain damage repair and treat chronic inflammatory neurodegenerative diseases.

6.2 Introduction

In the adult brain two canonical neurogenic niches are known, the subgranular zone (SGZ) and the subventricular zone (SVZ), where new dentate granule cells and olfactory bulb interneurons are formed, respectively. Adult neurogenesis is a multistep process involving proliferation of neural precursor cells (NPs), their differentiation into lineage-restricted immature neurons, and the progressive maturation of these newborn cells into fully functional and integrated neurons. Neurons are originated from neural stem cells (NSC) throughout the entire lifespan,

and might continuously replace dying neurons or contribute to specific brain functions and plasticity (Merkle et al., 2007; Toni et al., 2007; van Praag et al., 2002).

Microglia cells have been recently recognized as a component of the neurogenic niche, releasing soluble factors that promote the proliferation, migration and differentiation of neural precursor cells (Aarum et al., 2003; Deierborg et al., 2010; Walton et al., 2006). In healthy brain microglia monitor the environment and respond to the functional state of synapses by sensing and eliminating defunct synapses (Wake et al., 2009), controlling developmental synaptogenesis (Bessis et al., 2006) and clearing newborn adult hippocampal neuroprogenitors (Sierra et al., 2010). As a hallmark, microglia are the first cells in the brain to react to acute injury and recruit astrocytes by secreting acute-phase proteins and also by releasing cytokines and reactive oxygen species (ROS) (Kettenmann et al., 2011). Unlike their role in phagocytosis, activated microglia are classified as antigen-presenting cells, as they upregulate the major histocompatiblity complex class II (MHC-II) after activation. Under certain conditions following brain inflammation or injury, reactive microglia contribute to brain repair by providing a potent neuroprotection (Carson et al., 2006) and a proneurogenic influence that supports the different steps of neurogenesis (Ekdahl et al., 2009). Indeed, microglia reaction is emerging as a dynamic and complex process characterized by unique functional patterns, depending on the nature, intensity and persistence of the activating agent and on changes in the microenvironment (Perry et al., 2010; Schwartz et al., 2006). Therefore, microglia activity is not proneurogenic or antineurogenic per se, but it likely depends on the balance of secreted molecules that define the net outcome.

Phenotypic changes of microglia reaction involve fostering of K_{ATP}-channel expression (Ortega et al., 2012a). This channel is assembled as a hetero-octameric complex (Clement et al., 1997; Mikhailov et al., 2005; Proks and Ashcroft 2009; Wheeler et al., 2008) from two structurally distinct subunits: the regulatory sulfonylurea receptor (SUR), and the pore forming inwardly rectifying K^+ channel (Kir6.x) subunit. Sulfonylureas such as glibenclamide (Gbc) close the channel by interaction with two drug-binding sites on SUR subunits (Mikhailov et al., 2001) and present neuroprotective activity after ischemia (Ortega et al., 2012a; Simard et al., 2006). SUR forms the regulatory subunit of the K_{ATP}- (Mikhailov et al., 2005) and

the NC$_{Ca-ATP}$-channels (Chen and Simard 2001). It has been proposed that the astroglial NC$_{Ca-ATP}$-channel mediates the Gbc-induced prevention of edema after cerebral ischemia (Simard et al., 2006), however SUR1 assembling with TRPM4 to form NC$_{Ca-ATP}$-channels has recently been questioned (Sala-Rabanal et al., 2012). K$_{ATP}$ channel is expressed by neurons, oligodendrocytes, capillaries (Simard et al., 2006), and microglia (Ortega et al., 2012a) under ischemic conditions. Previous studies showed that microglia K$_{ATP}$-channel is a drug target that regulate cell reactive state, controls the release of a diversity of inflammatory mediators, such as nitric oxide, interleukine-6 (IL-6) or tumor necrosis alpha (TNFα), or even modifies its phagocytic activity (Ortega et al., 2012a; Virgili et al., 2011). However, the importance of the microglial K$_{ATP}$-channel on NPs physiology is still unknown.

Regenerative processes triggered by brain insults such as traumatic injury, cerebral ischemia or epilepsy, is limited by the poor survival of newly formed neurons. This is mainly attributed to the detrimental effect of the inflammatory events sustained by microglia and infiltrating immune cells, which determine an unfavorable environment for mature and newborn neurons (Ekdahl et al., 2003; Liu et al., 2007a; Monje et al., 2003). However, early inflammatory response has beneficial effects on neurogenesis and brain repair processes, were control of the microglia neuroinflammatory activity may offer clinical therapeutics for inflammation-related brain pathologies. Several groups have established that microglia instruct neurogenesis through secreted factors, which are essential for neuroblast and neuron generation (Aarum et al., 2003; Walton et al., 2006). For all these reasons, our study focus in the mechanism whether the microglial K$_{ATP}$-channel can influence NPs activity, thus, neurogenesis *in vivo*.

In order to further characterize the influence of microglia on the NPs physiology through the K$_{ATP}$-channel, we employed the neurosphere assay. *In vitro* assays have proven invaluable to examine the effects of inflammatory factors on precursor cell proliferation, self-renewal and differentiation (Reynolds and Weiss 1992) with the neurosphere assay being the predominant method to quantify the numbers of neural precursors present in the neurogenic niches. In the present study we used the neurosphere assay to analyzed whether the blockade of the microglial K$_{ATP}$-channel blockade influences NPs activity. We also assessed the microglial-secreted molecules

that could be potentially involved in the process. We herein revealed that the microglial K_{ATP}-channel regulates NP proliferation under immunologically challenged conditions.

6.3 Material and Methods

Mice mixed glial and primary microglial cultures were activated with 0.1 mg/mL of LPS and 0.05 ng/mL of IFNγ and treated with Gbc 1pM. Conditioned media from these cultures were used to evaluate the neurosphere formation capacity.

To assess the direct effect of Gbc on NPs, we added a wide range of Gbc doses (form 10fM to 1μM) in the culture media used to perform the neurosphere assay, in presence or absence of microglial cells (eGFP-positive population was depleted by cell sorting, using eGFP–Csf1r mice, in which the microglia have been shown previously to be labeled with eGFP). We then evaluated the differentiation potential of undissociated neurospheres using the βIII-tubulin marker. We also quantified the potential neuroprotective effect of Gbc on hippocampal neuronal cultures, through the expression of the βIII-tubulin marker.

To investigate whether microglial K_{ATP} could be implicated in the modulation of the SVZ precursor activity, we added Gbc to neurosphere cultures containing LPS+IFNγ in presence or absence of microglia. Microglial depletion was allowed after collection of the eGFP-positive population from the SVZ by cell sorting. On the other hand, we collected the eGFP-positive from the cortex and we plated them on top of the neurosphere culture, as a result we obtained an enriched microglial. We also confirmed the activation of these eGFP-positive cells caused by LPS+IFNγ exposition, and MHCII expression was measured by flow cytometry.

To know whether microglia can modulate NPs proliferation, we used conditioned media from isolated eGFP-positive population from the SVZ collected by cell sorting. We cultured these cells for 24 h in presence of LPS+IFNγ, Gbc and/or diazoxide (Dz), and neurosphere assay was done using microglial-conditioned media.

Inflammatory cytokines production, such as IL-6, IL-10, IL-12p70, IFNγ, TNFα and CCL2/MCP-1, were evaluated performing a cytometric bead array using the microglial culture supernatants.

6.4 Results

6.4.1 Microglia provides better outcome when stimulated with LPS+IFNγ

To establish that the blockade of the microglial K_{ATP} could modify NPs proliferation, neurosphere cultures were performed using conditioned media from primary mixed glial and pure microglia cultures. In control conditions, cells present highly ramified morphology and thin processes, however, after activation with LPS+IFNγ astrocytes turns into activated morphology, with bigger processes and cytoplasm, whereas microglia adopt the classical amoeboid shape (**Fig. 6.1A**). Gbc did not cause morphology change in any case. To assess the purity microglial cultures, we carried out double-immunofluorescence against Iba1 and GFAP (>98% were Iba1-positive cells). NPs proliferation derived from mixed glial cultures supernatant that underwent activation with LPS+IFNγ was 75% reduced and Gbc did not modify this effect (*P*<0.05 and *P*<0.001 respectively when compared to pure-microglia (**Fig. 6.1B**). Interestingly, when the astrocytes were removed from the cultures, the inhibition of neurosphere formation capacity was less severe (45%; P<0.001 compared to control) and activation of NPs was restored to basal levels when cultured with pure microglial-conditioned media in presence of Gbc.

We cannot assume that the inhibition of the NPs proliferation from the mixed glial cultures is only due to the presence of astrocytes. Is worthwhile to remark that in mixed glial cultures more proliferative microglia are present, moreover astrocytes are strengthening microglial activation in a positive feedback manner (Saura et al. 2003a). Therefore, its possible that the inhibition of the NPs observed in mixed glial cultures could be attributed to an excess of proinflammatory signals released by microglia, although if this were the case, a Gbc-derived response would be also expected.
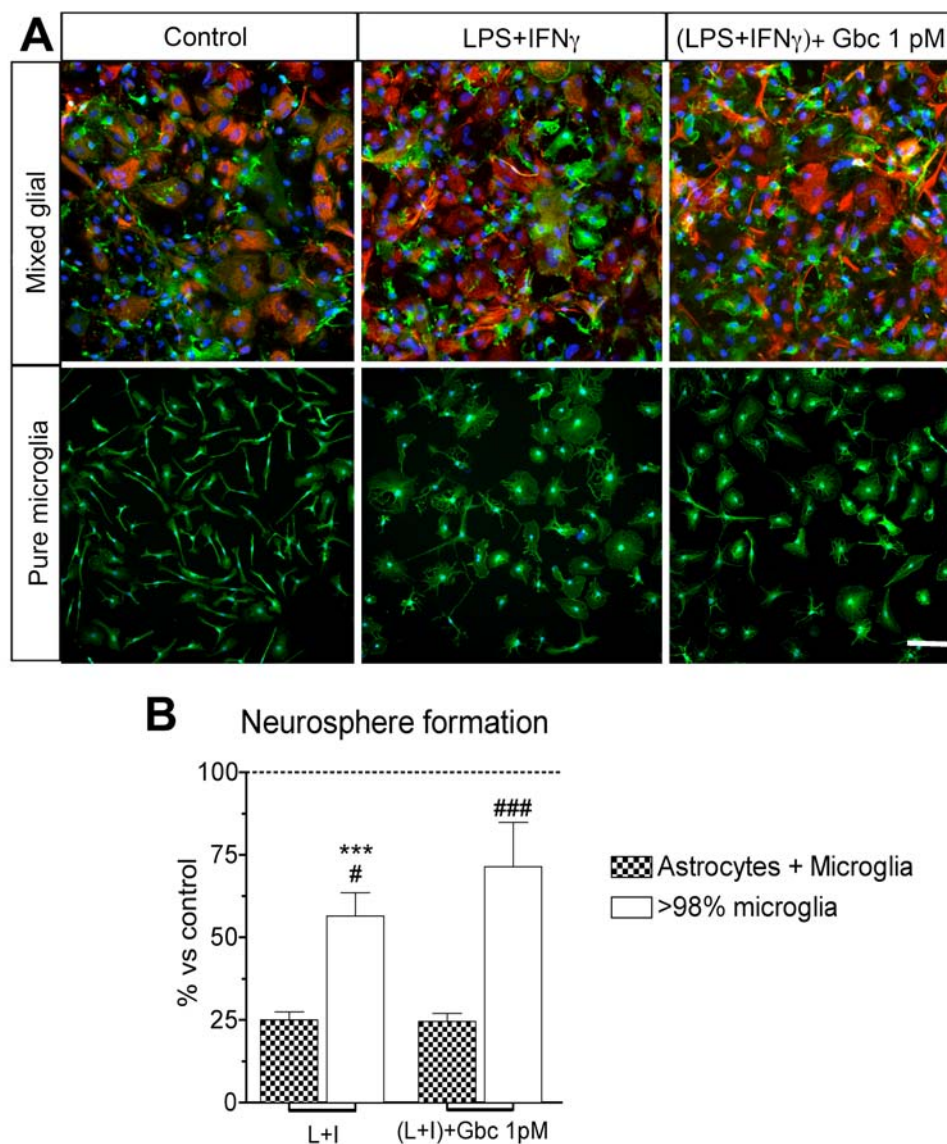
Fig. 6.1. Absence of astrocytes increases neural precursor proliferation. (**A**) Photomicrographs of mixed glial or pure microglial culture after 24 h of LPS+IFNγ (L+I) and Gbc (Gbc) 1pM treatment. Microglial cells were immunostained for Iba1 (green), astrocytes for GFAP (red), and stained for DAPI (blue). (**B**) Neurosphere formation using glial cultures conditioned media, where in absence of astrocytes, neuronal precursors showed better outcome and responded to K_{ATP} blockade by Gbc. Statistics: ***$P<0.001$ vs control and #$P<0.05$ and ###$P<0.001$ vs mixed glial cultures. Scale bar 50μm.

6.4.2 Microglia appears to influence differentiation

Yasuda et al (2008) have established that Kir6.x is not present in adult neural progenitor cells, and no K_{ATP} channel expression is expected, we want to assess if Gbc has a direct effect on NPs. To address this question, we used the neurosphere assay with a wide range of Gbc doses (form 10fM to 1μM) added directly on the culture media at plating. After 7 DIV for SVZ culture and 14 DIV on hippocampal culture, Gbc could not modify neurosphere number or neurosphere size (**Fig. 6.2**).
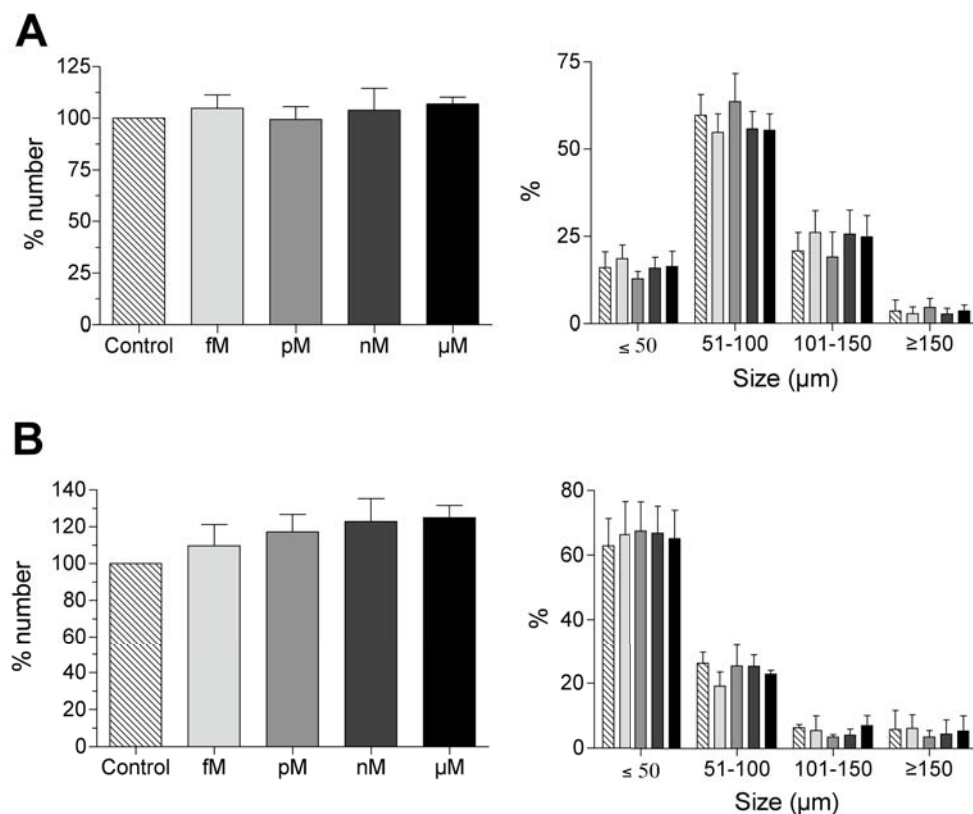
Fig. 6.2. Expanded cultures maintain NP-forming cells. (**A**) SVZ or (**B**) Hippocampal dissociates contain similar numbers of NPs cells that in presence of Gbc do not differ in number (left column), or size (right column).

We then performed the differentiation assay with the neurospheres from the SVZ in the absence of growth factors but in presence of fresh Gbc in the media. Those NPs plated out from the Gbc-treated group increased the number of beta-III tubulin positive cells from <25 to 25-100 cells/sphere (**Fig. 6.3A)**. To determine whether microglial cells directed this effect, we performed the same approach but we removed all eGFP-positive microglia from the culture. In these conditions, cultures exposed to Gbc did not modify NPs differentiation profile, suggesting a role of microglia cells in the observed effect (**Fig. 6.3B)**.

As Gbc can be neuroprotective after brain injury (Ortega et al., 2012a), we studied whether Gbc was directly protecting NPs from death programs rather than stimulating their differentiation to neural lineage. We then cultured embryonic hippocampal neurons in presence of increasing doses of Gbc, its noteworthy that due to the technique manipulation a percentage of the neurons plated are determined to die. We therefore quantified the total number of neurons expressing

beta-III tubulin after 7 DIV, and found no Gbc effect in cell numbers, which indicates that Gbc does not directly modify neuronal survival (**Fig. 6.3C**).
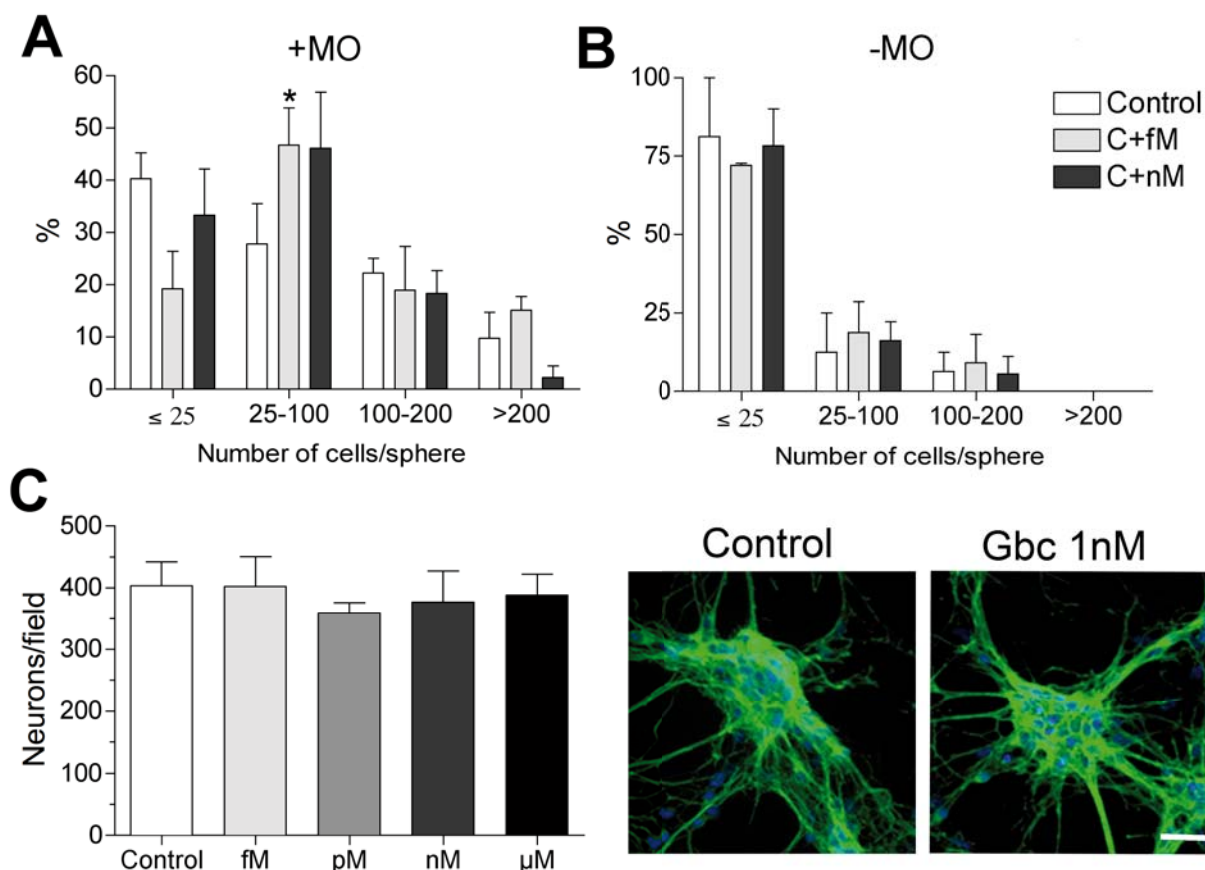
Fig. 6.3. K_{ATP} channel blockade appears to shift the differentiation profile to a neuronal lineage. Bar graphs showing quantification differentiating SVZ dissociates for beta-III tubulin in (**A**) presence (+MO) or (**B**) absence (-MO) of microglial cells. Following differentiation, mature cell types emerge in neurospheres, where Gbc (Gbc) increased the number of beta-III tubulin/sphere only in presence of microglial cells. (**C**) Survival of hippocampal neurons did not differ in presence of Gbc. Statistics: *$P<0.05$ vs ≤25 cells/sphere. Scale bar 100µm.

6.4.3 Role of microglial K_{ATP} channel in activation of SVZ precursors

Given that it has been shown previously that IFNγ induced inhibition of the precursor proliferation and that microglia modulate this inhibitory effect (Li et al. 2010), and that the exposition of microglial cells to LPS+IFNγ trigger an overexpression of the microglial K_{ATP} channel (Ortega et al., 2012a), we wanted to investigate whether microglial K_{ATP} could be implicated in the modulation of the SVZ precursor activity. To do that we added Gbc to neurosphere cultures containing

LPS+IFNγ in presence or absence of microglia. Microglial depletion was allowed after collection of the eGFP-positive population from the SVZ by cell sorting using eGFP–Csf1r mice, in which microglia is labeled with eGFP (Sasmono et al., 2003).

To assess whether LPS+IFNγ activates microglia in the adult SVZ after 24 hours, we measured the MHC-II expression, a canonical reactive phenotype marker, on microglia isolated from the SVZ of Csf1r-GFP mice (**Fig. 6.4A)**. We observed that 21.4% of the cells expressing high levels of eGFP where MHC-II positive in control cultures, whereas, this value increased to 44.8% 24 h after LPS+IFNγ treatment.

To evaluate the influence of microglia activation in the neurosphere formation we exposed SVZ cultures, where 7-10% of the cells are eGFP-positive (+MO), to LPS+IFNγ stimulation. The results revealed that LPS+IFNγ induced a 35% of decrease in neurosphere formation compared with that observed in control cultures. We then removed the eGFP-positive population by cell sorting and assessed the neurosphere formation of the remaining eGFP-negative population (-MO) in presence of LPS+IFNγ. In these conditions, the neurosphere formation presented a higher reduction (49%). Then, using the same approach, we collected the eGFP-positive from the cortex and we plated them on top of the SVZ neurosphere culture. The resulted enriched cortical microglial culture induced a much greater decrease on neurosphere formation than that observed in presence or absence of SVZ microglia (77% vs 35% or 49%, *P*<0.05 or *P*<0.001 respectively) (**Fig. 6.4B**).

We then determined whether the microglial K_{ATP}-channel is implicated in SVZ neurogenesis. Using the same approach described above, we activated the cultures with LPS+IFNγ and exposed them to 1pM and 1nM Gbc and we did not observe Gbc effect for +MO nor –MO cultures. However, when added to the enriched cortical microglia cultures, Gbc showed a dose dependent recovery of neurosphere formation capacity, reaching values equivalent to +MO (**Fig. 6.4B**). This result indicates that an exacerbated microglial activity could be detrimental for NPs proliferation. Moreover control of microglia activity through K_{ATP}-channel blockade would partially restore the proliferation capacity of NPs, which is critical for brain repair.
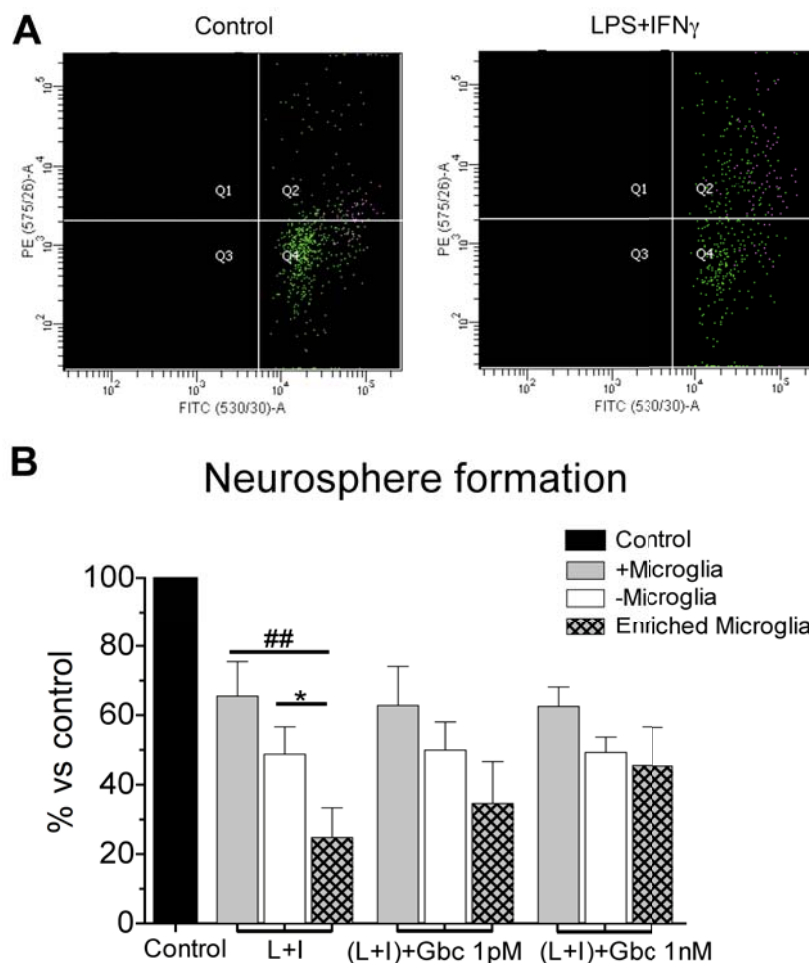
Fig. 6.4. Loss of inducible neurogenesis is reversed by microglial K_{ATP} channel blockade in proliferating cultures. (**A**) Flowcytometric analysis of MHCII expression from rapidly isolated microglia. Microglial cells were identified based on eGFP-labeling (FITC axis) in control cultures or after 24 h of LPS+IFNγ exposition, in which MHCII expression increased (PE axis). (**B**) Neurosphere formation after 7 DIV. Cultures were performed in presence or absence of microglia, where the eGFP-positive population was removed by cell sorting and the remaining eGFP-negative population was harvested. We also collected the eGFP-positive population from the cortex and we plated them on top of the neurosphere culture, as a result we obtained an enriched microglial culture. All these cultures were exposed to LPS+IFNγ (L+I) and glibenclamide (Gbc). Note that in presence of microglia the inhibition of precursors proliferation is reduced in presence of LPS+IFNγ and that Gbc treatment is able to revert that effect in enriched microglial culture. Statistics: *$P<0.05$ and ##$P<0.01$.

6.4.4 Microglia K_{ATP}-channel potentiates SVZ neurogenesis through a soluble factor

Based on the previous results we aimed to know whether microglia could modulate NPs proliferation through a cell-cell contact or through a released soluble factor. We collected and cultured the eGFP-positive population into tubes **(Fig. 6.5A)**, to then challenge the cells with LPS+IFNγ and treat them with Gbc. To determine the reversibility of the process we used diazoxide, a K_{ATP}-channel opener. After 24h incubation we counted the number of cells in the tube and collected the

conditioned media to perform a neurosphere assay. The number of the remaining eGFP-positive microglia in the tubes exposed to LPS+IFNγ were significantly reduced (10-40%) compared to controls. However, Gbc 1pM treatment promoted cytoprotection to LPS+IFNγ exposition (*P*<0.05) (**Fig. 6.5B**).

When we harvested the neurosphere cultures with microglial-conditioned media, the microglial K$_{ATP}$-channel blockade by Gbc stimulated neurosphere formation (*P*<0.01), and this effect was blocked with diazoxide (**Fig. 6.5C**). However, Gbc did not modify the neurosphere size, since we only observed an overall size reduction due to LPS+IFNγ (*P*<0.05) (**Fig. 6.5D**).
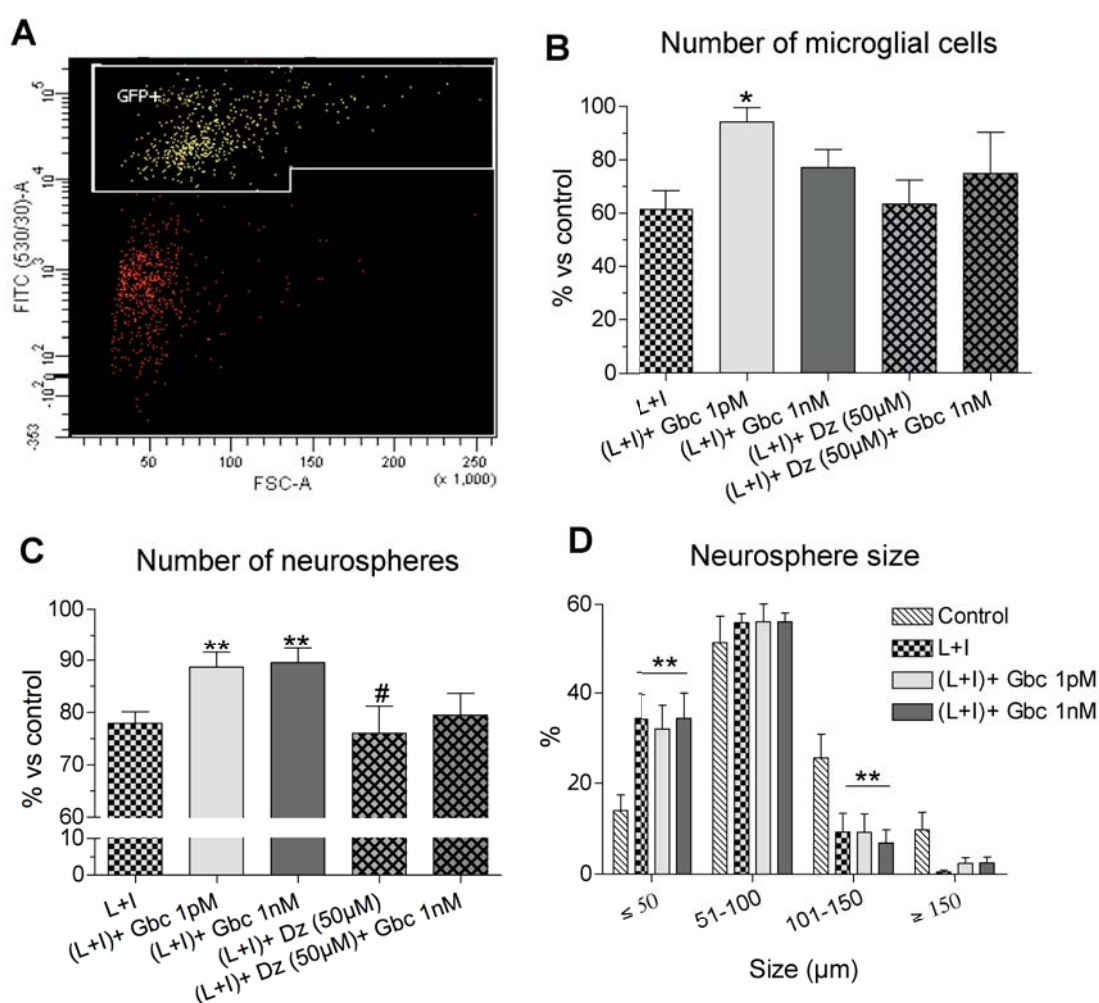
Fig. 6.5. Microglial cells modulate inducible neurogenesis through a soluble factor(s). (**A**) eGFP-positive population (FITC axis) was collected by cell sorting and cultured 24 h in presence of LPS+IFNγ (L+I), Gbc (Gbc) and/or Diazoxide (Dz). (**B**) Microglial cells counting 24 h after treatments. (**C**) Neurosphere formation with eGFP-positive conditioned culture media 24 h after treatments. Please note that conditioned media from eGFP-positive cells in presence of Gbc is able to reverse the loss of inducible neurogenesis caused by L+I, and that treatment with Dz blocked the Gbc effect. (**D**) Neurosphere size measurement. Note that there is only an overall size reduction caused by the exposition to LPS+IFNγ. Statistics: *P<0.05 and **P<0.01 vs (L+I), #P<0.05 vs (L+I)+ Gbc 1nM.

6.4.5 Microglial released factors are modified by the K_{ATP} channel

Inflammatory cytokines, such as IFNγ, TNFα and IL-6, are known to be potent immunomodulators (Muñoz-Fernández and Fresno 1998). It has been established that proinflammatory cytokines are detrimental for neurogenesis, whereas, antiinflammatory cytokines or chemokines are beneficial (Das and Basu 2008; Das et al., 2011; Ekdahl et al., 2009; Whitney et al., 2009). We therefore tested whether K_{ATP} channel blockade modify microglia inflammatory activity. To do that, we collected supernatants from the pure microglia cultures stimulated with LPS+IFNγ and treated with Gbc (**Fig. 6.1A**), then we perform a cytometric bead array. As expected we observed that activation of microglia increased the concentration of TNFα, IL-6 and MCP-1 ($P<0.001$) (**Fig. 6.6**). Incubation of cell cultures with diazoxide, a specific K_{ATP}-channel opener with anti-inflammatory activity (Virgili et al., 2011), decreased the production of TNFα, IL6 and MCP-1 ($P<0.01$) (**Fig. 6.6**). Interestingly, incubation with Gbc, a specific K_{ATP}-channel blocker with neuroprotective actions (Ortega et al., 2012a), boosted the microglial production of the MCP-1 ($P <0.05$).
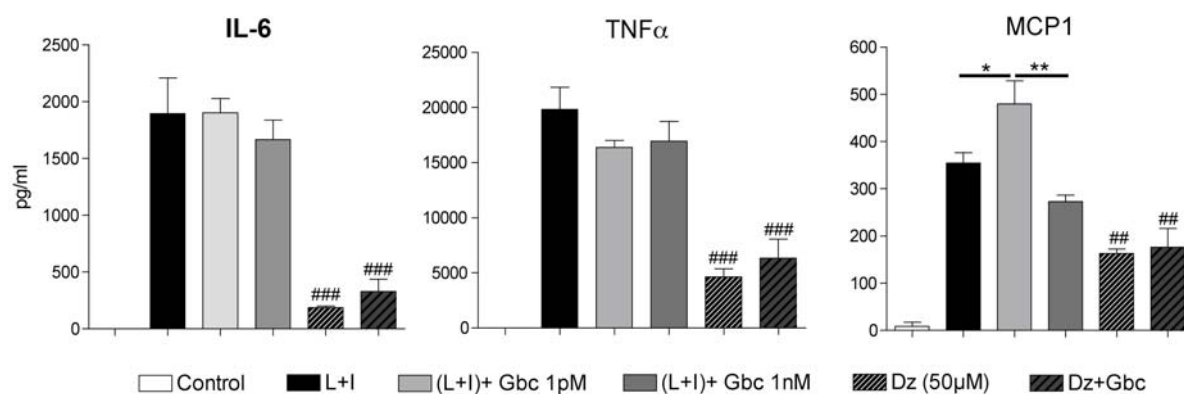
Fig. 6.6. Microglial K_{ATP} channel blockade increased the production of the MCP-1. Analysis of released microglial factor levels of some of the cytokines reported to be involved in the inflammatory pathway. Please note that there is an overall overproduction of inflammatory mediators caused by the exposure to LPS+IFNγ. The addition of diazoxide (Dz) reversed that effect and Gbc 1pM boosted the production of the MCP-1. Statistics: *$P<0.05$ and **$P<0.01$ or ##$P<0.01$ and ###$P<0.001$ vs (L+I).

6.5 Discussion

Recent evidence points for an immunological control of neural stem cells physiology, thus the present study explored the possibility that microglia has the capacity to promote precursor activation through the blockade of the K_{ATP} channel.

We here showed that after inhibition of NPs proliferation with the addition of LPS+IFNγ, the specific blockade of the microglial K_{ATP} channel cause a release of a soluble factor that enhance the activation of these NPs. Moreover, progenitor cells cultured with non-challenged microglia and treated with Gbc gave rise to a higher number of beta-III tubulin-positive cells, however did not influence precursor activation. Among the panel of the microglia releasing cytokines characterized, Gbc treatment only boosted the production of MCP-1. In this regard, it has been shown that activated microglia has a role in the attraction, mediated by inflammatory-associated releasing factors where MCP-1 has a critical importance (Tran et al. 2006).

Reactive microglia present different phenotypes in the acute and chronic phases of CNS injury and during neurodegeneration (Kettenmann et al., 2011). These different phenotypes may have different repercussions on NPs properties and on neurogenesis processes. In our experimental conditions, after challenging neurospheres with LPS+IFNγ during 7 DIV, we found an inhibition of neuron differentiation with only a few scattered beta-III tubulin-positive cells in some neurospheres. This data correlates with previous studies where precursor cells were found to migrate preferentially to sites of chronic inflammation in animal models of multiple sclerosis, and that these new cells preferentially differentiate into oligodendrocytes (Ben-Hur et al., 2003; Picard-Riera et al., 2002; Pluchino et al., 2003). On the contrary, experimental models of acute damage leading to massive neuronal loss found that both transplanted and endogenous precursor cells migrate towards the damaged area and differentiate into neurons (Aboody et al., 2000; Arvidsson et al., 2002; Nakatomi et al., 2002; Thored et al., 2006). Microglia activation mediates inflammation at the site of injury and is firmly established in these lesions (Aloisi 2001; Graeber and Streit 2010). Our data suggest that acutely activated microglia are detrimental for cell survival and neurogenesis as also described elsewhere (Butovsky et al., 2006; Cacci et al., 2008; Liu et al., 2005; Monje et al., 2003). However, we found that specific blockade of the microglial K_{ATP} channel endows microglia to a clearly distinct phenotype that, similarly to non-reactive microglia, does not impair cell survival and allows activation of NPs **(Fig. 6.5C)**.

In a model of irradiation injury, Hellstrom and colleagues found that the SVZ was more resistant to injury than the hippocampus and that microglia from the

irradiated hippocampus had a gene expression profile that was less indicative of factors that would promote stem cell recovery and maintenance than in the SVZ, while the precursor cells themselves showed no difference in the two regions (Hellström et al. 2011). This result is a first indication how different microglial properties might determine differential plastic responses in the two neurogenic regions. In our case we established that only SVZ-derived microglia was able to respond to Gbc, changing the fate of the undifferentiated progenitors.

The pro- or anti-neurogenic niche may depend on the degree of microglia activation and the balance between the pro- and anti-inflammatory cytokines produced (Battista et al., 2006). Interestingly, microglial K_{ATP} channel appears to be target to regulate cell reactive state, controlling the release of a diversity of inflammatory mediators, such as nitric oxide, IL-6 or TNFα, or even modifying microglia phagocytic activity (Ortega et al., 2012a; Virgili et al., 2011). Although diazoxide is a potent anti-inflammatory agent decreasing the concentration of TNFα and IL-6, and these mediators are known to be potent anti-neurgenic factors (Cacci et al., 2008; Ekdahl et al., 2009), in our study microglial-conditioned supernatant treated with diazoxide did not foster NPs activation. Nonetheless, we herein found microglial K_{ATP} channel blockade with Gbc turns microglia on a distinct phenotype, more likely to be beneficial as the chronically activated, rather than the classical acute pro-inflammatory phenotype that is detrimental for NPs proliferation. Thus, the microglial K_{ATP} blockade with Gbc enhances NPs activation and differentiation through the release of pro-neurogenic factors. This raises the possibility that in a chronically altered environment, persistently activated microglia may display protective functions that favor rather than attenuate brain repair processes, as found with Gbc treatment. In line with this, other studies indicate that acutely activated microglia induce an inflammatory response detrimental for neurogenesis, whereas chronically activated microglia is permissive to neuronal differentiation and cell survival (Cacci et al., 2008; Ekdahl et al., 2009).

Several recent studies have shown that microglia is able to release soluble factors influencing the proliferation, migration and differentiation of NPs (Aarum et al., 2003; Deierborg et al., 2010; Walton et al., 2006). In healthy brain microglia shape hippocampal SGZ neurogenesis through an apoptotic-phagocytic mechanism (Sierra

et al., 2010), whereas in pathogenic brain, they produce trophic factors with a positive effect on neurogenesis *in vivo* (Butovsky et al., 2006); and also on oligodendrocytic differentiation (Nicholas et al., 2001) and astrogliogenesis (Zhu et al., 2008). Several mitogens are also known to positively regulate the proliferation of NPs in the SVZ, for example erythropoietin (Wang et al., 2004), insulin growth factor I (Yan et al., 2006), vascular endothelial growth factor (Jin et al., 2002) and endocannabinoids (Aguado et al., 2005). These results are in line with a recent *in vivo* study describing whether minocycline treatment suppresses the microglial response in stroke-injured rats and leads to a reduction in cell proliferation in the SVZ (Kim et al., 2009). Conversely, we herein found that Gbc conditioned medium from Gbc-treated microglia activated with LPS+IFNγ is able to stimulate neurogenesis. This evidences that reactive microglia can exert a stimulatory effect in enhancing neurogenesis, where cell contact is not required, with an important role of K_{ATP} channels in the control of this microglial activity.

Direction of microglial functions towards the proneurogenic chronic phenotype could represent a new strategy to enforce endogenous brain regenerative processes. The characterization of releasing cytokines determined that the blockage of the K_{ATP} channel increased the secretion of MCP-1, which does not directly activate an inflammatory response in microglia or cause neuronal damage (Hinojosa et al., 2011). Numerous studies demonstrate that neuroinflammation processes involve upregulation of MCP-1 in activated microglia and astrocytes (Mahad and Ransohoff 2003) and with important roles of this chemokine in neurogenesis and neuroprotection. NPs express CCR5 and CCR2 receptors of MCP-1 (Ji et al., 2004), which induces NPs migration towards the lesion (Liu et al., 2007b; Widera et al., 2004). In addition, MCP-1 protects neurons against inflammatory damage caused by NMDA-mediated (Eugenin et al., 2003). Our results suggest that microglial K_{ATP} channel blockade after brain injury increase MCP-1 secretion, which may enhance the migration of precursors to the site of injury, and stimulate lesion repair and neuron protection.

To sum up, our findings provide evidence that soluble factors released by microglia enhance NPs proliferation, and differentiation to neurons, and points out to the K_{ATP} channel as a player in the control of the microglial release of MCP-1.

Thus it may present a potential new drug target stimulate brain damage repair to treat chronic inflammatory neurodegenerative diseases. Further *in vivo* experiments are needed to validate the role of microglial K_{ATP} channel in MCP-1 secretion and neurogenesis processes during CNS neurodegeneration.

6.6 Sources of funding

6.7 Disclosures

The authors report no disclosures.

6.8 References

Aarum J, Sandberg K, Haeberlein SL, Persson MA. 2003. Migration and differentiation of neural precursor cells can be directed by microglia. Proc Natl Acad Sci USA 100(26):15983-15988.

Aboody KS, Brown A, Rainov NG, Bower KA, Liu S, Yang W, Small JE, Herrlinger U, Ourednik V, Black PM and others. 2000. Neural stem cells display extensive tropism for pathology in adult brain: evidence from intracranial gliomas. Proc Natl Acad Sci USA 97(23):12846-51.

Aguado T, Monory K, Palazuelos J, Stella N, Cravatt B, Lutz B, Marsicano G, Kokaia Z, Guzman M, Galve-Roperh I. 2005. The endocannabinoid system drives neural progenitor proliferation. FASEB Journal 19(12):1704-1706.

Aloisi F. 2001. Immune function of microglia. Glia 36(2):165-179.

Arvidsson A, Collin T, Kirik D, Kokaia Z, Lindvall O. 2002. Neuronal replacement from endogenous precursors in the adult brain after stroke. Nature Medicine 8(9):963-970.

Battista D, Ferrari CC, Gage FH, Pitossi FJ. 2006. Neurogenic niche modulation by activated microglia: transforming growth factor beta increases neurogenesis in the adult dentate gyrus. European Journal of Neuroscience 23(1):83-93.

Ben-Hur T, Ben-Menachem O, Furer V, Einstein O, Mizrachi-Kol R, Grigoriadis N. 2003. Effects of proinflammatory cytokines on the growth, fate, and motility of multipotential neural precursor cells. Mol Cell Neurosci 24(3):623-31.

Bessis A, Béchade C, Bernard D, Roumier A. 2006. Microglial control of neuronal death and synaptic properties. Glia 55(3):233-238.

Butovsky O, Ziv Y, Schwartz A, Landa G, Talpalar AE, Pluchino S, Martino G, Schwartz M. 2006. Microglia activated by IL-4 or IFN-gamma differentially induce neurogenesis and oligodendrogenesis from adult stem/progenitor cells. MolCell Neurosci 31(1):149-160.

Cacci E, Ajmone-Cat MA, Anelli T, Biagioni S, Minghetti L. 2008. In vitro neuronal and glial differentiation from embryonic or adult neural precursor cells are differently affected by chronic or acute activation of microglia. Glia 56(4):412-425.

Carson MJ, Doose JM, Melchior B, Schmid CD, Ploix CC. 2006. CNS immune privilege: hiding in plain sight. Immunol Rev 213:48-65.

Chen M, Simard JM. 2001. Cell swelling and a nonselective cation channel regulated by internal Ca2+ and ATP in native reactive astrocytes from adult rat brain. J Neurosci. p 6512-21.

Clement JP, Kunjilwar K, Gonzalez G, Schwanstecher M, Panten U, Aguilar-Bryan L, Bryan J. 1997. Association and stoichiometry of K(ATP) channel subunits. Neuron 18(5):827-38.

Das S, Basu A. 2008. Inflammation: a new candidate in modulating adult neurogenesis. J Neurosci Res. p 1199-208.

Das S, Dutta K, Kumawat KL, Ghoshal A, Adhya D, Basu A. 2011. Abrogated Inflammatory Response Promotes Neurogenesis in a Murine Model of Japanese Encephalitis. PLoS ONE 6(3):e17225.

Deierborg T, Roybon L, Inacio AR, Pesic J, Brundin P. 2010. Brain injury activates microglia that induce neural stem cell proliferation ex vivo and promote

differentiation of neurosphere-derived cells into neurons and oligodendrocytes. Neuroscience 171(4):1386-1396.

Ekdahl CT, Claasen J-H, Bonde S, Kokaia Z, Lindvall O. 2003. Inflammation is detrimental for neurogenesis in adult brain. Proc Natl Acad Sci USA 100(23):13632-7.

Ekdahl CT, Kokaia Z, Lindvall O. 2009. Brain inflammation and adult neurogenesis: the dual role of microglia. Neuroscience 158(3):1021-1029.

Eugenin EA, D'Aversa TG, Lopez L, Calderon TM, Berman JW. 2003. MCP-1 (CCL2) protects human neurons and astrocytes from NMDA or HIV-tat-induced apoptosis. J Neurochem 85(5):1299-311.

Graeber MB, Streit WJ. 2010. Microglia: biology and pathology. Acta Neuropathol 119(1):89-105.

Hellström NAK, Lindberg OR, Ståhlberg A, Swanpalmer J, Pekny M, Blomgren K, Kuhn HG. 2011. Unique gene expression patterns indicate microglial contribution to neural stem cell recovery following irradiation. Mol Cell Neurosci 46(4):710-719.

Hinojosa AE, Garcia-Bueno B, Leza JC, Madrigal JLM. 2011. CCL2/MCP-1 modulation of microglial activation and proliferation. J Neuroinflammation 8:77.

Ji JF, He BP, Dheen ST, Tay SSW. 2004. Expression of chemokine receptors CXCR4, CCR2, CCR5 and CX3CR1 in neural progenitor cells isolated from the subventricular zone of the adult rat brain. Neurosci Lett 355(3):236-40.

Jin K, Zhu Y, Sun Y, Mao XO, Xie L, Greenberg DA. 2002. Vascular endothelial growth factor (VEGF) stimulates neurogenesis in vitro and in vivo. ProcNatlAcadSciUSA 99(18):11946-11950.

Kettenmann H, Hanisch U-K, Noda M, Verkhratsky A. 2011. Physiology of Microglia. Physiol Rev 91(2):461-553.

Kim BJ, Kim M-J, Park J-M, Lee S-H, Kim Y-J, Ryu S, Kim YH, Yoon B-W. 2009. Reduced neurogenesis after suppressed inflammation by minocycline in transient cerebral ischemia in rat. J Neurol Sci 279(1-2):70-5.

Li L, Walker TL, Zhang Y, Mackay EW, Bartlett PF. 2010. Endogenous interferon gamma directly regulates neural precursors in the non-inflammatory brain. J Neurosci 30(27):9038-50.

Liu X, Wang Q, Haydar TF, Bordey A. 2005. Nonsynaptic GABA signaling in postnatal subventricular zone controls proliferation of GFAP-expressing progenitors. Nature Neuroscience 8(9):1179-1187.

Liu XS, Zhang ZG, Zhang RL, Gregg SR, Meng H, Chopp M. 2007a. Comparison of in vivo and in vitro gene expression profiles in subventricular zone neural progenitor cells from the adult mouse after middle cerebral artery occlusion. Neuroscience 146(3):1053-61.

Liu XS, Zhang ZG, Zhang RL, Gregg SR, Wang L, Yier T, Chopp M. 2007b. Chemokine ligand 2 (CCL2) induces migration and differentiation of subventricular zone cells after stroke. J Neurosci Res 85(10):2120-5.

Mahad DJ, Ransohoff RM. 2003. The role of MCP-1 (CCL2) and CCR2 in multiple sclerosis and experimental autoimmune encephalomyelitis (EAE). Semin Immunol 15(1):23-32.

Merkle FT, Mirzadeh Z, Alvarez-Buylla A. 2007. Mosaic organization of neural stem cells in the adult brain. Science 317(5836):381-384.

Mikhailov MV, Campbell JD, De Wet H, Shimomura K, Zadek B, Collins RF, Sansom MSP, Ford RC, Ashcroft FM. 2005. 3-D structural and functional characterization of the purified K_{ATP} channel complex Kir6.2-SUR1. EMBO J 24(23):4166-75.

Mikhailov MV, Mikhailova EA, Ashcroft SJ. 2001. Molecular structure of the glibenclamide binding site of the β-cell K_{ATP} channel. FEBS Lett 499(1-2):154-60.

Monje ML, Toda H, Palmer TD. 2003. Inflammatory blockade restores adult hippocampal neurogenesis. Science 302:1760-1765.

Muñoz-Fernández MA, Fresno M. 1998. The role of tumour necrosis factor, interleukin 6, interferon-gamma and inducible nitric oxide synthase in the development and pathology of the nervous system. Prog Neurobiol 56(3):307-40.

Nakatomi H, Kuriu T, Okabe S, Yamamoto S, Hatano O, Kawahara N, Tamura A, Kirino T, Nakafuku M. 2002. Regeneration of hippocampal pyramidal neurons after ischemic brain injury by recruitment of endogenous neural progenitors. Cell 110(4):429-441.

Nicholas RS, Wing MG, Compston A. 2001. Nonactivated microglia promote oligodendrocyte precursor survival and maturation through the transcription factor NF-kappa B. Eur J Neurosci 13(5):959-67.

Ortega FJ, Gimeno-Bayon J, Espinosa-Parrilla JF, Carrasco JL, Batlle M, Pugliese M, Mahy N, Rodríguez MJ. 2012. ATP-dependent potassium channel blockade strengthens microglial neuroprotection after hypoxia-ischemia in rats. Exp Neurol Feb 24 (*Epub ahead of print*). doi: 10.1016/j.expneurol.2012.02.010

Perry VH, Nicoll JAR, Holmes C. 2010. Microglia in neurodegenerative disease. Nat Rev Neurol 6(4):193-201.

Picard-Riera N, Decker L, Delarasse C, Goude K, Nait-Oumesmar B, Liblau R, Pham-Dinh D, Evercooren AB-V. 2002. Experimental autoimmune encephalomyelitis mobilizes neural progenitors from the subventricular zone to undergo oligodendrogenesis in adult mice. Proc Natl Acad Sci USA 99(20):13211-6.

Pluchino S, Quattrini A, Brambilla E, Gritti A, Salani G, Dina G, Galli R, Del Carro U, Amadio S, Bergami A and others. 2003. Injection of adult neurospheres induces recovery in a chronic model of multiple sclerosis. Nature 422(6933):688-94.

Proks P, Ashcroft FM. 2009. Modeling K_{ATP} channel gating and its regulation. Prog Biophys Mol Biol 99(1):7-19.

Reynolds BA, Weiss S. 1992. Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. Science 255(5052):1707-10.

Sala-Rabanal M, Wang S, Nichols CG. 2012. On the potential interactions between Non-Selective cation channel TRPM4 and the sulfonylurea receptor SUR1. J Biol Chem.

Sasmono RT, Oceandy D, Pollard JW, Tong W, Pavli P, Wainwright BJ, Ostrowski MC, Himes SR, Hume DA. 2003. A macrophage colony-stimulating factor

receptor-green fluorescent protein transgene is expressed throughout the mononuclear phagocyte system of the mouse. Blood 101(3):1155-63.

Schwartz M, Butovsky O, BrÅck W, Hanisch UK. 2006. Microglial phenotype: is the commitment reversible? Trends in Neuroscience 29 (2):68-74.

Sierra A, Encinas JM, Deudero JJP, Chancey JH, Enikolopov G, Overstreet-Wadiche LS, Tsirka SE, Maletic-Savatic M. 2010. Microglia Shape Adult Hippocampal Neurogenesis through Apoptosis-Coupled Phagocytosis. Cell Stem Cell 7(4):483-495.

Simard JM, Chen M, Tarasov KV, Bhatta S, Ivanova S, Melnitchenko L, Tsymbalyuk N, West GA, Gerzanich V. 2006. Newly expressed SUR1-regulated NC_{Ca-ATP} channel mediates cerebral edema after ischemic stroke. Nat Med 12(4):433-440.

Thored P, Arvidsson A, Cacci E, Ahlenius H, Kallur T, Darsalia V, Ekdahl CT, Kokaia Z, Lindvall O. 2006. Persistent production of neurons from adult brain stem cells during recovery after stroke. Stem Cells 24(3):739-747.

Toni N, Teng EM, Bushong EA, Aimone JB, Zhao C, Consiglio A, van Praag H, Martone ME, Ellisman MH, Gage FH. 2007. Synapse formation on neurons born in the adult hippocampus. Nat Neurosci 10(6):727-34.

Tran PB, Banisadr G, Ren D, Chenn A, Miller RJ. 2006. Chemokine receptor expression by neural progenitor cells in neurogenic regions of mouse brain. J Comp Neurol 500(6):1007-1034.

van Praag H, Schinder AF, Christie BR, Toni N, Palmer TD, Gage FH. 2002. Functional neurogenesis in the adult hippocampus. Nature 415(6875):1030-1034.

Virgili N, Espinosa-Parrilla JF, Mancera P, Pastén-Zamorano A, Gimeno-Bayon J, Rodríguez MJ, Mahy N, Pugliese M. 2011. Oral administration of the KATP channel opener diazoxide ameliorates disease progression in a murine model of multiple sclerosis. J Neuroinflammation 8:149.

Wake H, Moorhouse AJ, Jinno S, Kohsaka S, Nabekura J. 2009. Resting microglia directly monitor the functional state of synapses in vivo and determine the fate of ischemic terminals. J Neurosci 29(13):3974-80.

Walton NM, Sutter BM, Laywell ED, Levkoff LH, Kearns SM, Marshall GP, Scheffler B, Steindler DA. 2006. Microglia instruct subventricular zone neurogenesis. Glia 54(8):815-825.

Wang H, Zhang Y-l, Tang X-C, Feng H-S, Hu G. 2004. Targeting ischemic stroke with a novel opener of ATP-sensitive potassium channels in the brain. Mol Pharmacol 66(5):1160-8.

Wheeler A, Wang C, Yang K, Fang K, Davis K, Styer AM, Mirshahi U, Moreau C, Revilloud J, Vivaudou M and others. 2008. Coassembly of different sulfonylurea receptor subtypes extends the phenotypic diversity of ATP-sensitive potassium (KATP) channels. Mol Pharmacol 74(5):1333-44.

Whitney NP, Eidem TM, Peng H, Huang Y, Zheng JC. 2009. Inflammation mediates varying effects in neurogenesis: relevance to the pathogenesis of brain injury and neurodegenerative disorders. Journal of Neurochemistry 108(6):1343-1359.

Widera D, Holtkamp W, Entschladen F, Niggemann B, Zänker K, Kaltschmidt B, Kaltschmidt C. 2004. MCP-1 induces migration of adult neural stem cells. Eur J Cell Biol 83(8):381-7.

Yan Y-P, Sailor KA, Vemuganti R, Dempsey RJ. 2006. Insulin-like growth factor-1 is an endogenous mediator of focal ischemia-induced neural progenitor proliferation. Eur J Neurosci 24(1):45-54.

Yasuda T, Bartlett PF, Adams DJ. 2008. K(ir) and K(v) channels regulate electrical properties and proliferation of adult neural precursor cells. Mol Cell Neurosci 37(2):284-97.

Zhu P, Hata R, Cao F, Gu F, Hanakawa Y, Hashimoto K, Sakanaka M. 2008. Ramified microglial cells promote astrogliogenesis and maintenance of neural stem cells through activation of Stat3 function. FASEB J 22(11):3866-77.