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

Fco. Javier Ortega González

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

Fco. Javier Ortega González

PhD Thesis

Barcelona, March 2012

Chapter 5.
RESULTS BLOCK II

Glibenclamide Enhances Neurogenesis and Improves Long-term Functional Recovery after Transient Focal Cerebral Ischemia

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Stroke (Submitted)

5.1 Summary

Glibenclamide (Gbc) is neuroprotective against cerebral ischemia in rats. Here we studied whether Gbc enhances long-term brain repair and improves behavioral recovery after transient cerebral ischemia in rats. Special emphasis was placed on the contribution of microglia to these processes. Male Wistar rats were subjected to transient middle cerebral artery occlusion (MCAO) for 90 min. A low dose of Gbc (total 0.6 μg) was administered i.v. 6, 12 and 24 h after reperfusion. The behavioral outcome was assessed by the limb-placing, tapered ledged beam-walking, and cylinder tests, and the Morris water-maze during a 30-day follow-up. The rats were then perfused for histology to evaluate infarct size, apoptosis, neurogenesis and angiogenesis. Primary rat microglial cultures were used to determine whether Gbc binds specifically to microglial K_{ATP}-channels. Pro-inflammatory stimuli increased Gbc-labeling in the microglial cell cultures. Gbc did not decrease corticostriatal infarct size of MCAO rats, however increased migration of doublecortin-positive cells from the subventricular zone towards the ischemic lesion. Gbc also increased the number of NeuN-positive and BrdU-positive neurons in the peri-infarct cortex, as well as BrdU-positive neurons in the intact cortex and hippocampus. Gbc enhanced angiogenesis in the hippocampus of MCAO rats. Consequently, performance in the limb placement test on postoperative days 22 to 29 and in the cylinder test on postoperative day 29 improved in Gbc-treated MCAO rats. Therefore, acute blockade of K_{ATP}-channels by Gbc did enhance ischemia-induced neurogenesis and angiogenesis in MCAO rats, which was associated with improved sensorimotor outcome.

5.2 Introduction

Low doses of glibenclamide (Gbc) reduce cerebral edema and infarct volume and decrease mortality in experimental stroke models.(Simard et al. 2010; Simard et al. 2009) A retrospective study has also shown that patients with diabetes mellitus taking Gbc have a better neurological outcome,(Kunte et al. 2007) although this finding has recently been challenged.(Favilla et al. 2011)

Gbc blocks sulfonylurea receptor 1 (SUR1), which is expressed in neurons, astrocytes, oligodendrocytes and capillaries under ischemic conditions.(Simard et al. 2006) SUR1 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 to TRPM4 to form NC_{Ca-ATP}-channels has also recently been questioned (Sala-Rabanal et al. 2012). The function of the K_{ATP} channel is however unclear.

Microglial cells are brain-resident macrophages that present electric currents similar to the K_{ATP}-channels.(McLarnon et al. 2001) Cerebral ischemia leads to an accumulation of microglia in the subventricular zone (SVZ),(Thored et al. 2009) from where newly formed neuroblasts migrate towards the ischemic boundary zone. Besides these canonical neurogenic niches, recent data provided new insights of other brain regions with the same capacity, such as, the cortex. One example are the studies where cortical progenitors proliferation in situ after brain injury have been found (Shimada et al. 2010; Zhang et al. 2001), and more importantly, that stroke-induced cortical neurogenesis has also been found in the adult human brain, even in advanced age patients (Jin et al. 2006; Macas et al. 2006; Minger et al. 2007). Nonetheless, whether microglial cells can mediate all these processes and the fate of the newborn neurons is still controversial, but microglial role had been strongly pointed out as a neurogenesis regulator (Aarum et al. 2003; Ekdahl et al. 2009; Walton et al. 2006).

We hypothesized that early blockade of microglial K_{ATP}-channel by Gbc after cerebral ischemia enhances ischemia-induced neurogenesis in the SVZ, thus leading to an improved functional outcome. To test this hypothesis, we first showed that Gbc binds to microglial K_{ATP}-channels by analyzing the binding of fluorescently tagged Gbc in primary rat microglial cultures. Next, we treated rats with low doses of Gbc early after focal cerebral ischemia and evaluated the long-term neuroprotection, neurogenesis, angiogenesis and functional recovery.

5.3 Material and Methods

Rat primary microglial cultures were activated with 0.1 mg/mL of LPS and 0.05 ng/mL of IFNγ. To assess if rat primary microglia express the K_{ATP} channel, 48 h after activation, cells were incubated for 30 min HBSS supplemented with 500 nM Gbc–BODIPY-FL (green fluorescent dye). We visualized immunolabeled microglial cells against CD11b directly on the plate.

Focal ischemia of 90 min was produced by tMCAO using the intraluminal filament technique (Van Groen et al., 2005). We divided 34 rats into the following three groups:

- 10 sham rats
- 12 tMCAO rats treated with vehicle (0.01 M PBS) i.v.
- 12 tMCAO rats treated with Gbc 0.2 µg i.v. 6, 12 and 24 h after the onset of reperfusion (Total dose 0.6µg).

In order to analyze the long-term cell proliferation, we administered intraperitoneal injections of BrdU (50 mg/kg in PBS 50 mmol/L) to 90 min tMCAO rats from postoperative days 4 to 8.

We evaluated behavioral outcome using the limp-placing test, forelimb asymmetry test and the tapered/ledged beam-walking test (1 month follow-up groups). Moreover, at the end of the study, what corresponds with postoperative days 27 to 29, rats were subjected to the Morris water maze test.

To quantify lesion volume, animals were deeply anesthetized 30 days after reperfusion, sacrificed for histological and inmmunohistochemical analysis. Sections stained with Haematoxylin-Eosin (H&E) were randomly selected to determine the total infarct volume whereas degenerating neurons were detected by Fluorojade B staining.

Were measured the areas of astrogliosis and microgliosis on GFAP-immunostained and IB4-stained sections. Moreover, stereological methods were used to quantify immunolabeled neurons (NeuN) at the core of the lesion (-0.8 to -1.8 of bregma).

To detect apoptotic cells in tMCAO animals, we performed double immunofluorescence labeling using specific antibodies against Caspase-3 and neuronal, astroglial or microglial markers.

To detect neuroblasts in migration we used doublecortin immunohistochemistry and proliferating cells were determined by labeling of the BrdU-positive cells.

We studied angiogenesis in several brain regions by measuring vessel diameter on RECA-1 immunolabeled samples.

5.4 Results

5.4.1 Primary Cultured Rat Microglial Cells

We analyzed the binding of the fluorescently tagged Gbc (glibenclamide BODIPY FL; green fluorescence) in primary rat microglial cultures to determine whether Gbc binds specifically to microglial K_{ATP}-channels. When microglial cells were in resting state, Gbc labeling was localized in the perinuclear space (**Fig. 5.1**). Forty-eight hours after LPS+IFNγ activation, Gbc-labeling was present in the plasmalemmal membrane, even in those that were pre-incubated with 1 nmol/L Gbc for 30 min before LPS+IFNγ activation. This finding indicates that not all the K_{ATP}-channel binding sites were blocked (**Fig. 5.1**).

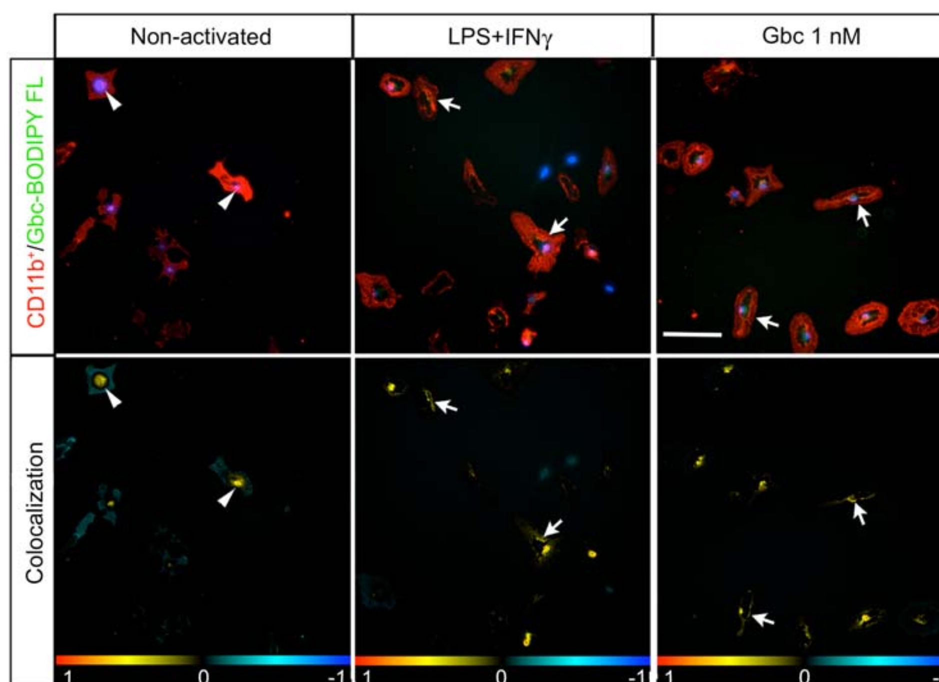

Fig. 5.1. Localization of Gbc (Gbc BODIPY FL; green fluorescence) in rat microglial primary culture. Non-activated, cultures activated with LPS+IFNγ for 48 h or cultures pre-treated (30 min) with 1 nmol/L Gbc before activation are shown in the upper row. Microglial cells were labeled with an anti-CD11b (red) antibody and Hoechst (blue) to stain the nuclei. Lower row shows respective colocalization of the red and green channels, where the yellow denotes the presence of the Gbc binding in microglial cells. Arrowhead denotes peri-nuclear colocalization and arrows shows surface labeling. Bar=20 μm.

5.4.2 Infarct Size, Gliosis and Apoptosis

There was no difference in infarct volume size between vehicle- and Gbc-treated MCAO rats (**Fig. 5.2**A). However, stereological counting showed higher number of NeuN-positive cells in the peri-infarct area in Gbc-treated MCAO rats (P<0.05) (**Fig. 5.2B**).

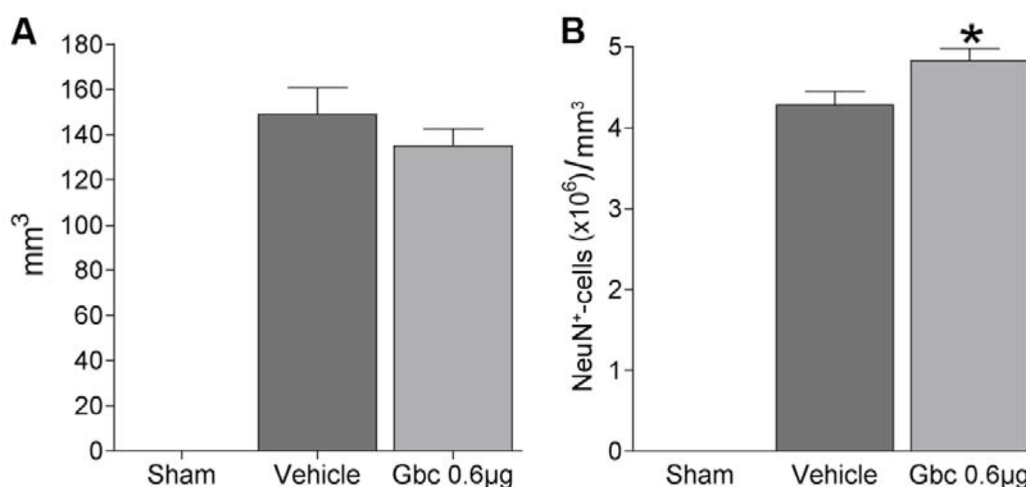

Fig. 5.2. Gbc does not affect corticostriatal infarct size in MCAO rats (**A**). Quantification of NeuN-positive cells in the peri-infarct cortex (**B**). Results are mean±SEM (n=9-12). **P*<0.05 (compared with vehicle-treated MCAO rats).

The pattern and cell density of microglia/macrophages (IB4-stained) and astrocytes (GFAP-immunopositive) in ischemic animals showed activation, as evidenced by their reactive morphology (**Fig. 5.3C**). Gbc did not modify MCAO-induced astrogliosis or microgliosis in the cortex (**Fig. 5.3A, B**).

Only scattered Fluoro-Jade B (FJB)-positive neurons were detected in the thalamus (**Fig. 5.4A**). Apoptosis was not detected by cleaved caspase-3 staining in any of the brain sections analyzed (**Fig. 5.4B, C).** However, cleaved caspase-3 staining co-localized with the microglial/macrophage marker CD11b in the peri-infarct region (**Fig. 5.4D**).

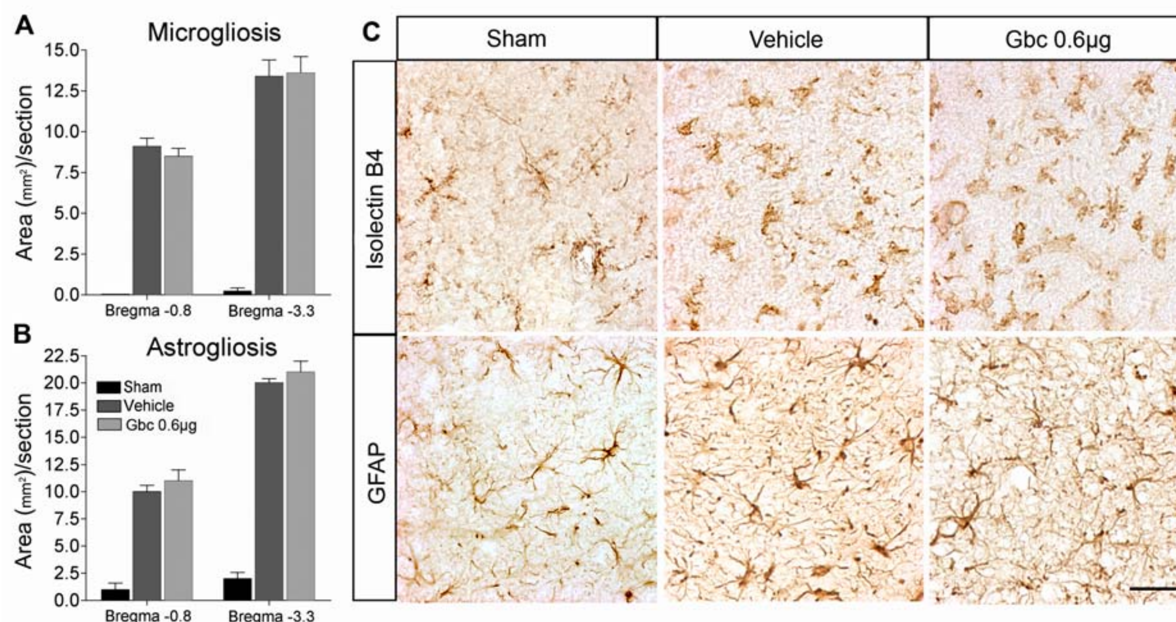

Fig. 5.3. Quantitative data for IB4 (microglia) and GFAP (astrocytes) immunoreactivity in the cortex (**A, B**). Gbc treatment did not modify microgliosis or astrogliosis in MCAO rats. Representative micrographs showing IB4 and GFAP immunoreactivity in sham-operated rat, vehicle- and Gbc-treated MCAO rats (**C**). Bar 25 µm.

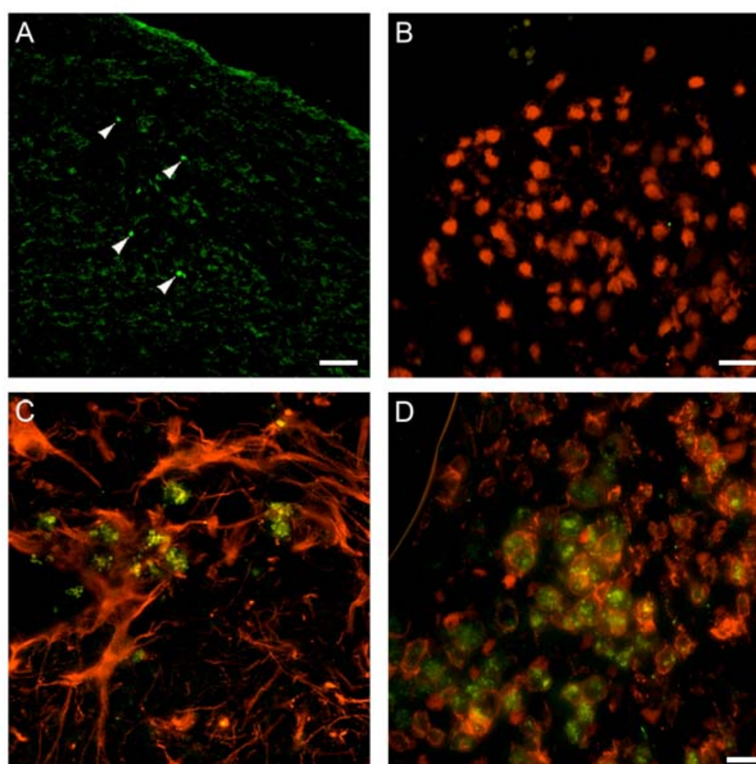

Fig. 5.4. Representative micrographs showing neurodegenerative neurons and apoptotic cells in the peri-infarct zone of MCAO rats. Fluorojade B-positive neurons in the thalamus (**A**), and double staining of anti-caspase-3 (green) and NeuN (in red) (**B**), GFAP (**C**) and CD11b (**D**). No differences between experimental groups were observed. Bar 60 µm (**A**), 20 µm (**B**), 10 µm (**C-D**).

5.4.3 SVZ Cell Proliferation

A group of rats (n=3-6) were sacrificed three days after MCAO for DCX immunohistochemistry. Results showed increased migration of neural progenitor cells from the SVZ toward the infarct in MCAO rats **(Fig. 5.5A, B)** as reported previously (Arvidsson et al. 2002; Parent et al. 2002). We found more migrating neuroblasts in Gbc-treated than in vehicle-treated MCAO rats (P<0.001, **Fig. 5.5A, B).** Neuroblasts migration towards the infarct core was still active 30 days after MCAO (**Fig. 5.5C**). Microglia from the SVZ became reactive after ischemia, but Gbc treatment did not modify their morphology (**Fig. 5.5A).**

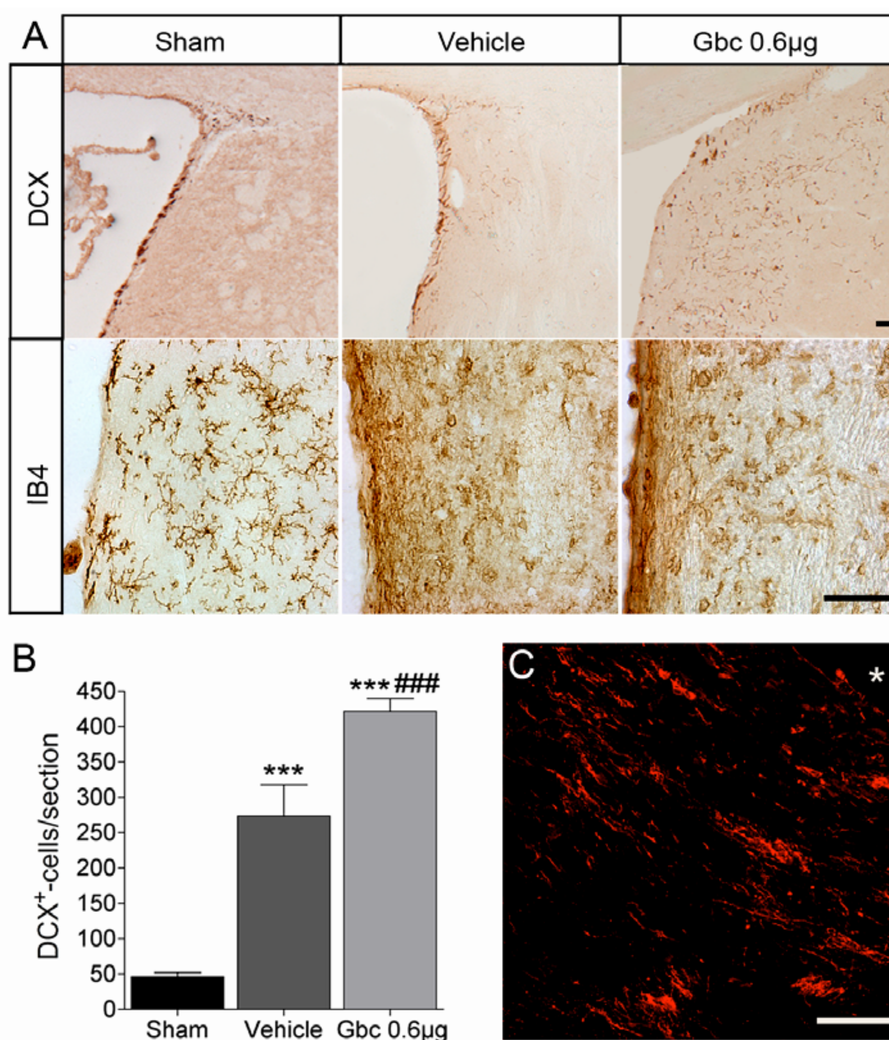

Fig. 5.5. Representative micrographs of doublecortin (DCX) and microglia/macrophage (IB4) immunostaining in the SVZ of sham-operated rat and vehicle- and Gbc- (0.6μg) treated MCAO rats three days after ischemia (**A**). Quantitative analysis of the migrating neuroblasts/section (**B**). DCX-positive cells (red) were still present in the peri-infarct tissue 30 days after MCAO (asterisk denotes the striatal lesion core) (**C**). Results are mean±SEM (n=3-6). ****P*<.001 (compared with sham rats); ###*P*<.001 (compared with vehicle-treated MCAO rats). Bar=50 μm.

5.4.4 Neurogenesis

Vehicle-treated MCAO rats showed more BrdU-positive cells in the cerebral cortex ($P<0.001$) and hippocampal parenchyma ($P<0.05$) than sham-operated rats (**Fig. 5.6A).** Gbc treatment increased the number of BrdU-positive cells in the cerebral cortex ($P<0.001$), in the CA1 pyramidal layer ($P<0.05$; Gbc vs. sham) and in the hippocampal parenchyma ($P<0.05$; Gbc vs. sham) (**Fig. 5.6A).**

Double immunohistochemistry (**Fig. 5.6A, B**) showed an increased number BrdU/NeuN-positive cells in the cerebral cortex in vehicle-treated MCAO rats ($P<0.001$) and this was further boosted by Gbc treatment ($P<0.001$; Gbc vs. vehicle-treated MCAO rats). A slight increase in the number of BrdU/NeuN-positive cells was found in the CA1 region and in the SGZ of the hippocampus, although this increase was not statistically significant compared to the vehicle group. In addition, the other neuronal population markers (e.g., parvalbumin, tyrosine hydroxylase, calbindin) did not co-localize with BrdU (data not shown).

The number of BrdU/GFAP-positive cells increased in the cortex of vehicle-treated MCAO rats ($P<0.001$) (**Fig. 5.6A, B**). This observation points to astroglial proliferation caused by the lesion. Gbc-treated MCAO rats showed increased astroglial proliferation in the cortex ($P<0.001$) and in the hippocampal parenchyma ($P<0.01$) compared to sham-operated rats and vehicle-treated MCAO rats.

An increased number of BrdU/IB4-stained cells was observed in the cortex ($P<0.001$), in the CA1 pyramidal layer ($P<0.001$) and in hippocampal parenchyma ($P<0.001$) in MCAO rats (**Fig. 5.6A, B**).

To study whether Gbc modifies the fate of the neuroblasts as they migrate through the rostral migratory stream (RMS), we quantified the number of BrdU-positive cells in the olfactory bulb 30 days after MCAO. No differences in the number of these cells were found between MCAO groups in the olfactory bulb (542±26 BrdU-positive cells/section for sham, 518±37 for vehicle-treated group and 580±28 for Gbc-treated group). These observations indicate that Gbc strengthens intrinsic neurogenic processes.

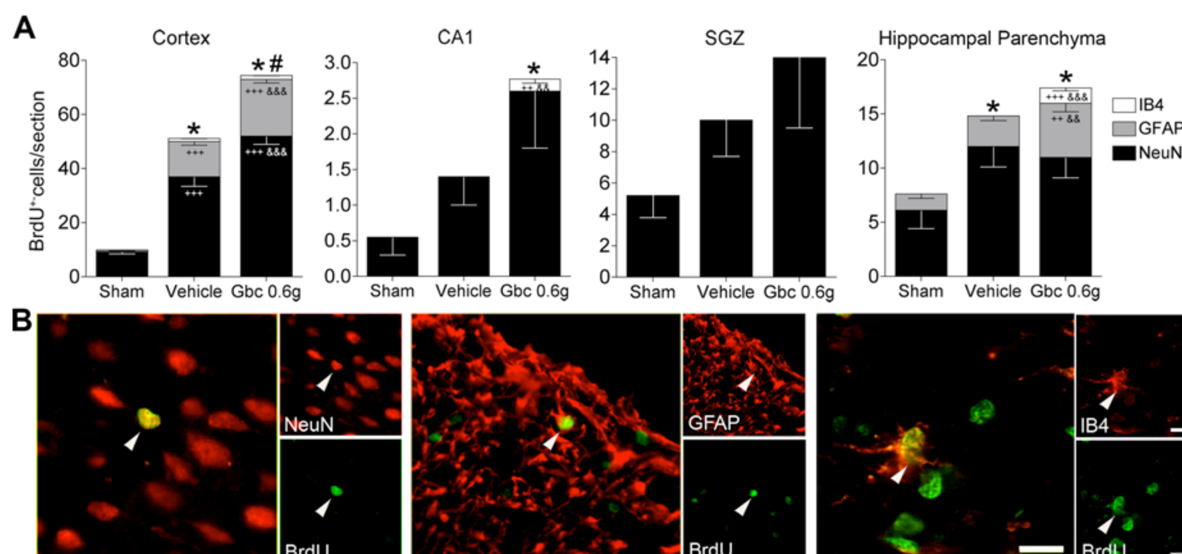

Fig. 5.6. Quantitative analysis of the total number of BrdU-positive cells/section and relative number of neurons (NeuN), astrocytes (GFAP) and microglia (IB4) double stained BrdU-positive cells in the cortex, CA1 pyramidal cell layer, subgranular zone (SGZ) and hippocampal parenchyma (**A**). Confocal photomicrographs of the rat cortex showing colocalization of BrdU (in green) with NeuN, GFAP and IB4 (**B**). Gbc increased the total number of BrdU-positive cells after MCAO. These cells preferentially co-localized with markers of neurons and astrocytes. Bar 10 µm. Results are mean±SEM (n=9-10). **P*<.05 (total BrdU-positive cells compared with sham rats); #*P*<.05 (total BrdU-positive cells compared with vehicle-treated MCAO rats); ++*P*<.01,+++*P*<.001 (cell type compared with sham rats); &&*P*<.01, &&&*P*<.001 (cell type as compared with vehicle-treated MCAO rats).

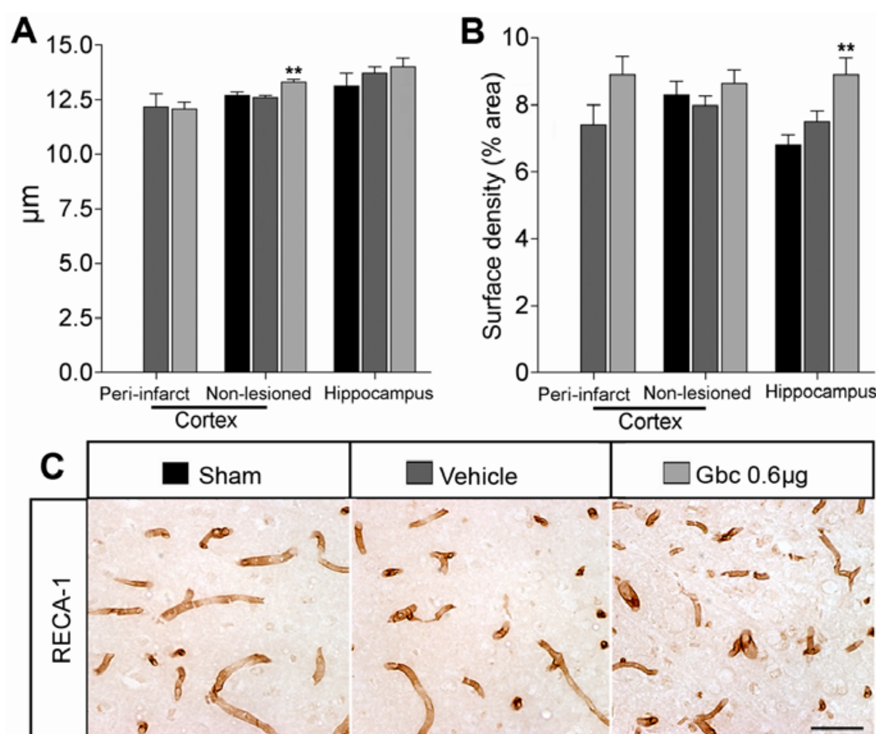

Fig. 5.7. Quantitative analysis of the microvessel diameter (**A**) and RECA-1 immunoreactivity (**B**) in the cortex and hippocampus after MCAO. Gbc administration increased microvessel diameter in the non-lesioned cortex and RECA-1 immunoreactivity in the hippocampus. Representative micrographs of RECA-1 immunoreactive microvessels in the cortex of sham-operated rat, vehicle- and Gbc-treated (0.6µg) MCAO rats (**C**). Bar 50 µm. Results are mean±SEM (n=6-11). ***P*<.01 (compared to sham and vehicle-treated MCAO rats).

5.4.5 Angiogenesis

We studied angiogenesis in several brain regions by measuring vessel diameter and RECA-1 immunohistochemistry 30 days after ischemia (Czéh et al. 2010). The diameter of vessels increased in the ipsilateral cortex of Gbc-treated MCAO rats **(Fig. 5.7A, C)**. Quantitative analysis of RECA-1 immunoreactivity showed increased microvessel density in the ipsilateral hippocampus of Gbc-treated MCAO rats ($P<0.01$) (**Fig. 5.7B, C**). This difference was not detected in the peri-infarct cortex, although a slight increase was found.

5.4.6 Recovery of Sensorimotor and Cognitive Functions

All ischemic animals presented initial severe impairment in the limb-placing test after MCAO, followed by spontaneous recovery during the 29-day follow-up (**Fig. 5.8A**). Limb-placing scores between sham-operated and MCAO rats were different in all post-operative time points ($P<0.01$). MCAO rats treated with Gbc showed an improved score compared to the vehicle group on postoperative days 22 to 29 ($P<0.05$; $P<0.01$).

Spontaneous forelimb use was analyzed by the cylinder test (**Fig. 5.8B**). MANOVA showed an overall group effect ($P<0.05$), but no significant group x time interaction, thereby indicating that forelimb use differed between groups. A *post hoc* analysis revealed that forelimb use between sham-operated and vehicle-treated MCAO rats was different ($P<0.05$), but not between sham-operated and Gbc-treated MCAO rats. Forelimb use in the MCAO groups was different from that in sham-operated rats on postoperative day 7 ($P<0.01$). Impaired forelimb use in MCAO rats treated with Gbc was recovered and these animals showed similar performance to that of sham-operated rats at the end of the follow-up period.

The effect of Gbc on recovery of hindlimb function in MCAO rats was analyzed by tapered/ledged beam-walking. MANOVA showed an overall group effect ($P<0.01$) and a group x day interaction ($P<0.01$). However, there was no difference between Gbc-treated and vehicle-treated MCAO rats ($p=0.81$).

Results from the Morris water-maze on post-operative days 26 to 28 showed that escape latency, length and swimming speed did not differ between groups. No significant differences were observed in the number of passes over the removed

platform or the time rats spent in the target quadrant (probe trial). However, vehicle-treated MCAO rats spent more time in the outermost zone (zone 3; P<0.05) and less time in the middle zone (zone 2; P<0.01) compared to sham-operated animals (**Fig. 5.8C, D**). The search strategy in Gbc-treated MCAO rats was similar to that of sham-operated animals.

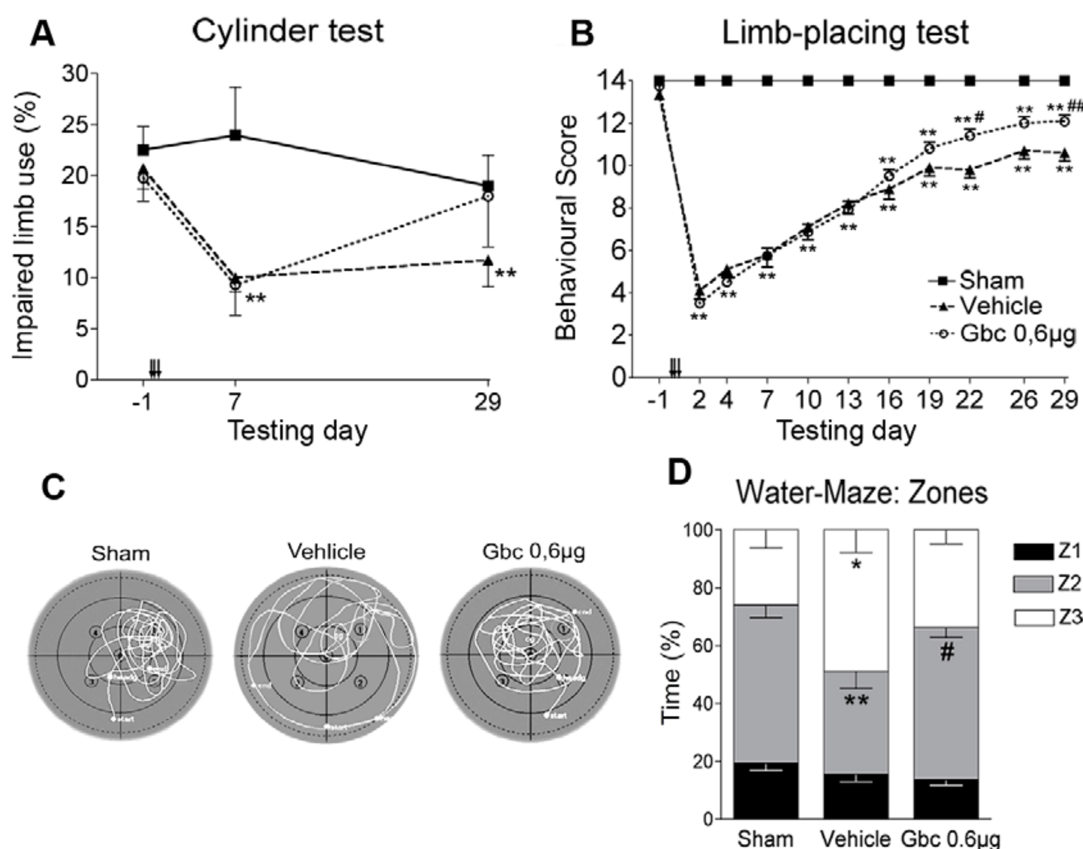

Fig. 5.8. Contralateral forelimb use (cylinder test) (**A**) and limb-placing scores (**B**) during a 29-day follow-up after MCAO. Swimming strategy in the probe trial test (Morris water-maze) on postoperative day 28 (**C, D**). Here the values are shown as a percentage of time spent in equal zones of the pool. Zone 1 refers to the inner annulus and zone 3 the outermost annulus. Results are mean±SEM (n=9-12). **P*<.05; ***P*<.01 (compared with sham rats); #*P*<.05; ##*P*<.01 (compared with vehicle-treated MCAO rats).

5.5 Discussion

Previous preclinical and clinical data suggest neuroprotective role for Gbc (Kunte et al., 2007; Simard et al., 2009). Here we showed that Gbc also enhanced long-term brain repair in MCAO rats possibly through blockade of microglial K_{ATP}-channel and this was associated with improved behavioral recovery.

We recently reported that 72 h after MCAO, activated microglia/macrophages in

the ischemic hemisphere express the K_{ATP}-channel. Furthermore, we showed that 48 h after inflammatory stimuli the murine microglial BV2 cell line increases K_{ATP}-channel expression (Ortega et al., 2012a). The subunit SUR1 has particularly high affinity for Gbc and ATP (Dörschner et al., 1999; Matsuo et al., 2000). We confirmed now these results in primary rat microglial cultures. When microglial cells became activated after pro-inflammatory stimuli, they showed higher specific Gbc-labeling and the signal extended to the plasmalemmal membrane. This suggests that after microglial activation, K_{ATP}-channel expression is increased and translocated to the cell surface where it may regulate phagocytic activity and release of cytokines/chemokines (Ortega et al., 2012a; Virgili et al., 2011). Although Gbc possibly also blocks the SUR1 subunits in K_{ATP}- or $NC_{Ca\text{-}ATP}$-channels expressed in neurons (Toulorge et al., 2010), astrocytes(Simard et al., 2009) and capillary endothelial cells (Simard et al., 2010), the present data strongly suggest a crucial role for microglia in activation of protective processes and brain repair after cerebral ischemia.

Focal cerebral ischemia promotes neurogenesis in the SVZ and the SGZ of the dentate gyrus and induces SVZ neuroblast migration towards the ischemic boundary (Arvidsson et al., 2002; Thored et al., 2009). Recent data have provided new evidence that also the cerebral cortex has the same capacity (Shimada et al., 2010). Interestingly, stroke-induced cortical neurogenesis has been found in the adult human brain, even in the elderly patients (Jin et al., 2006; Macas et al., 2006). Whether microglial cells influence neurogenesis and the fate of the newborn neurons is still controversial. In the present study, ischemia increased neuroblast proliferation and migration towards the lesion 3 days after ischemia/reperfusion and this persisted up to one month. Inhibition of the microglial K_{ATP}-channel using Gbc led to a further increase in the number of migrating neuroblasts, thereby indicating that Gbc modifies the cell lineage choice or enhances progenitor cell proliferation and migration. It has been proposed that microglial cells participate in modifications of stem cell proliferation, migration and/or differentiation into neurons after stroke and status epilepticus through producing trophic factors and inflammatory cytokines/chemokines that enhance neurogenesis (Butovsky et al. 2006; Ekdahl et al. 2009). The co-culture of neural stem cells with microglial cells or

microglia-conditioned medium has also demonstrated that microglia provide secreted factors that are essential for neuroblast and neuron generation (Aarum et al., 2003; Walton et al., 2006).

To study the fate of proliferative cells, we injected BrdU into the animals from days 4 to 8 after ischemia/reperfusion. We found that the number of BrdU-labeled cells co-expressing NeuN, a mature neuronal marker, increased in the peri-infarct area of the cortex 30 days after reperfusion and this was potentiated by Gbc. Although we cannot rule out the possibility that neural progenitors migrate from the SVZ and establish themselves in the ipsilateral cortex network, we found no co-localization of BrdU-positive cells with the classical the rostral migratory stream derived neuronal markers (i.e., calbindin, calretinin, tyrosine hydroxylase, parvalbumin). These newborn cortical neurons may have originated from potential resident neural stem cells within the cortex (Shimada et al., 2010). Several authors have proposed that these endogenous quiescent neural stem cells are present in the cerebral cortex and that their proliferation and differentiation to mature neurons is induced by ischemic insults (Gu et al., 2000; Jiang et al., 2001). Although the number of these cells is very small, strategies to foster the intrinsic neurogenesis would be highly relevant for clinical approaches to facilitate neural repair and functional recovery.

Also angiogenesis is activated after cerebral ischemia. Interestingly, Gbc further increased the diameter of microvessels in the non-lesioned cortex and RECA-1 immunoreactivity in the hippocampus. In both regions we also found stimulated glial proliferation. These observations are consistent with those of Fantin and colleagues (2010), who reported tissue macrophages/microglia are associated with angiogenesis in various developing organs. Vasculature attracts microglial cells and stimulates them to release angiogenic factors (Rymo et al., 2011), with subsequent growth stimulation of neural stem cells (Androutsellis-Theotokis et al., 2010). Furthermore, angiogenesis is considered a key feature of ischemic stroke recovery and neuronal post-stroke re-organization (Slevin et al., 2006).

In summary, here we demonstrated that early K_{ATP}-channel blockade by Gbc after stroke strengthens the microglial response and enhances neurogenesis in the cortex following cerebral ischemia, which was temporally associated with improved long-

term sensorimotor recovery and memory. These data identify K_{ATP}-channels as a multifaceted target to modulate microglia and promote both neuroprotective and brain repair mechanisms.

5.6 Sources of funding

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5.7 Disclosures

NM and MJR hold an EU patent (No. WO2006/000608). The other authors report no disclosures.

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