

# Blockade of microglial $K_{ATP}$ -channel abrogates suppression of inflammatory-mediated inhibition of neural precursor cells

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**Abstract**

Microglia positively affect neural progenitor cell physiology through the release of inflammatory mediators or trophic factors. We previously demonstrated that reactive microglia foster  $K_{ATP}$ -channel expression, and that blocking this channel using glibenclamide administration enhances striatal neurogenesis after stroke. In the present study, we investigated whether the microglial  $K_{ATP}$ -channel directly influences the activation of neural precursor cells from the subventricular zone by using transgenic *Csf1r-GFP* mice. *In vitro* exposure of neural precursor cells to lipopolysaccharide and interferon-gamma resulted in a significant decrease in precursor cell number. The complete removal of microglia from the culture or exposure to enriched microglia culture also decreased the precursor cell number. The addition of glibenclamide rescued the negative effects of enriched microglia on neurosphere formation, and promoted a ~20% improvement in precursor cell number. Similar results were found using microglial-conditioned media from isolated microglia. Using primary mixed glial and pure microglial cultures glibenclamide specifically targeted reactive microglia to restore neurogenesis and increased the microglial production of the chemokine monocyte chemoattractant protein-1. These findings provide the first direct evidence that the microglial  $K_{ATP}$ -channel is a regulator of the proliferation of neural precursor cells under inflammatory conditions.

## Introduction

Adult neurogenesis is a multistep process involving the proliferation of neural precursor cells (NPCs), their differentiation into lineage-restricted immature neurons, and their progressive maturation into fully functional and integrated neurons. There is a constant turnover of neurons in the two niches of the adult brain necessary for the maintenance of specific functions and ongoing brain plasticity, namely the subgranular zone of the hippocampus and the subventricular zone (SVZ) of the lateral ventricle (Merkle et al. 2007; Toni et al. 2007).

Microglia are the primary immune effector cells in the brain that release soluble factors promoting the proliferation, migration and differentiation of NPCs (Aarum et al. 2003; Deierborg et al. 2010; Walton et al. 2006). When microglia become reactive in response to injury, they are generally considered to be anti-neurogenic, as they release pro-inflammatory cytokines; however, under inflammatory conditions, they have been shown to be neuroprotective (Carson et al. 2006) and to enhance neurogenesis (Ekdahl et al. 2009). It is therefore likely that the microglial activity state depends on the balance of secreted molecules within the microenvironment (Perry et al. 2010).

The sulphonylurea receptor 1 (SUR1) subunit can assemble either with the inward rectifying  $K^+$  ion channel subunits Kir6.1 or Kir6.2 to form the  $K_{ATP}$ -channel (Mikhailov et al. 2005) or with the transient receptor potential melastatin (Trpm4) to form the  $NC_{Ca-ATP}$ -channel (Simard et al. 2012). SUR1-regulated channels have been shown to be expressed in neurons, astrocytes, oligodendrocytes and endothelial cells under ischemic conditions (Simard et al. 2006; Simard et al. 2012). We recently demonstrated that reactive microglia increased the expression of SUR1 and the  $K_{ATP}$ -channel after cerebral ischemia or after a pro-inflammatory stimuli (Ortega et al. 2012, Ortega et al. 2013). Indeed, this and other *in vitro* studies have demonstrated that the  $K_{ATP}$ -channel regulates the cell reactive state, controls the release of a diversity of inflammatory mediators, such as nitric oxide, interleukin-6 (IL-6) and

tumor necrosis factor-alpha (TNF $\alpha$ ), and modifies phagocytic activity (Ortega et al. 2012; Virgili et al. 2011). Post-ischemic administration of the SUR1 blocker glibenclamide has been proposed to prevent cytotoxic edema (Simard et al. 2006; Simard et al. 2012), promote neuroprotection (Ortega et al. 2012), enhance migration of SVZ neuroblasts towards the lesion core, and assist long-term cortical neurogenesis (Ortega et al. 2012; Ortega et al. 2013). It is clear that the K<sub>ATP</sub>-channel is a major component of the inflammatory response after brain injury; however, its role in NPC physiology under inflammatory conditions is still largely unknown. In the current study, we used the neurosphere assay (Reynolds and Weiss 1992) to evaluate whether specific blockade of the microglial K<sub>ATP</sub>-channel in response to pro-inflammatory stimuli contributes to NPC activation.

## Materials and methods

### *Animals*

To elucidate the role of the microglial  $K_{ATP}$ -channel in NPC activity we used adult (3 to 4-month-old) C57BL/6 mice or *Csflr*-GFP (MacGreen) transgenic mice, as previously described (Vukovic et al. 2012). All experiments were conducted according to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, with approval from The University of Queensland Animal Ethics Committee. Animals were maintained on a 12 h light/dark cycle with food and water provided *ad libitum*.

### *Neurosphere culture*

Mice were sacrificed by cervical dislocation. Brains were removed immediately and the SVZ dissected. Four to six SVZs were digested with 0.05% trypsin-EDTA (Invitrogen) at 37°C for 7 min and the reaction was quenched with 0.014% (w/v) trypsin inhibitor (type I-S from soybean; Sigma-Aldrich). Following centrifugation at 100 g for 7 min, the resulting pellet was resuspended and washed twice in neurosphere growth medium comprised of mouse NeuroCult neural stem cell (NSC) basal medium, 10% mouse NeuroCult NSC proliferation supplement (Stem- Cell Technologies), 2% bovine serum albumin (Roche), and 2 µg/ml heparin (Sigma-Aldrich), 20 ng/ml purified mouse receptor-grade epidermal-like growth factor (BD Biosciences), and 10 ng/ml recombinant bovine fibroblast growth factor (Roche). The suspension was then mechanically triturated until smooth and filtered through 40 µM cell sieve (Falcon™; BD Bioscience) to obtain a single cell suspension. Some of the suspensions were further examined using fluorescence-activated cell sorting (FACS) before being plated. The number and size of the SVZ neurospheres were determined after 7 days *in vitro* (DIV).

*Treatment of primary neurosphere cultures.* To assess the effect of glibenclamide on

NPC activity, fresh glibenclamide solution, ranging from fM to  $\mu$ M in concentration, was added directly to the culture medium upon plating.

### ***Differentiation of primary neurospheres***

Neurospheres established from the SVZ were plated onto glass coverslips coated with poly-L-ornithine (Sigma) and laminin (natural mouse; Invitrogen) before being cultured in neurosphere differentiation medium containing mouse NeuroCult NSC basal medium, 10% mouse NeuroCult NSC proliferation supplements, and 100 U/ml penicillin/streptomycin (Invitrogen). To quantitatively analyze the multipotentiality of the neurospheres, individual neurospheres were selected from cultures and were allowed to differentiate for 6 days before analysis of differentiation markers by immunohistochemistry.

*Immunocytochemistry.* Coverslips containing the differentiated neurospheres were rinsed gently with PBS and fixed with 4% paraformaldehyde (PFA) for 15 min at 4°C. Fixed samples were for immunocytochemistry, as described previously (Vukovic et al. 2012). Briefly, samples were blocked (5% FCS and 5% normal goat serum in PBS containing 0.1% Triton X-100) for 1 h at 37°C. After rinsing, fresh solution containing anti- $\beta$ -III tubulin mouse IgG monoclonal antibody (diluted 1:2000; Promega) or monoclonal rabbit anti-doublecortin (DCX) antibody (1:500; Abcam) was added, and coverslips were kept at 4°C overnight. Samples were then incubated with Alexa Fluor-488 conjugated anti-mouse antibody (1:1000; Invitrogen) or Alexa Fluor-555 conjugated anti-rabbit antibody (1:1000; Invitrogen) for 30 min at 37°C. The slides were then coverslipped with fluorescence mounting medium (Dako) and DAPI (0.5  $\mu$ g/ml; Invitrogen) to label nuclei. The total number of differentiated neurospheres containing  $\beta$ -III tubulin and DCX expressing cells were counted. Data were expressed as the percentage of total neurospheres containing <25, 25–100, 100–200 or >200  $\beta$ -III tubulin-positive or DCX-positive cells present in each

differentiated neurosphere.

***Fluorescence-activated cell sorting and generation of primary neurosphere culture***

To either deplete or collect microglia from SVZ cultures, dissociated cells from *Csf1r*-GFP transgenic mice were sorted using a FACS Vantage SE DiVa sorter (BD Bioscience). Four to six SVZs were used per preparation to obtain a single-cell suspension, as described above.

Snapshots of GFP<sup>pos</sup> SVZ microglia were obtained using AMNIS ImageStream 100. To assess the effect of glibenclamide on neurosphere cultures in either the presence or absence of microglial cells, we used three different approaches: (1) GFP<sup>pos</sup> cells (microglia) were removed from the cell culture (microglia-depleted cultures; -MO) and plated in 200  $\mu$ l/well of complete neurosphere medium ( $\sim$ 5,000 events/well or  $\sim$ 16,000 events/cm<sup>2</sup>); (2) the control SVZ preparations were passed through the cell sorter and all cell populations were collected (containing  $\sim$ 10% of microglial cells; +MO) and plated in 200  $\mu$ l/well of complete medium ( $\sim$ 16,000 events/cm<sup>2</sup>); (3) Enriched microglial cultured was obtained by combination of control SVZ preparations ( $\sim$ 16,000 events/cm<sup>2</sup>) plated in 100  $\mu$ l/well of complete medium with 100  $\mu$ l of the GFP<sup>pos</sup> cells ( $\sim$ 16,000 events/cm<sup>2</sup>) derived from cortical samples. Primary neurospheres were incubated for 7 days to permit neurosphere formation and the number and size of the SVZ neurospheres were measured.

*Activation and treatment of fluorescence-activated cell sorting cultures.* To characterize whether blockade of the microglial K<sub>ATP</sub>-channel instructs SVZ neurogenesis, we performed primary neurosphere cultures either in the presence (+MO) or absence (-MO) of microglia, or, alternatively, in an environment with an increased number of microglia. Cultures were activated using 0.1  $\mu$ g/mL recombinant lipopolysaccharide (LPS, Sigma-Aldrich) and 0.05 ng/mL recombinant mouse interferon gamma (IFN $\gamma$ ; R&D), both of which were diluted in complete culture media. At the same time, all cultures were co-treated with fresh

glibenclamide solution diluted in complete culture media and added until the desired final concentration was achieved (DMSO final concentration <0.05%).

*MHCII staining.* To assess microglial activation, MHCII-positive cells were quantified, analyzed, and sorted using FACS. In brief, cells were incubated with unconjugated rat anti-CD16/32 (1:100; BD PharMingen) for 5 min at RT, followed by incubation with anti-I-A/I-E antibody (1:200) for 15 min at RT. The cells were washed once in fresh medium before analysis and/or FACS.

#### ***Microglial-conditioned media and generation of primary neurosphere culture***

To obtain the microglial-conditioned media, cortical GFP<sup>POS</sup> cells were collected (n = 4 to 6) by cell sorting, as described above, and harvested in 15 mL Falcon™ tubes to a final concentration of 100,000 cells/mL. FACS-isolated GFP<sup>POS</sup>-microglia were cultured for 24 h in the presence of either LPS+IFN $\gamma$ , glibenclamide (K<sub>ATP</sub>-channel blocker) or diazoxide (K<sub>ATP</sub>-channel opener), all of which were diluted in complete culture media until the desired final concentration was achieved (DMSO final concentration <0.05%). Diazoxide was added at a concentration of 50  $\mu$ M (Virgili et al. 2011) either alone or in combination with glibenclamide. Following centrifugation at 100 g for 15 min, we collected the microglial-conditioned media to perform the neurosphere assay, as described above. Aliquots of conditioned media were kept at -20°C to determine the concentration of soluble cytokine/chemokine. Primary neurosphere cultures in the presence of microglial-conditioned media were incubated for 7 days in humidified 5% CO<sub>2</sub> to allow neurosphere formation. After incubation, the number and size of the neurospheres were measured.

#### ***Primary glial cultures***



Primary glial cultures were obtained as described previously (Saura et al. 2003b). Mixed glial cultures from 2 to 4-day-old C57BL/6 mice were obtained from the cerebral cortex (n = 6 to 8) and digested using 0.25% trypsin-EDTA solution (Invitrogen) for 30 min at 37°C. Trypsinization was stopped by adding an equal volume of culture medium, consisting of DMEM:F12 (Invitrogen) supplemented with 10% FBS, 0.1% penicillin-streptomycin (Invitrogen), 0.5 mg/mL amphotericin B (Fungizone®, Invitrogen), and 0.04% deoxyribonuclease I (Sigma-Aldrich). Cells were pelleted (8 min, 200 g), resuspended in culture medium, and brought to a single cell suspension by pipetting vigorously and filtering through a 105 µm-pore mesh. Cells suspensions were seeded in 24-well plates at a density of  $3 \times 10^5$  cells/mL ( $\sim 78,000$  cells/cm<sup>2</sup>) and the medium was replaced every 7 days.

To assess the effect of astrocytes on neurospheres, half of the wells were processed as mixed glial cultures ( $\sim 250,000$  cells/cm<sup>2</sup>) and half were sorted to obtain pure-microglial cultures using the mild trypsinization method after 19–21 DIV, as described by Saura and colleagues (2003b). After the astroglial layer was removed, microglia were cultured for 24 h in complete medium. To assess the purity of the microglial culture, cells were fixed with 4% PFA for 15 min at 4°C and immunocytochemistry was carried out with an anti-Iba1 (ionized calcium binding adaptor molecule 1; 1:500; Wako Chemical) primary antibody and a goat anti-mouse Alexa Fluor-488 secondary antibody (1:500; Molecular Probes). The purity of the cultures was  $98 \pm 1\%$  (n = 5 cultures), based on the number of Iba1-positive cells ( $\sim 20,000$  Iba1-positive cells/cm<sup>2</sup>).

*Activation of primary glial cultures.* Primary mixed glial or primary microglial cultures were activated for 24 h in the presence of LPS+IFN $\gamma$ . At the same time, cultures were co-treated for 24 h with fresh glibenclamide or diazoxide solution, as described above.

*Immunocytochemistry to detect the K<sub>ATP</sub>-channel.* To assess the expression of the K<sub>ATP</sub>-channel in the pure microglia, immunocytochemistry was carried out on 4% PFA fixed cells

with a rat anti-CD68 antibody (1:500; Abcam) combined with a goat anti-SUR1 (1/100; Santa Cruz), rabbit anti-Kir6.1 or anti-Kir6.2 (1/400; Alomone Labs) primary antibodies. As secondary antibodies, we used goat anti-rat Alexa Fluor-488 and anti-rabbit/goat Alexa Fluor-488 (1:500; Molecular Probes).

### ***Neuronal Culture***

We used embryonic neuronal cultures to determine whether glibenclamide influenced NPC survival. Hippocampi from E16 to E18 C57BL/6 mice were isolated (n = 6 to 8), minced and digested using 0.05% trypsin-EDTA solution (Invitrogen) for 15 min at 37°C. Digestion was halted by adding an equal volume of trypsin-inhibitor (Invitrogen) and spinning the solution for 5 min at 104 g. The cell pellet was then suspended in 1 ml DMEM:F12 media (Gibco), and cells were passed through a 19-gauge needle and then a 23-gauge needle. The cell suspension was filtered through a mesh 40  $\mu$ M in diameter before being diluted in complete culture media containing glibenclamide. Cells were plated onto glass coverslips previously coated with poly-L-ornithine (Sigma) to a final density of 100,000 cells/well. Culture media supplemented with glibenclamide was changed after 24 h. Immunofluorescence was carried out after 7 DIV to detect  $\beta$ -III tubulin- and DCX-positive cells as described above. To count the number of  $\beta$ -III tubulin and DCX-expressing cells, five random pictures/well were taken using a Zeiss inverted microscope, and images were analyzed using ImageJ 1.39u (NIH, USA).

### ***Cytometric Bead Array (CBA)***

To quantify the concentration of releasing cytokines in the conditioned media derived from the pure primary microglia cultures, IL-6, TNF $\alpha$ , IL-12p70, IFN $\gamma$  and CCL2 were assayed using the cytometric bead array (CBA) reagents (BD Biosciences) with four-color FACS

Calibur flow-cytometer (BD Biosciences), following manufacturer instructions. The analysis of BD CBA data was performed using the FCAP array software provided by the manufacturer (BD Bioscience).

### **Statistical analysis**

Data were subjected to statistical analysis using the GraphPad Prism software (version 5.0c). All experiments were subjected to statistical analyses using a one-way ANOVA followed by the Bonferroni *post hoc* test for pair wise group comparisons. Values are presented as mean  $\pm$  standard error of the mean (SEM), with significance determined at  $p < 0.05$ .

## Results

### Microglial $K_{ATP}$ -channel blockade restores neural precursor cell activation after inflammation

Using the *Csflr-GFP* transgenic mouse, we selectively isolated microglia using fluorescence-activated cell sorting (FACS) (**Figure 1A**). Examples of individual snapshots of GFP<sup>pos</sup> cells taken during flow cytometry are shown in **Figure 1B**. To confirm microglial activation by LPS+IFN $\gamma$ , MHC-II expression was measured in GFP<sup>pos</sup> isolated microglia (**Figure 1C**, panel a). Of the control GFP<sup>pos</sup> microglia, 21.4 $\pm$ 2% were also MHC-II positive and when treated with LPS+IFN $\gamma$  this percentage increased to 44.8 $\pm$ 5% (**Figure 1C**, panel b), which indicated microglial activation.

We evaluated the influence of microglial activation on NPC activity by assessing the neurosphere formation frequency in the presence or absence of microglia. After SVZ cultures containing ~10% microglia (+MO) were treated with LPS+IFN $\gamma$ , NPC number was significantly reduced, by 35.4 $\pm$ 8%, which was in agreement with our previous findings (Li et al. 2010) (**Figure 1D and E**). A further significant decrease in neurosphere number was observed in cultures depleted of microglia (-MO) (49.2 $\pm$ 6%;  $p < 0.05$  when compared to +MO). It therefore appeared probable that the presence of microglia at endogenous levels was protective of neurosphere formation. To create a similar situation to that seen after brain injury, where microglia are present in greater concentrations, a higher ratio (1:1) of GFP<sup>pos</sup> (microglia) to GFP<sup>neg</sup> cells was added to the SVZ neurosphere culture. After LPS+IFN $\gamma$  treatment, the formation of neurospheres from microglial-enriched cultures was significantly reduced (75.6 $\pm$ 8%;  $p < 0.05$  and  $p < 0.01$  when compared to -MO and +MO, respectively), indicating that an environment with enriched microglia negatively affects NPC activation. When the  $K_{ATP}$ -channel blocker glibenclamide (1 pM and 1 nM) was added to the

enriched microglial culture, we were able to almost completely overcome the inhibitory effect of LPS+IFN $\gamma$  treatment. In the presence of 1 nM glibenclamide there was no difference between the three conditions treated with LPS+IFN $\gamma$ . Our data indicate a trend toward improved recovery with increasing concentrations of glibenclamide.

### **Microglia release a soluble factor to enhance neurogenesis**

As glibenclamide positively influences NPC formation in situations of increased microglial concentration, we first checked whether primary microglia expressed the K<sub>ATP</sub>-channel. We performed double immunolabeling using anti-SUR1, anti-Kir6.1 and anti-Kir6.2 antibodies combined with anti-CD68 antibody as microglia/macrophage marker (**Figure 2A**). Colocalization of Kir6.1, Kir6.2 and SUR2 with CD68 labeling indicated that non-activated microglia (control, left panel) and also activated microglia (LPS+IFN $\gamma$ , right panel) expressed the K<sub>ATP</sub>-channel, making these cells a putative target for glibenclamide *in vitro*.

We then looked at whether soluble factors had a direct effect on neurosphere formation and size. Conditioned media from isolated GFP<sup>pos</sup> cells treated with LPS+IFN $\gamma$  for 24 h significantly reduced the number of neurospheres when added to neurosphere cultures by 22.2 $\pm$ 3% ( $p$ <0.01) (**Figure 2B**). Conversely, cultures treated with LPS+IFN $\gamma$  and 1  $\mu$ M or 1 nM glibenclamide produced significantly more neurospheres (11.4 $\pm$ 3% and 10.3 $\pm$ 3% respectively;  $p$ <0.01 in both cases) than the cultures treated with LPS+IFN $\gamma$  alone. The opposite effect was observed when the K<sub>ATP</sub>-channel opener diazoxide was used alone (23.8 $\pm$ 5%;  $p$ <0.05 compared to glibenclamide 1 nM). Diazoxide partially reversed the effect of glibenclamide; however, this reduction was not significant. These data suggest that glibenclamide treatment triggers microglia to release soluble neurogenesis-promoting factors.

Conditioned media from microglial cultures treated with LPS+IFN $\gamma$  caused an overall reduction in neurosphere diameter, irrespective of whether or not they had received

glibenclamide treatment ( $p < 0.01$ , **Figure 2C**). Neuronal differentiation was almost completely inhibited when neurospheres were cultured with conditioned media from microglia treated with LPS+IFN $\gamma$ , with only a few  $\beta$ -III tubulin-positive cells observed (data not shown). It therefore appears that long-term exposure to pro-inflammatory signals blocks neuronal differentiation.

### **Neural precursor cell activation is microglia-dependent**

To study whether the effect of glibenclamide was mediated solely by microglia, or whether other cells, such as astrocytes, could also be implicated in the process, we used primary mixed glial cultures (microglia and other cell types) and pure microglial cultures, both treated with LPS+IFN $\gamma$  and glibenclamide for 48 h. Mixed glial cultures showed both GFAP- and Iba1-positive cells (75% $\pm$ 2 for GFAP, 20% $\pm$ 2 for Iba1 and 5% $\pm$ 1 for others), whereas >98 $\pm$ 1% of cells in pure microglial cultures were positive only for Iba1 (**Figure 3A**). Visual analysis of cell morphology showed that, compared to control conditions, LPS+IFN $\gamma$  treatment resulted in astrocytes with a greater number of wider processes and an extended cytoplasm, and microglia with a classical amoeboid shape (**Figure 3A**, right vs center and left panels). Cell morphology was not affected by glibenclamide (**Figure 3A**, right panels).

Neurosphere cultures treated with conditioned supernatant from mixed glial cultures challenged with LPS+IFN $\gamma$  showed a 75 $\pm$ 3% reduction in neurosphere formation frequency compared to control ( $p < 0.001$ ; **Figure 3B**). This effect could not be rescued by treatment with either glibenclamide or diazoxide alone, nor in combination ( $p < 0.001$  compared to control). Treatment with conditioned supernatant derived from pure microglial cultures and LPS+IFN $\gamma$  resulted in a less severe, but still significant, inhibition of neurosphere formation (45.3 $\pm$ 8%;  $p < 0.001$  compared to control or  $p < 0.01$  compared to mixed glial). Conditioned

media from microglial cultures treated with 1 pM glibenclamide restored neurosphere formation in pure microglial cultures to near control levels ( $25.6 \pm 11\%$ ;  $p > 0.05$  compared to control and  $p < 0.001$  compared to mixed glial). Treatment with diazoxide alone or in combination with glibenclamide blocked this effect ( $p < 0.001$  compared to control). It therefore appears that other cell types, including astrocytes, are not mediating the glibenclamide-derived recovery in neurosphere formation.

### **K<sub>ATP</sub>-channel modulates the release of microglial inflammatory factors**

Having established a critical role for microglia in mediating the beneficial effects of K<sub>ATP</sub>-channel blockade after inflammatory conditions, we next sought to unravel the underlying molecular mechanism by which microglia regulate NPC activation under inflammatory conditions. To do so, we tested a panel of cytokines/chemokines that affect NPC physiology and found that supernatant from primary pure microglial cultures treated with LPS+IFN $\gamma$  showed an overall increase in concentrations of TNF $\alpha$ , IL-6 and CCL2 ( $p < 0.001$ ; **Figure 4**). Detailed *post hoc* analysis indicated that diazoxide induced an anti-inflammatory response and decreased production of TNF $\alpha$ , IL6 and CCL2 ( $p < 0.001$  or  $p < 0.01$  compared to LPS+IFN $\gamma$ ), whereas glibenclamide treatment enhanced microglial production of CCL2 ( $p < 0.05$  compared to LPS+IFN $\gamma$ ) had no observable effect on IL10 or IL12p70 (data not shown). These results indicate that the microglial K<sub>ATP</sub>-channel mediates the release of cytokines/chemokines or other neurotrophic factors that can modulate neurogenesis.

### **K<sub>ATP</sub> signaling does not stimulate precursor activation under physiological conditions**

We next wanted to elucidate whether neurosphere formation under physiological conditions is affected by glibenclamide as observed under inflammatory conditions. We treated SVZ-derived neurosphere cultures (containing ~10% microglia) with glibenclamide or diazoxide

and found that neither neurosphere number (**Figure 5A**) nor size (**Figure 5B**) were affected. We also assessed whether glibenclamide affected NPC differentiation, and found that in the presence of resting microglia, the total number of neurospheres containing either  $\beta$ -III tubulin-positive cells or DCX-positive cells was similar in glibenclamide treated and control cultures (data not shown). Glibenclamide-treated groups (10 fM or 1 nM) moderately increased the number of neurospheres containing a higher number of  $\beta$ -III tubulin-positive cells (25 to 100 cells/neurosphere) by  $17.8\pm 5\%$  and  $17.3\pm 7\%$ , respectively ( $p < 0.05$ ), and also increased the number of neurospheres containing DCX-expressing cells by (100 to 200 cells/neurosphere) by  $14.6\pm 4\%$ ; ( $p < 0.05$ ) (**Figure 5C and G – left panel**). To ensure that resting microglial cells were directing this effect, we performed another set of experiments removing all GFP<sup>pos</sup> microglia using FACS. In the absence of microglia, the NPC differentiation profile was unaltered for cultures treated with glibenclamide (**Figure 5D**). Due to the known neuroprotective characteristics of glibenclamide *in vivo* (Ortega et al. 2012), we wanted to confirm that the increased number of  $\beta$ -III tubulin- and DCX-positive cells was not due to activation of neuronal survival mechanisms. We therefore cultured embryonic hippocampal neurons in the presence of increasing doses of glibenclamide; however, after 7 DIV, no influence on the total number of neurons expressing  $\beta$ -III tubulin or DCX was observed (**Figure 5E, F and G – right panel**).



## Discussion

It has been suggested that immunological control of neural stem cell physiology and the dual role of microglia is paramount in mediating neurogenesis after brain injury. In the present study we explored whether the microglial  $K_{ATP}$ -channel influences adult NPC activity under pro-inflammatory conditions. We used a novel *ex vivo* model system using *Csf1r-GFP* transgenic mice, as described by Vukovic and colleagues (2012), whereby microglia could be selectively depleted or isolated and subsequently added to neurosphere cultures. We were able to show that blockade of the microglial  $K_{ATP}$ -channel restored the reduction in NPC number normally observed after inflammation and that this recovery was a consequence of the release of soluble factors.

### **The $K_{ATP}$ -channel has a key role in the degree of microglial-derived inflammation**

After brain injury, reactive microglia present a variety of phenotypes that each influence NPC physiology and neurogenic processes in different ways (Kettenmann et al. 2011). Systemic or central administration of LPS is a common method to activate microglia, as well an inflammatory response, and LPS-activated microglia and its conditioned medium has been shown to reduce neural stem cells survival and inhibit neural differentiation *in vitro* (Cacci et al., 2008; Monje et al., 2003). Thus, we used LPS+IFN $\gamma$  to activate microglia, which in turn caused a reduction in NPC activation and neuronal differentiation. Interestingly, high inhibition of NPC activation was seen as a result of exposure to cultures with enriched concentrations of microglia, most likely due to an excessive production of pro-inflammatory mediators, which are detrimental for NPC proliferation. Similar findings were described by Picard-Riera and colleagues (2002) who observed that an exacerbated inflammatory response is complicated, strengthening the inhibition of NPC proliferation, stimulating migration, and enhancing astro- or oligodendrogenesis (Picard-Riera et al. 2002). Our results using

conditioned media derived from the primary microglial culture (>98% purity) or pure isolated GFP<sup>POS</sup>-microglia (100% purity) also point toward activated microglia exerting the observed effect. Our data confirm that neurogenesis is a microglial-dependent process, where microglia are required for NPC activation, and uncontrolled microgliosis is detrimental for neurogenesis.

### **Microglial K<sub>ATP</sub>-channel blockade fosters neural precursor cell activation through a soluble factor**

Microglia have positive and negative regulatory effects as constituents of the neurogenic niche (Morrens et al., 2012). In the pathogenic brain, microglia produce trophic factors with a positive effect on neurogenesis (Butovsky et al. 2006). As LPS+IFN $\gamma$  does not enhance microglial proliferation *in vitro* (Kloss et al. 1997; Palwinder et al. 2006), and after a brain insult, microglia proliferate and migrate from the surrounding tissue towards the site of injury, increasing their cell density in the affected area, we used enriched microglial cultures to resemble this inflammatory situation. Given that in our neurosphere cultures, approximately 10% of the cells are microglia (Vukovic et al. 2012) and that conditioned media from primary reactive microglia treated with glibenclamide and microglial enriched cultures reversed the inhibition of neurogenesis caused by LPS+IFN $\gamma$  treatment, it seems that at least a certain number of microglial cells is necessary to respond to glibenclamide and produce enough trophic factors to reverse the inhibition of neurogenesis. Thus, by controlling the reactive phenotype of reactive microglia through the blockade of the K<sub>ATP</sub>-channel, we were able to restore neurogenesis through a process that did not require cell-cell contact. Walton and colleagues (2006) and Deierborg and colleagues (2012) each demonstrated that microglial-conditioned media restores SVZ neurogenesis and directs their differentiation into mature neurons. Furthermore, several studies have shown that microglia secrete a number of

mitogens that positively regulate the proliferation of NPCs in the SVZ (Yan et al. 2006; Jin et al. 2002; Aguado et al. 2005), which can have autocrine and paracrine actions. By characterizing cytokine release from primary microglial cultures, we found that blocking the  $K_{ATP}$ -channel increased the secretion of MCP-1. This chemokine does not directly activate an inflammatory response in microglia nor causes neuronal damage (Hinojosa et al. 2011); however, it can enhance NPC differentiation (Turbic et al. 2011; Liu et al. 2007). Our previous findings *in vivo* demonstrate that glibenclamide increased migration of doublecortin-positive striatal cells toward the lesion after cerebral ischemia (Ortega et al. 2013), where MCP-1 has a major role. The enhanced NPC activation observed in the current study is unlikely to be a consequence of MCP-1 action alone and further cytokine analysis is necessary to reveal the mechanism by which the microglial  $K_{ATP}$ -channel blockade enhances NPC activation.

As glibenclamide binds to SUR1, which forms both the  $K_{ATP}$ -channel and the astroglial  $NC_{Ca-ATP}$ -channel expressed under pathological conditions (Ortega et al. 2012; Ortega et al. 2013; Simard et al. 2006), we wanted to further explore the relationship between enhanced neurogenesis and the microglial  $K_{ATP}$ -channel and the astroglial  $NC_{Ca-ATP}$ -channel. We found that conditioned media from mixed glial cultures markedly blocked NPC activation, an effect that could not be rescued by glibenclamide treatment. These results are in line with studies using experimental cerebral ischemia models, where the  $NC_{Ca-ATP}$  channel was involved in the resolution of the cytotoxic edema (Simard et al. 2006; Simard et al. 2012). It appears that the astroglial  $NC_{Ca-ATP}$  channel, although capable of reversing the cytotoxic edema after ischemia, does not contribute to the glibenclamide-dependent rescue of neurogenesis (Ortega et al. 2013). Also, as more proliferative microglia were present in mixed glial cultures than in microglial cultures, it is therefore possible that astrocytes in mixed glial cultures use a positive feedback mechanisms to strengthen the pro-inflammatory

response derived from microglia (Losciuto et al, 2013; Saura et al. 2003a). Indeed, NPC inhibition could be a consequence of an excessive production of pro-inflammatory factors mediated by microglia; however, as reactive microglia express the  $K_{ATP}$ -channel (Ortega et al. 2012; Ortega et al. 2013), a glibenclamide-derived response would have also been expected in this scenario.

### **Treatment of microglia with diazoxide does not correlate with enhanced precursor cell activation *in vitro***

Previous studies established a pro-neurogenic role for microglia in both the SVZ and subgranular zone (Mcperson et al. 2011; Thored et al. 2009). In our study, microglial-conditioned supernatant treated with diazoxide, a well-known anti-inflammatory  $K_{ATP}$ -channel opener (Virgili et al. 2011; Zhou et al. 2008), presented a lower concentration of inflammatory mediators but did not foster NPC activation. Our results align with previous studies showing that anti-inflammatory minocycline treatment, does not cause any changes in neurogenesis (Ng et al., 2012), or lead to a reduction in NPC proliferation of SVZ progenitors after inflammation (Kim et al. 2009; Deierborg et al., 2010). On the contrary, although glibenclamide did not modify the release of IL-6 and  $TNF\alpha$ , it was able to enhance activation of NPCs. Thus, rather than modifying the microglial inflammatory phenotype, the glibenclamide effect most likely resulted from the modification of the release of specific pro-neurogenic factors. Studies of the specific role of inflammatory cytokines have shown conflicting results in central nervous system pathogenesis and neurogenesis, supporting the view that the effects of inflammatory cytokines are dose- and context-dependent (Bernardino et al. 2008; Bowen et al. 2011; Lambertsen et al. 2009; Veroni et al. 2010). In addition, the finding that cyclooxygenase-2 inhibitors, which are highly expressed by reactive microglia, are potent suppressors of neurogenesis (Goncalves et al., 2010) indicates that anti-

inflammatory therapies may not all be beneficial for restoring neurogenesis. Our findings reinforce the idea that some pro-inflammatory modulators, such as IL-6 and TNF $\alpha$ , do not act as major negative regulators of adult neurogenesis *per se*. Thus, as microglial activation mediates inflammation at the site of injury and in the lesion (Graeber and Streit 2010) and NPCs from the SVZ respond positively to inflammatory mediators (Ekdahl et al. 2009; Macas et al. 2006; Zhang et al. 2008), under some conditions, therapies focusing on abrogating inflammation would be detrimental for NPC proliferation.

In summary, our findings provide evidence that blockade of the microglial K<sub>ATP</sub>-channel causes the release of soluble factors that enhance NPC proliferation and partially modifies cell fate determination. Also, we demonstrate that the microglial K<sub>ATP</sub>-channel can modulate microglial release of MCP-1. Therefore, modulation of the reactive microglial state and the release of inflammatory cytokines and trophic factors through pharmacological manipulation of the K<sub>ATP</sub>-channel represent a possible alternative for the treatment of brain and central nervous system injury.

### **Acknowledgements**

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**Author Disclosure Statement**

MJR holds an EU patent for glibenclamide (No. WO2006/000608) and has applied for a PCT application for diazoxide (PCT/EP2011/050049). The other authors report no conflict of interest.

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## Figure Legend

**Figure 1. Inhibition of neurosphere formation is reversed by microglial  $K_{ATP}$ -channel blockade *in vitro*.** *Csfr1*-GFP transgenic mice were used and (A) the GFP<sup>pos</sup> population (microglia) was sorted using FACS. (B) Photomicrographs of single GFP<sup>pos</sup> cells from dissociated *Csfr1*-GFP cortex taken during flow cytometry. (C) FACS analysis of MHC-II expression levels from isolated microglia (a) or after 24 h LPS+IFN $\gamma$  treatment (b). (D) An overall reduction in neurosphere number was observed after LPS+IFN $\gamma$  (L+I) treatment in all conditions when compared to control ( $p < 0.01$ ;  $n = 3$  to 4 per experimental condition). Glibenclamide (Gbc) reversed the inhibitory effect of the enriched microglial culture in a dose-dependent manner. (E) Representative image of SVZ neurospheres. (Results are shown as mean  $\pm$  SEM; one-way ANOVA followed by the Bonferroni *post hoc* test;  $*p < 0.05$  and  $**p < 0.01$ ; scale bar in E = 150  $\mu$ m, and E insert = 50  $\mu$ m).

**Figure 2. Microglia express the  $K_{ATP}$ -channel and modulate neurogenesis through a soluble factor.** (A) Representative photomicrographs of pure microglial culture in control conditions (left panel) or after 24 h of activation with LPS+IFN $\gamma$  (right panel). The  $K_{ATP}$ -channel components Kir6.1, Kir6.2 and SUR1 (red) were colocalized with microglial cells CD68 (green), and counterstained with DAPI (blue). The GFP<sup>pos</sup> cell population was collected from *Csfr1*-GFP transgenic mice by cell sorting and cultured for 24 h in the presence of LPS+IFN $\gamma$  (L+I), glibenclamide (Gbc) and/or diazoxide (Dz). The conditioned-media from these cells was then used to perform neurosphere cultures. (B) Conditioned media of microglia treated with glibenclamide was able to restore the inhibition of neurosphere formation caused by L+I ( $p < 0.01$  at 1 pM and 1

nM Gbc). Diazoxide reversed this effect ( $p < 0.05$  compared to Gbc 1 nM). At 1 nM glibenclamide, this reduction was not significant ( $n = 3$  to  $7$  per experimental condition). Dotted line represents control group. (C) Overall reduction in neurosphere size after treatment with L+I. Glibenclamide treatment did not affect neurosphere size ( $n = 5-7$  per experimental condition). (Results are shown as mean  $\pm$  SEM; one-way ANOVA followed by the Bonferroni *post hoc* test;  $*p < 0.05$  and  $**p < 0.01$ ; scale bar =  $20 \mu\text{m}$ ).

**Figure 3. Absence of astrocytes increased neural precursor cell activation.** (A) Representative photomicrographs of mixed glial or pure microglial culture after 24 h of LPS+IFN $\gamma$  (L+I) and glibenclamide (Gbc; 1 pM) treatment. Microglial cells were immunostained for Iba1 (green), astrocytes for GFAP (red), and counterstained with DAPI (blue). (B) Conditioned supernatant from mixed glial cultures challenged with LPS+IFN $\gamma$  caused a  $75 \pm 3\%$  reduction in neurosphere formation frequency, while supernatant derived from pure microglial cultures and LPS+IFN $\gamma$  caused a  $45.3 \pm 8\%$  decrease ( $p < 0.001$  compared to control in both cases). Pure microglial-conditioned media treated with 1 pM glibenclamide restored neurosphere formation to near control levels. This positive effect was blocked by diazoxide alone and when combined with glibenclamide ( $p < 0.001$  compared to control in both cases;  $n = 5$  per experimental condition). (Results are shown as mean  $\pm$  SEM; one-way ANOVA followed by the Bonferroni *post hoc* test;  $***p < 0.001$  (vs control conditions),  $##p < 0.01$ ,  $###p < 0.001$  (vs pure microglia cultures); dotted line in B represents control group = 100%; scale bar =  $50 \mu\text{m}$ ).

**Figure 4. Microglial K<sub>ATP</sub>-channel blockade increased the production of the MCP-**

Quantification of microglial-released cytokines and inflammatory mediators from primary pure microglial-conditioned media showed that LPS+IFN $\gamma$  (L+I) treatment increased the overall production of IL-6, TNF $\alpha$  and MCP-1 ( $p < 0.001$ ) and the addition of diazoxide (Dz) reversed this effect. Glibenclamide at 1 pM boosted the production of MCP-1 when compared to LPS+IFN $\gamma$  and 1 nM glibenclamide ( $n = 4$  per experimental condition). (Results are shown as mean  $\pm$  SEM; one-way ANOVA followed by the Bonferroni *post hoc* test; \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ ).

**Figure 5. In the presence of microglia, neurosphere cultures produce more neurons after treatment with glibenclamide.** (A) Neurosphere formation and (B) neurosphere size is unaffected by glibenclamide (Gbc) treatment ( $n = 4$  per experimental condition). (C & D) Percentage of total neurospheres containing  $\beta$ -III tubulin-positive and DCX-positive cells in the presence (+MO; C) or absence (-MO; D) of microglia. Glibenclamide treatment resulted in more neurospheres containing a higher number of  $\beta$ -III tubulin or DCX expressing cells only in the presence of microglial cells ( $n = 4$  per experimental condition). (E & F) Survival of hippocampal neurons labeled with  $\beta$ -III tubulin (E) or DCX (F) was unaltered in the presence of glibenclamide ( $n = 6$  per experimental condition). (G) Representative photomicrographs of  $\beta$ -III tubulin or DCX-positive cells in differentiated neurospheres in presence of microglia (left panel) and neuronal cultures (lower panel). (Results are shown as mean  $\pm$  SEM; one-way ANOVA followed by the Bonferroni *post hoc* test; \* $p < 0.05$ . Scale bar left panels = 120  $\mu$ m, right panels = 100  $\mu$ m).

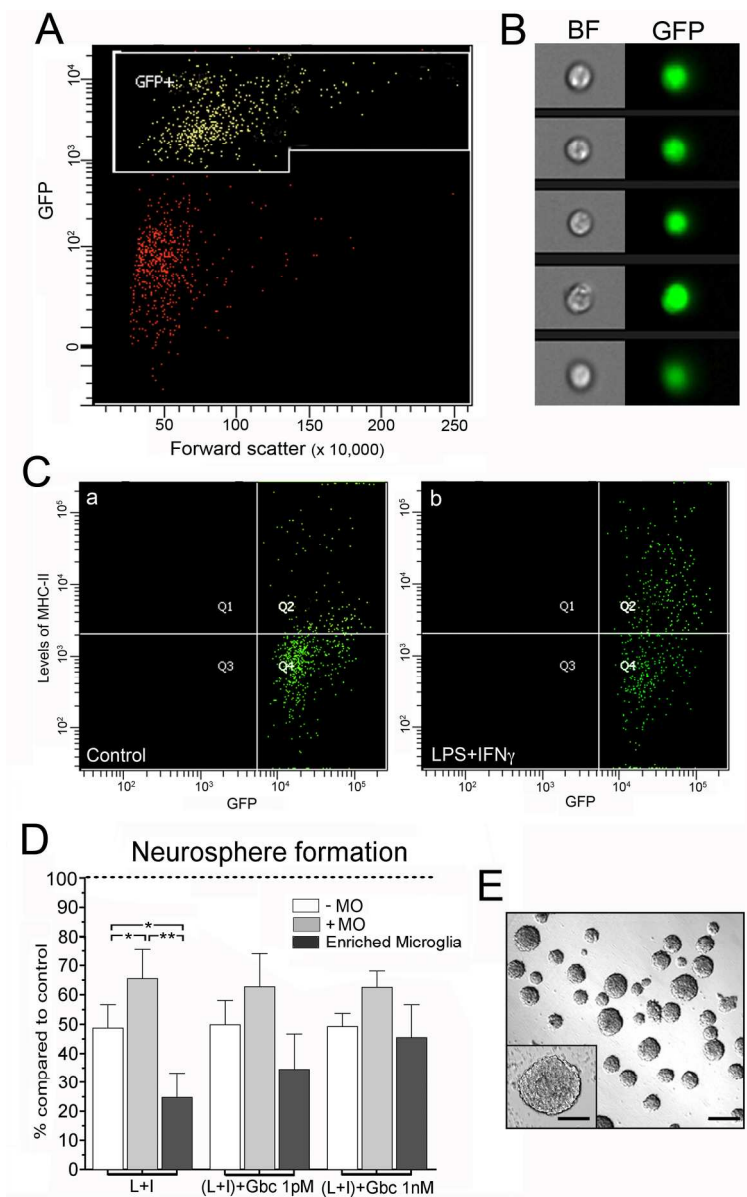


Figure 1. Inhibition of neurosphere formation is reversed by microglial KATP-channel blockade in vitro. Csf1r-GFP transgenic mice were used and (A) the GFPpos population (microglia) was sorted using FACS. (B) Photomicrographs of single GFPpos cells from dissociated Csf1r-GFP cortex taken during flow cytometry. (C) FACS analysis of MHC-II expression levels from isolated microglia (a) or after 24 h LPS+IFN $\gamma$  treatment (b). (D) An overall reduction in neurosphere number was observed after LPS+IFN $\gamma$  (L+I) treatment in all conditions when compared to control ( $p < 0.01$ ;  $n = 3$  to 4 per experimental condition). Glibenclamide (Gbc) reversed the inhibitory effect of the enriched microglial culture in a dose-dependent manner. (E) Representative image of SVZ neurospheres. (Results are shown as mean  $\pm$  SEM; one-way ANOVA followed by the Bonferroni post hoc test; \* $p < 0.05$  and \*\* $p < 0.01$ ; scale bar in E = 150  $\mu$ m, and E insert = 50  $\mu$ m). 157x249mm (300 x 300 DPI)



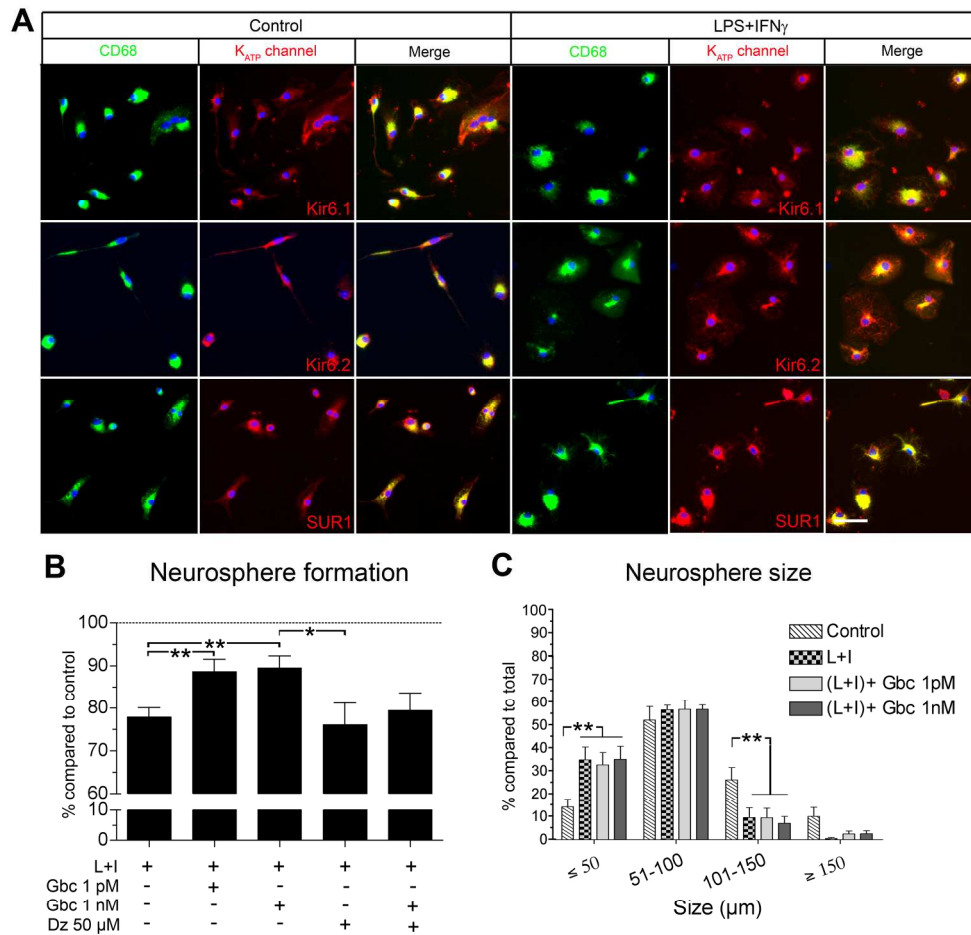


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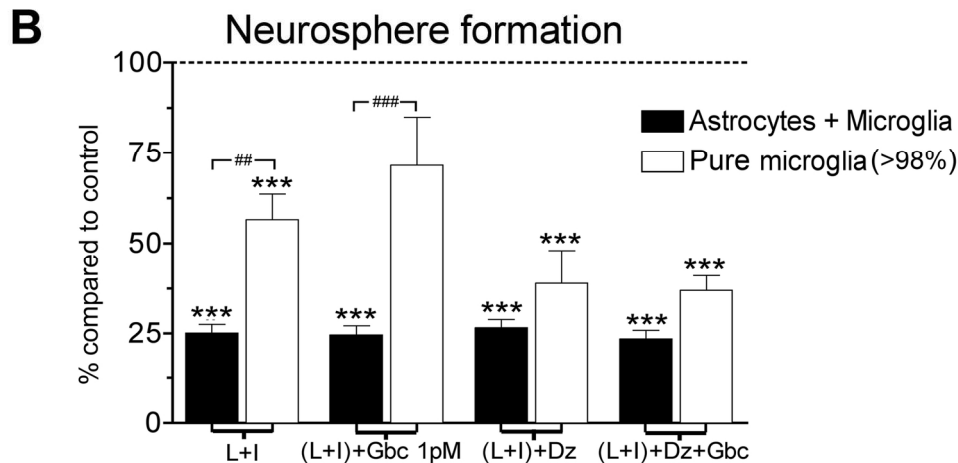
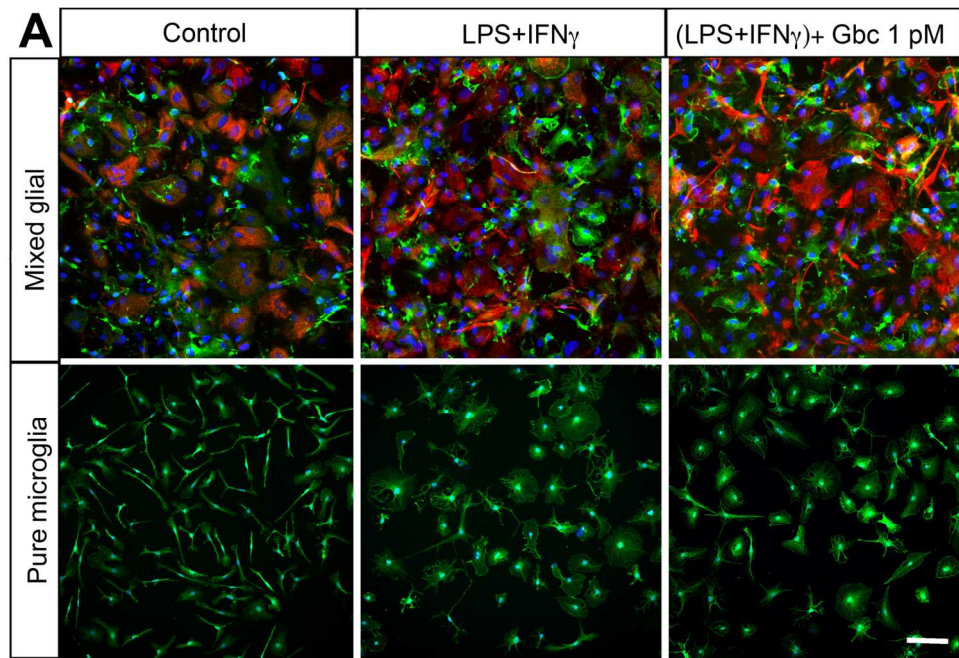


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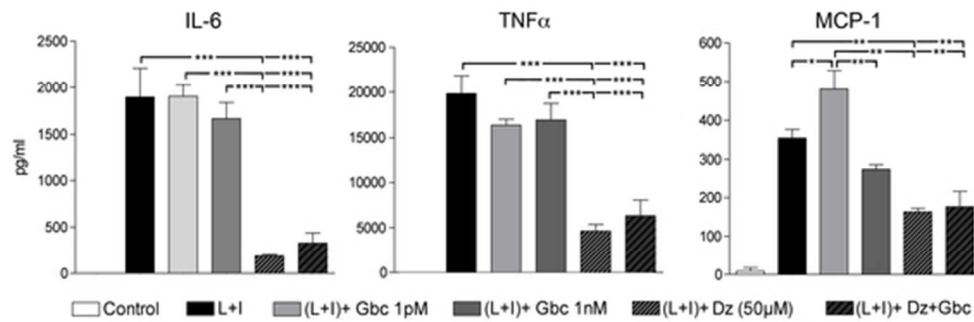


Figure 4. Microglial KATP channel blockade increased the production of the MCP-1 (A) The concentration of microglial-released cytokines and inflammatory mediators from primary pure microglial-conditioned media as a result of treatment with LPS+IFN $\gamma$  (L+I), glibenclamide (Gbc; KATP-channel blocker) and/or diazoxide (Dz; KATP-channel opener) was quantified ( $n = 4$  per experimental condition). LPS+IFN $\gamma$  treatment increased the overall production of IL-6, TNF $\alpha$  and MCP-1 ( $p < 0.001$ ) and the addition of diazoxide reversed this effect. Glibenclamide at 1 pM boosted the production of MCP-1 when compared to LPS+IFN $\gamma$  and 1 nM glibenclamide. Values are given as mean  $\pm$  SEM. For pairwise group comparisons, a one-way ANOVA followed by the Bonferroni post hoc test was used. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .  
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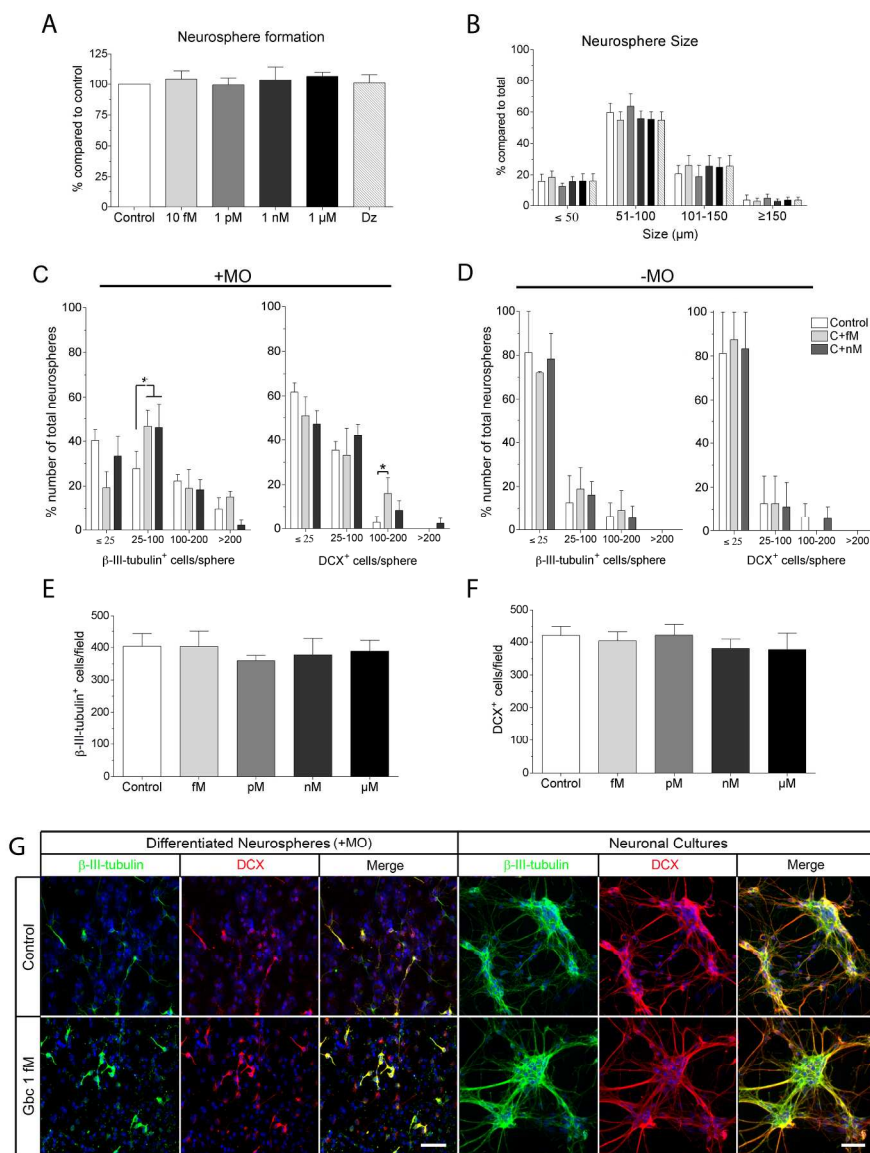


Figure 5. In the presence of microglia, neurosphere cultures produce more neurons after treatment with glibenclamide. (A) Neurosphere formation and (B) neurosphere size is unaffected by glibenclamide (Gbc) treatment at a range of concentrations ( $n = 4$  per experimental condition). (C & D) Percentage of total neurospheres containing  $\beta$ -III-tubulin and DCX positive cells in presence (+MO; C) or absence (-MO; D) of microglia. Note that glibenclamide treatment presented more neurospheres containing higher number of  $\beta$ -III-tubulin or DCX expressing cells only in the presence of microglial cells ( $n = 4$  per experimental condition). (E & F) Survival of hippocampal neurons labeled with  $\beta$ -III-tubulin positive cells (E) or DCX (F) was unaltered in the presence of glibenclamide ( $n = 6$  per experimental condition). (G) Representative photomicrographs of  $\beta$ -III-tubulin or DCX-positive cells in differentiated neurospheres in presence of microglia (left panel) and neuronal cultures (lower panel). Statistics: one-way ANOVA followed by the Bonferroni post hoc test.  $*p < 0.05$ . Scale bar for left panels = 120  $\mu$ m; right panels = 100  $\mu$ m. 219x279mm (300 x 300 DPI)

