SOCIETÀ ITALIANA DI FARMACOLOGIA

MODELLO PER INVIO RELAZIONE DI METÀ E FINE PERIODO

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TIPOLOGIA DI BORSA RICEVUTA: Borsa SIF-MSD

TIPOLOGIA DI RELAZIONE (es.: metà periodo o finale): FINALE

TITOLO DELLA RELAZIONE: Role of the new GPR17 receptor in the reparative processes after ischemia

RELAZIONE:

Background

Stroke is the 2nd most common cause of death in the European Union and a leading cause of disability (Wittchen et al., 2011) for which there are currently no effective therapies.

The discovery that oligodendrocyte precursor cells (OPCs) are present within the adult brain and are recruited in the penumbra has raised the hypothesis of repairing ischemic injury by promoting endogenous remyelination (Li et al., 2010; Zhang et al., 2013). Previous data indeed showed that these cells are activated in the peripheral area of the ischemic "core" and spontaneously differentiate into mature oligodendrocytes (OL), the myelin-repairing cells, thus helping re-establishing cell-to-cell communication within the lesioned area (Li et al., 2009). Unfortunately, this process is "per se" normally insufficient to allow complete recovery and, for this reason, new remyelination strategies are required.

In this respect, our laboratory has identified and characterized a new P2Y-like receptor, GPR17 and has also demonstrated that its activation by endogenous ligands (e.g. UDP-glucose or LTD_4) promotes OPC maturation (Fumagalli et al., 2011). However, none of these ligands is selective for GPR17 and, therefore, suitable for *in vivo* administration. To overcome this limitation, through an in silico screening performed in collaboration with Prof. Ivano Eberini at our Department, we have identified new GPR17 ligands (Eberini et al., 2011) and during the first six months of the SIF-MSD fellowship, we demonstrated, through *in vitro* experiments, that the new GPR17 ligand compound Asinex promotes OPC differentiation and myelination.

In order to test this compound in an animal model of brain ischemia, we induced stroke by the permanent middle cerebral artery occlusion (pMCAO) in GPR17iCreER^{T2}:CAG-eGFP transgenic mouse line. In these mice, upon tamoxifen administration, the enhanced Green Fluorescence Protein (eGFP) is expressed in OPCs in which the Gpr17 promoter is active, without affecting the physiological expression and function of the receptor. In this way, all GPR17+ cells can be traced by fluorescence microscopy throughout animal's life (Vigano et al., 2016).

By using this mouse line, we recently showed that GPR17 expressing cells constitute a subpopulation of OPCs that is reluctant to differentiate under healthy conditions. On the contrary, after stab wound-induced cerebral injury, GPR17+ cells rapidly react–to the damage, suggesting that they represent a 'reserve pool' of adult progenitors maintained for repair purposes.

During the first six months of the fellowship, we showed that, also after brain ischemia, the number of GPR17 expressing cells (that are the GFP+ cells) was significantly increased in the regions surrounding the lesion compared to the contralateral side starting from 72 h after MCAo and that this increase can be, at least partially, ascribed to their increased proliferation rate.

During these last six months, we:

- analysed the final fate of GFP+/BrdU+ cells in order to discriminate between cells which continue to proliferate and cells which, likely, undergo differentiation;
- assessed the possibility that the increased number of GFP+ cells can also be a consequence of an augmented migratory capacity of these cells which are recruited to the injured area;
- completed the fate mapping analysis of the GFP+ cells mainly focusing at later time points after ischemia.

Methods

Permanent middle cerebral artery occlusion (MCAo)

11 weeks old GPR17-iCreER^{T2}:CAG-eGFP report mice received 10 mg tamoxifen, three times by gavage once every second day. After 3 weeks of wash-out from tamoxifen, the mice were anesthetized and underwent permanent MCAo, as previously described (Sironi et al., 2003). Starting from 1 day before surgery, mice received BrdU (1 mg/ml) in drinking water supplemented with 2% sucrose for 15 days. Mice were sacrificed at 72 hours and 1, 2, 4 and 8 weeks after MCAo (n=3-5 mice for each time point). In parallel, sham-operated mice were included in each experimental group.

Immunofluorescence analysis

Mice were perfused with phosphate-buffered saline and then 4% paraformaldehyde in phosphate-buffered saline for at least 25 minutes. Brains were removed, post-fixed 1 hour in the same solution and cryoprotected in 30% sucrose solution until precipitation at 4°C. Coronal section of 20-µm were incubated with the following primary antibodies: chicken anti-GFP, rat anti-BrdU rabbit anti-NG2, rabbit anti-GPR17 and rabbit anti-GSTpi. Incubation with primary antibodies was made overnight at 4°C in PBS with 1% normal goat serum and 0.1%-0.3% Triton-X 100. The sections were then exposed to secondary fluorescent antibodies. For triple GFP/NG2/BrdU labelling, staining of BrdU was performed last incubating them with HCl 2N for 45 minutes at 37° C. Nuclei were labelled with Hoechst 33258.

For the quantitative analysis, four anatomically defined regions of interest (ROIs) surrounding the ischemic lesion were identified (ventral and dorsal cortex, corpus callosum and striatum). Within the ROIs, specific fields were acquired at 40X magnification using a confocal microscope. Each image was the result of a Z-stack acquisition.

Sholl and Skeleton analysis

The Sholl and the Skeleton analysis method were used to quantify GFP+ cell morphology in immunofluorescent images of brain. Confocal images were acquired at each ipsilateral and contralateral dorsal cortex. Images were converted to a binary signal and, then, analysed using Simple Neurite Tracer plugin of the Fiji software.

Results

Effects of brain ischemia on proliferating GFP+ cells

During the first six months of the fellowship, we demonstrated that GPR17 expressing-OPCs actively responded to the ischemic insult increasing their proliferation rate (Vigano et al., 2016). In order to better characterize the pool of these proliferating cells and to monitor changes in their phenotype during the post-ischemic period, during these last six months we set up triple immunostainings with GFP, NG2 and BrdU antibodies to recognize recombinant cells, precursor cells and proliferating cells respectively (Fig. 1). This approach gave us the possibility to discriminate, within the recombined proliferating cell population, the cells that still express the early OPC marker NG2 from the cells that have already lost this marker and are likely proceeding into the differentiation process (Fumagalli et al., 2011). The analysis were, then, performed at 4 time points after MCAo (72h, 1w, 2w and 8w) to investigate whether the fate of these proliferating cells is modified by brain ischemia.

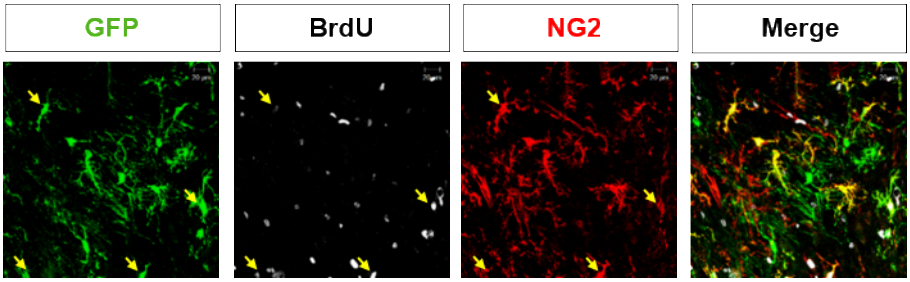

Figure 1. Set up of triple immunostaining with GFP/BrdU/NG2 antibodies.
Representative images of GFP (green), BrdU (white) and NG2 (red) immunofluorescence in the cortical cortex of mice subjected to MCAo. Arrows (yellow) show GFP^+-$BrdU^+$ cells positive for NG2. Scale bar=20 μm.

Effects of brain ischemia on GFP+ cells' morphology

In the regions surrounding ischemic lesion, OPCs have been reported to undergo early morphological alterations resembling monopolar and bipolar shape with elongated processes (Tanaka et al., 2003), which have been interpreted as a prerequisite for their migration (Schmidt et al., 1997). To investigate whether pMCAo induces changes of GFP+ cells' morphology, we set up a morphological analysis using the Simple Neurite Tracer plugin of the Fiji software. We performed this analysis only with well-identified or isolated GFP+ cells. Confocal images (merge of 8-µm z-stack at 2-µm intervals, LSM510 META Zeiss, 40X magnification) were acquired at each ipsilateral and contralateral cortical cortex. The local contrast of GFP channel was enhanced to optimize the visualization of cellular processes and the resulting images were converted to a binary signal. Cellular branches were manually traced using the Simple Neurite Tracer plugin.

For the Sholl analysis, the centre was pointed at the centroid of the nucleus. Concentric circles were automatically drawn beginning at 2 µm from the centre and increasing 2 µm with every circle and the number of intersections made by the extending cellular processes with each circle were automatic counted and used as a measure of process branching (Fig. 2 B and E).

For the Skeleton analysis, the Simple Neurite Tracer automatically generates a skeleton of the manually traced cells (Fig. 2 C and F). This analysis gave us information on the cell morphology complexity.

This morphological analysis was performed at 3 time points (72h, 1w and 2w). We analysed at least 18 cells for hemisphere of each mouse. For statistical analysis, we considered the Critical

Radius, the Critical Value and the Ramification Index obtained from the Sholl analysis and the number of branches, the average and the maximum branch length, the number of primary branches and the number of endpoints and of junctions from the Skeleton analysis.

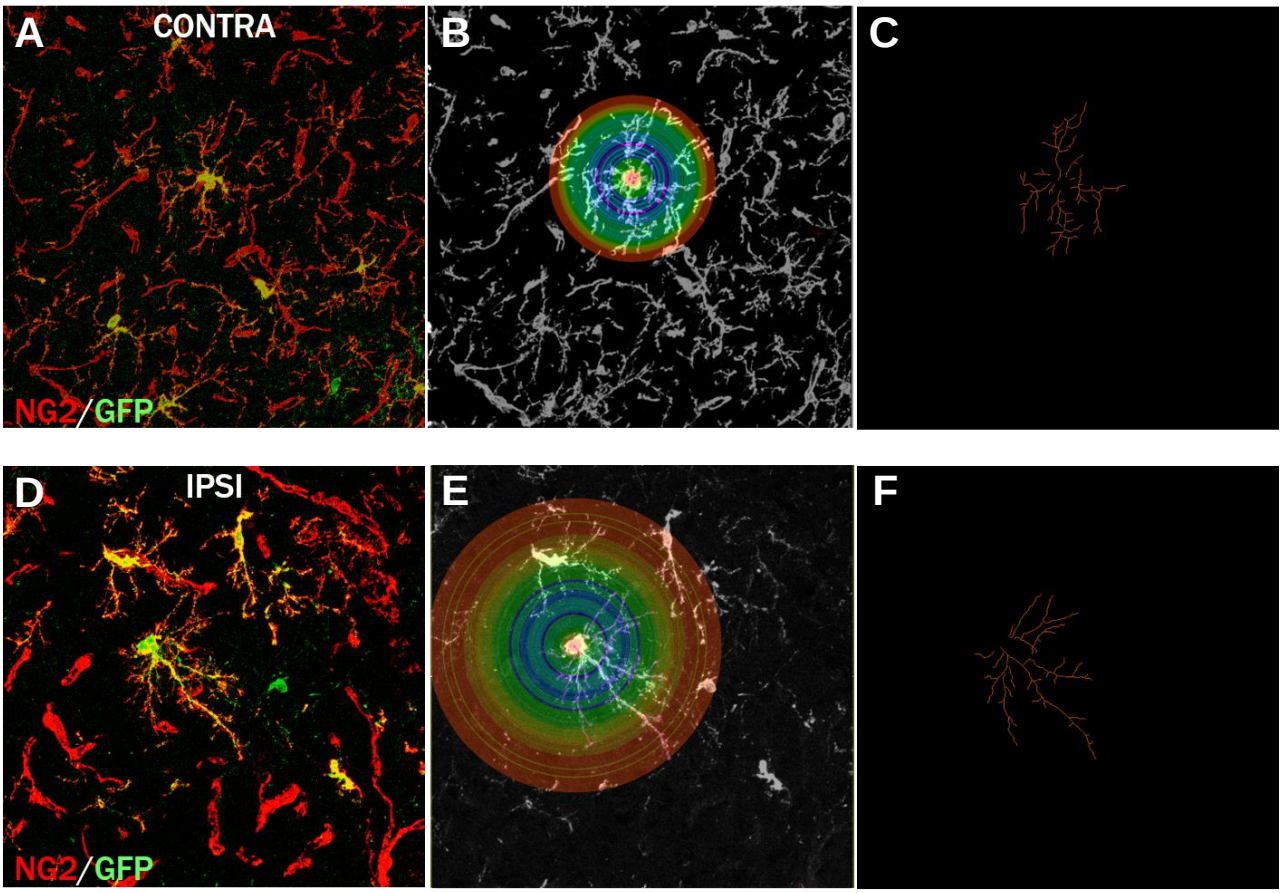

Figure 2. Morphological analysis of GFP+ cells.
(A, D) Representative images of a GFP+ (green) and NG2+ (red) cell in contralateral and ipsilateral dorsal cortex of mice 72h after MCAo selected for the morphological analysis. Scale bar=20 μm. **(B, E)** Illustrations of Sholl analysis used to quantify process branching. The number of intersections made by the extending processes with each circle were automatic counted and used as a measure of process branching. (**C, F)** Illustrations of skeletonized cells; Skeleton analysis was used to get information on the cell morphology complexity.

Effects of brain ischemia on the fate of GFP+ cells

To investigate how brain ischemia influenced the fate of GFP+ cells, we performed a detailed fate mapping analysis of the GFP+ cells at different times after MCAo. During these last six months, we set up double immunostainings with GFP antibody to identify recombinant cells and with antibodies for stage-specific oligodendrocyte markers: NG2 for OPCs, GPR17 for immature OL and GSTpi for mature OL (Fig. 3). Then, we counted the number of double positive cells and the number of cells which express only GFP. This analysis was performed at 3 time points after MCAo (2w, 4w and 8w) and gave us information on how the proportion of OPCs, immature OLs and mature OLs can be modified by brain ischemia over time.

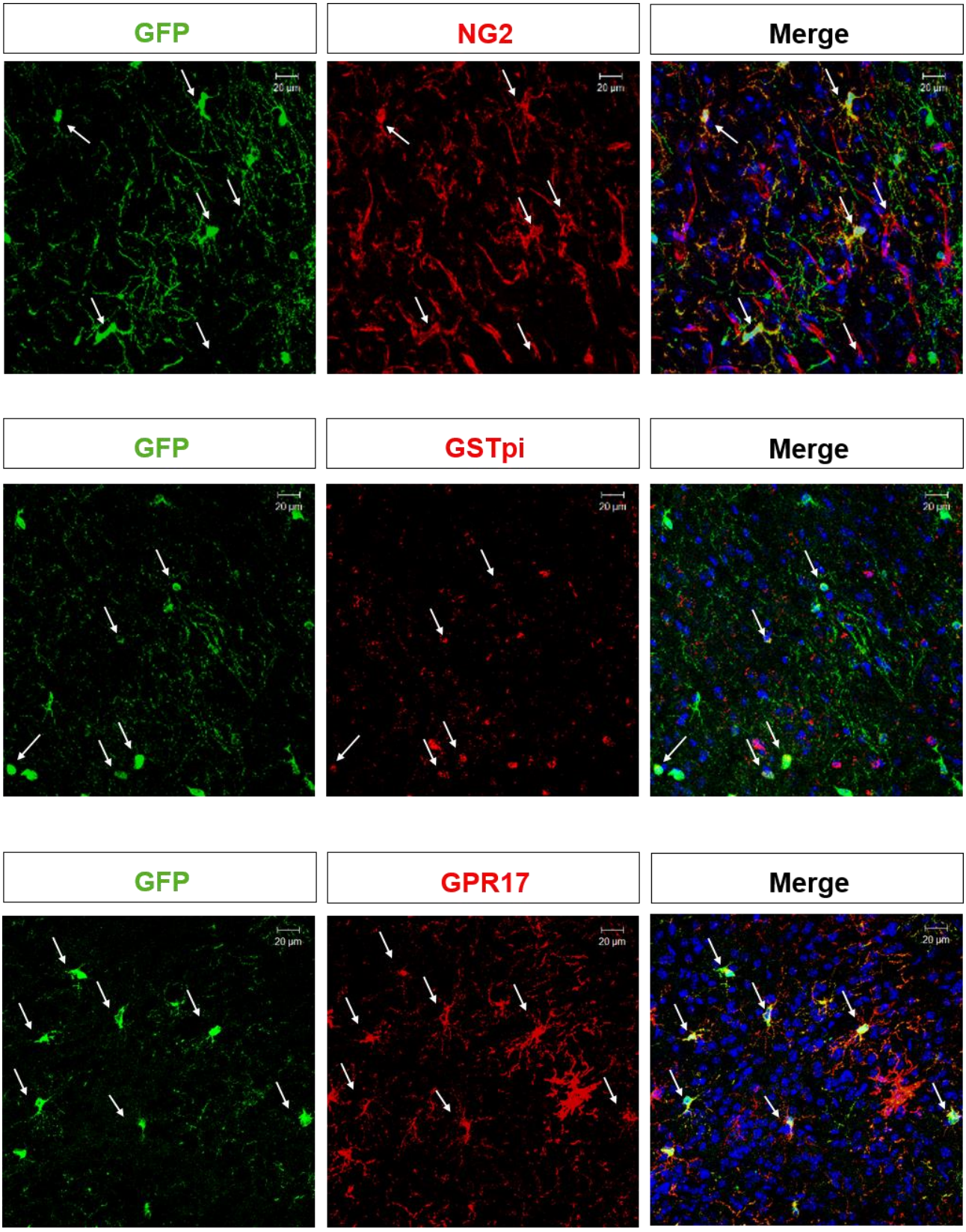

Figure 3. Set up of double immunostaining with GFP/NG2/GPR17/GSTpi antibodies.
Representative images of GFP (green) and NG2 (red) immunofluorescence, GFP (green) and GSTpi (red) immunofluorescence and of GFP (green) and GPR17 (red) immunofluorescence**.** Scale bar=20 µm.

A manuscript presenting the results obtained during this year of fellowship is currently in preparation for the peer-reviewed international journal "Brain".

Conclusions and future perspectives

The data obtained during this year clearly demonstrated that GPR17 expressing-OPCs actively and rapidly responded to the ischemic insult as indicated by their increased proliferation rate and their marked morphological changes, mainly during acute post-ischemic phase. We are now performing *in vivo* and *in vitro* experiments to assess the migratory capability of the GFP+ cells after MCAo or in presence of some inflammatory molecules.

Future experiments will be carried out in order to test the Asinex compound (Eberini et al., 2011) in GPR17-iCreERT2:GFP mice subjected to MCAo.

Overall, the detailed characterization of the GFP+ cells' behaviour after MCAo presented in this project, together with the future *in vivo* pharmacological experiments, will contribute to validate re-myelination via GPR17 as a new regenerative approach in stroke.

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