Expression of the new P2Y-like receptor GPR17 during oligodendrocyte precursor cell maturation regulates sensitivity to ATP-induced death

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EXPRESSION OF THE NEW P2Y-LIKE RECEPTOR GPR17 DURING OLIGODENDROCYTE PRECURSOR CELL MATURATION REGULATES SENSITIVITY TO ATP-INDUCED DEATH.

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Introduction: 911

Materials and Methods: 756

Results: 2583

Discussion: 1804

Bibliography: 1156

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Abstract SIAMO A 256, MAX 250. VEDETE SE VI SEMBRA PIU' CHIARO

The previously orphan GPR17 receptor is the first dualistic P2Y receptor, responding to both uracil nucleotides and cysteinyl leukotrienes, and it is crucially involved in brain reaction to injury. Interestingly, GPR17 is also one of the 3 genes exclusively expressed by adult neural stem cells from brain neurogenic areas, suggesting its possible role in cell fate determination. Since progenitor cells can be also found in brain parenchyma, we aimed at characterizing GPR17 expression and function in primary astrocyte/precursor cell cultures from mouse cerebral cortex. Both in vivo and in vitro, GPR17 is expressed by a subpopulation of oligodendrocyte precursor cells (OPCs), but not by astrocytes.When these cells are grown in vitro upon proliferating conditions, GPR17 expression is down regulated bythe presence of growth factors . Upon shifting to differentiating conditions, GPR17 is expressed by the totality of O4-positive immature oligodendrocytes in cultures originally grown with growth factors, but only by a subpopulation of O4-positive cells when cultures were originally grown without growth factors, suggesting that the latter are more strongly committed towards differentiation. In both cases, CNPase- and MBP-positive mature cells progressively loose GPR17 expression. Interestingly, GPR17 expression sensitizes cells to adenine nucleotide-induced cytotoxicity, whereas its activation promotes cell differentiation towards a more mature phenotype. Our results suggest that under physiological conditions GPR17 activation contributes to OPCs differentiation, but upon high extracellular nucleotide concentrations, as during trauma and ischemia, its expression sensitizes cells to cytotoxicity. This double-edged sword role of GPR17 might therefore be exploited to unveil new therapeutic approaches to acute and chronic brain disorders.

Introduction

The discovery of the endogenous potential of the adult brain to react to ischemic or traumatic events has open up new avenues in searching for effective approaches to acute and chronic neurodegenerative pathologies. It has been known for several years that parenchymal astrocytes act in response to deleterious events with the process named "reactive astrogliosis" (Buffo et al., 2010) mainly aimed at sealing the core of the damaged tissue to avoid spreading of toxic substances to the surrounding healthy tissue. More recently, it has emerged that when reacting to harmful conditions, astrocytes also reacquire some of the typical features of stem cells (i.e., proliferative ability, multipotency and self-renewal potential; Buffo et al., 2008), and can therefore contribute to the genesis of new cells.

Also oligodendrocyte progenitors (OPCs) reside in the adult brain parenchyma and remain undifferentiated after completion of the physiological myelination process (Chang and Chan, 2010). The question has recently risen whether they can be recruited to a lesion site (for example, in the case of demyelinating pathologies or ischemic brain damage), thus representing a possible source of newly generated cells (Chang and Chan, 2010).

In fact, despite this huge endogenous reparative potential and the initial repair response in the weeks and months after injury, data available so far indicate that very few newborn cells are still viable and integrate within the damaged brain area at longer time points, leading to very little functional improvement (Wiltrout et al., 2007). This suggests that the extracellular milieu is somehow restrictive or even abortive for such self-repair efforts (Buffo et al., 2010; Chang and Chan, 2010). Thus, understanding the nature of signals instructing or limiting the genesis of new cells from both OPCs and reactive astrocytes can help fostering brain capability to react to acute and chronic insults.

Extracellular purine and pyrimidine nucleotides (eNTs) exert their effects by activating 7 subtypes of ligand-gated and 8 subtypes of G protein-coupled receptors (named $P2X_{1\text{-}7}$ and $P2Y_{1,2,4,6,11,12,13,14}$ receptors, respectively; Burnstock, 2008). eNTs have all the

characteristics to fulfill a role as modulators of the endogenous reparative potential of the brain, since they induce cell proliferation, differentiation or even death (Neary and Zimmermann, 2009), play crucial roles during development (Dale, 2008), and are massively released under pathological conditions (Abbracchio et al., 2009).

The role of eNTs in the modulation of reactive astrogliosis following injury has been investigated for many years, leading to the demonstration of a synergy with growth factors in supporting astrocytic proliferation and the appearance of a reactive phenotype (for review, see Buffo et al., 2010). Very recently a similar synergic effect between uracil eNTs and growth factors has been also demonstrated on the proliferation of adult progenitor cells from the subventricular zone grown as neurospheres (Milosevich et al., 2006; Grimm et al., 2009), while conflicting results have been obtained for adenine eNTs (Mishra et al., 2005; Milosevich et al., 2006; Stafford et al., 2007).

The ability of extracellular nucleotides to promote increases in the intracellular calcium concentrations in oligodendrocytes at different stages of maturation through the activation of P2 receptors has been first demonstrated more than 15 years ago (Kirischuk et al., 1995). Nevertheless, to date the role of purines and pyrimidines in modulating OPCs functions is yet-to-be fully understood, especially under pathological conditions. The nucleoside adenosine, derived from breakdown of ATP released from astrocytes, has been demonstrated to promote axon myelination (Stevens et al., 2002). On the other hand, ATP can induce oligodendrocyte death by activating the $P2X_7$ ion channel receptors (Wang et al., 2009).

The previously orphan receptor GPR17 has been recently identified as an interesting modulator of oligodendrocyte functions. It can be activated by uracil nucleotides (i.e., UDP and UDPsugars; Ciana et al., 2006; Lecca et al., 2008; Pugliese et al., 2009; Temporini et al., 2009), and can therefore represent a new member of the P2Y receptor family. Also cysteinyl leukotrienes (i.e., LTC_4 and LTD_4) act as agonists at GPR17, which is therefore the first

"dual" non-peptidic receptor characterized so far (Parravicini et al., 2008). Both classes of GPR17 agonists are massively released at any site of injury following traumatic, ischemic or inflammatory conditions; indeed, our group has recently demonstrated a key role for this receptor in pathological situations, such as brain ischemia and traumatic spinal cord injury (Ciana et al., 2006; Lecca et al., 2008; Ceruti et al., 2009). Interestingly, GPR17 has been also recognized as one of the three genes exclusively expressed by human adult neural precursor cells in the hippocampus, when compared to embryonic cells (Maisel et al., 2007). This observation raises the possibility that GPR17 is also involved in precursor cell differentiation, maturation or lineage specification.

Our recent data also demonstrate GPR17 expression by OPCs in adult brain parenchyma and its key role in driving cell maturation and reaction to harmful conditions (Lecca et al., 2008). Following brain ischemia, parenchymal GPR17+ OPCs start proliferating in the peri-injured area, suggesting an attempt to initiate remyelination. Moreover, the in vitro exposure of cortical OPCs to the GPR17 endogenous ligands UDPglucose (UDPglc) and LTD_4 promoted their progression towards a more mature phenotype (Lecca et al., 2008).

Based on these premises, we deemed it interesting to evaluate the expression and function of GPR17 in primary mixed (astrocytes+OPCs) glial cultures grown in vitro under both standard culturing conditions or in conditions promoting cell reactivity, i.e. in the presence of growth factors (GFs) namely, EGF and bFGF (Williams et al., 2007), followed by a shift to a less permissive environment, able to promote the typical features of cell differentiation (Heins et al., 2002). Moreover, we exposed cultures to micromolar eNTs concentrations, as seen in vivo upon inflammatory of traumatic/ischemic injury, to understand whether GPR17 expression could confer specific survival or maturation behaviors.

Materials and Methods

Preparation of cultures

Primary astrocytic cortical cultures (also containing OPCs, see Results) were obtained from P5 mice, as described by Heins and coworkers (2002). Cultures were grown in Dulbecco's Modified Eagles Medium plus F12 nutrient (DMEM-F12) with 10% fetal calf serum (FCS) and 5% horse serum (HS) (without growth factors, GFs, and therefore named medium -GFs) or with the addition of 10ng/ml EGF and 10ng/ml bFGF (named medium +GFs). After 10 days, cells were collected and replated onto poly-D-lysine coated 13 mm diameter glass coverslips (6-8*10^4 cells/well) or 35 mm diameter petri dishes (2*10^5 cells/well), and allowed to attach for additional 24 hours in their original medium. Cultures were then switched to a chemically defined medium (Differentiating Medium; DM), containing DMEM-F12 and B27 mixture (Invitrogen, Italy), and fixed at various time points (see below). In selected experiments, cells originally grown in medium -GFs were switched to medium +GFs or cells were grown in the presence of either EGF or bFGF alone (see Results).

Pharmacological treatments

Cultures were subjected to pharmacological treatments with nucleotides when switched to DM. ATP, and UDP-glucose (UDPglc; Sigma Aldrich, Milan, Italy) were dissolved in sterile water at 10^{-2} M concentration and stored at -20^{o}C. 10^{-3} M solutions were freshly prepared in DM at the time of treatment and added to cultures to give a final concentration of 10^{-4} M. Due to high rate of hydrolysis upon culturing conditions, nucleotides were re-added to cultures every 24 hours. Cells were then fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) at various time points (24-72 hours).

Immunocytochemistry

Fixed cells were subjected to immunocytochemistry as previously described (Lecca et al., 2008). The following antibodies and final dilutions were used. Primary antibodies: rabbit anti-GPR17 (1:100; Ciana et al., 2006); mouse anti-glial fibrillary acidic protein (GFAP; 1:600, Cell Signaling, Euroclone, Milan, Italy); mouse anti-O4 (1:100; Chemicon, Millipore, Milan, Italy); mouse anti-2',3'-Cyclic Nucleotide 3'-Phosphodiesterase (CNPase; 1:100, Chemicon, Millipore); mouse anti-myelin basic protein (MBP; 1:100, Chemicon, Millipore). Primary antibodies were incubated at 37^{o}C for 1 hour or overnight at 4^{o}C. When double immunostaining for O4 and GPR17 was performed, a 10 min post-fixation with 4% PFA was applied after O4 primary and secondary antibodies, followed by immunostaining for GPR17. Secondary antibodies: goat anti –rabbit or -mouse IgG conjugated with either Alexa Fluor® 488 or 555 (1:600; Invitrogen, Milan, Italy; 1 hour at RT). Nuclear counterstaining was obtained by 20-min incubation with the Hoechst33258 dye (1:10,000 in PBS; Molecular Probes, Invitrogen, Milan, Italy). Coverslips were then mounted in Dako Fluorescence Mounting Medium (Dako Italia, Milano, Italy), and analyzed under a Zeiss Axiovert 8400 microscope (Carl Zeiss, Milan, Italy), equipped with a CCD camera module. Positive cells were counted in an identical area in each coverslip (2 diameters, corresponding to approximately 100 optical fields) at a 40X magnification. Results were replicated in at least 3 independent experiments with 3/4 coverslips for each condition.

Immunohistological procedures

For histological analysis, 2-4 month old C57BL/6 mice were anesthetized with an intraperitoneal administration of a ketamine/xylazine solution (ketamine, 100 mg/kg; Ketavet, and xylazine, 5 mg/kg; Rompun; Bayer, Milan, Italy ENRICA VA BENE? HO TOLTO BAYERN LEVERKUSEN), and transcardially perfused with 4% paraformaldehyde in phosphate buffer. All experiments were performed according to the NIH guidelines, the European Communities Council Directive of 24 November 1986 (86/609/EEC), the Italian

law for care and use of experimental animals (DL116/92) and approved by the Italian Ministry of Health and the Bioethical Committee of the University of Turin. Brains were post-fixed over-night, cryoprotected, and stained according to standard protocols (Buffo et al., 2005). Brains were cut coronally at -5°C with a cryostat in 30 μm-thick coronal slices collected in PBS. The sections were processed to detect the expression of GPR17 (with a rabbit in-house made affinity purified antibody; 1:10,000; Ciana et al, 2006), GFAP (rabbit anti-GFAP, 1:1,000; Dakopatts, Glostrup, Denmark) and MBP (mouse anti-MBP, 1:2,000; Covance, Princeton, NJ). Incubation with primary antibodies was performed overnight at 4°C in PBS with 1.5% normal serum and 0.25% Triton-X 100. The sections were then exposed for two hours at room temperature to secondary antibodies (Anti-rabbit AlexaFluor® 546, 1:500, Invitrogen; Anti-mouse Cy3, 1:500, Jackson ImmunoResearch Laboratories, West Grove, PA). GPR17 was detected by means of the high sensitivity tyramide signal amplification kit (Perkin Elmer, Monza, Italy), according to the manufacturer's instruction. The stained sections were mounted on microscope slides with Tris-glycerol supplemented with 10% Mowiol (Calbiochem, LaJolla, CA).

Images were collected with a Leica TCS SP5 confocal microscope (Leica Microsystems, Wetzlar, Germany). Adobe Photoshop 6.0 (Adobe Systems, San Jose, CA) was used to adjust image contrast.

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RNA extraction and RT-PCR analysis

For RNA isolation, cells were detached from culture dishes with PBS+4% EDTA, centrifuged, and lysed in TRIZOL® reagent (Invitrogen, Milan, Italy). Total RNA was extracted according to manufacturer's instructions, quantified spectrophotometrically, and

1μg was treated with RQ1 RNase-free- DNase (Promega, Milan, Italy) and then reverse-transcribed with Superscript II RNA H^- Reverse Transcriptase (200U/sample; Invitrogen), as previously described (Ceruti et al., 2005). cDNAs were amplified in each PCR assay with Platinum Taq DNA Polymerase (1.25 U/sample; Invitrogen) in a 25 μl reaction mixture containing 20 pmoles of 5' and 3' primers in a standard PCR buffer (50 mM KCl, 1,5 mM $MgCl_2$, 20 mM Tris-HCl, pH 8.4). Control samples lacking reverse transcriptase were processed in parallel with the same experimental protocol. All PCR reagents were purchased from Applied Biosystems (Milan, Italy)**.** Amplifications were performed in a GeneAmp 9700 thermal cycler (Applied Biosystems) for 35 cycles (typically for each cycle: 95ºC/45 sec, 45 sec at the selected annealing temperature; 72ºC/45 sec), after an initial denaturation at 95ºC for 2 min, by utilizing sense and antisense primers specifically designed for all the known murine P2X and P2Y receptor subtypes (Bianco et al., 2005) and for murine GPR17 receptor sequence (Ciana et al., 2006). cDNA from each sample was also amplified with β-actin primers to insure similar input of RNA and efficiencies of reverse transcription (Ceruti et al., 2005). See Supplementary Table 1 for primer sequences.

Real time RT-PCR

Total RNA was isolated and extracted as described above, and suspended in diethylpyrocarbonate-treated (DEPC; Sigma-Aldrich) water to avoid its degradation. Potential contaminating DNA was removed with DNase (Deoxiribonuclease I; Sigma-Aldrich) prior to the reverse transcription (RT) reaction. Concentration and purity of extracted RNA were evaluated prior and after the DNase treatment by spectrophotometry. RNA samples were stored at -80°C. 1 mg of total RNA was reverse-transcribed to single stranded cDNA using the commercially available High-Capacity cDNA Archive Kit (Applied Biosystems), according to the manufacturer's instructions. cDNA samples were stored at -20°C.

Quantitative Real Time PCR was carried out using the ABI Prism 7000 Sequence Detection System instrumentation (Applied Biosystems) in combination with commercial Taqman Gene Expression Assays (Applied Biosystems) to determine the amount of GPR17 (Mm02619401_s1), $P2Y_1$ (Mm00435471_m1) and the housekeeping gene beta-actin (Actβ, Mm00607939_s1). MBP??DECIDERE SE LO METTIAMO O NO, SI VEDANO RISULTATI REAL TIME PCR amplifications were performed on cDNA samples corresponding to a final RNA concentration of 200 ng. Negative controls consisting in no template or RT negative reactions were performed. Expression data were analysed by means of the 7000 v1.1 SDS instrument software (Applied Biosystems). A relative quantification approach was used, according to the 2^{-ddCT} method (Livak and Schmittgen, 2001).

Statistical analysis

All results are expressed as mean±S.E.M. of at least three independent experiments. Statistical significance between groups was derived from one-way ANOVA followed by the Dunnet Post Hoc Test performed with the SPSS17.0 program for MacOS X. A p value lower than 0.05 was considered as significant.

Results

Growth factors affect GPR17 expression in glial progenitor cells.

Immunohistochemical analysis of cortical sections confirmed our previously published results (Ciana et al., 2006; Lecca et al., 2008; Ceruti et al., 2009), showing that the new P2Y-like receptor GPR17 is not expressed in vivo by GFAP-positive astrocytes (Fig. 1A), but by a population of highly ramified cells, resembling oligodendrocyte precursors at a non-myelinating stage of maturation (as demonstrated by the lack of colocalization with the myelin marker MBP; Fig. 1B).

As a first step in the evaluation of the expression and function of GPR17 in vitro, we set up primary glial cultures from mouse cortex and grew them with 10% FBS and 5% HS either in the absence (medium -GFs) or in the presence of GFs for 11 days (medium +GFs).

Characterization of cultures with an anti-GFAP antibody clearly showed the positive staining for this astrocytic marker in the vast majority of cells in culture, either when grown for 11 days in medium -GFs (not shown) or in +GFs (green in Fig. 1A, T0). A significant difference in the total number of cells was detected between the two culturing conditions (3043±197 cells in cultures grown +GFs vs 1559±93 cells in cultures grown -GFs, *$P<0.05$ by ANOVA followed by Dunnett test; data from 3 independent experiments run in triplicate), indicating a mitogenic and pro-survival effect exerted by GFs on astrocytes in culture, as expected (Buffo et al., 2010). Shift to a sera- and GF-free medium (Differentiating Medium; DM) only slightly increased the total number of GFAP+ cells over time, but dramatically influenced their morphology, with the time-dependent emission of longer and thinner processes, indicative of differentiation (Fig. 1A).

Before assessing the presence of GPR17 in these cultures, we deemed it mandatory to determine the expression profile of all the already known P2 receptors (both the P2X ligand-operated channels and the P2Y G protein-coupled receptors; Burnstock, 2008). This was achieved by RT-PCR analysis in cultures grown for 11 days (T0) in either -GFs or +GFs

medium (Table 1). Although not absolutely quantitative, this technique allowed us to relatively compare receptor expression between the two different growing conditions. Among the 7 known P2X receptor subtypes, only $P2X_4$ and $P2X_7$ (and to a lesser extent $P2X_6$) are expressed, with no significant differences between cultures in medium -GFs and +GFs (Table 1). Among the 8 cloned members of the P2Y receptor family, $P2Y_{1,2}$ (and to a lesser extent $P2Y_4$) receptor expression was clearly detectable upon both culturing conditions, whereas significant changes were observed for the $P2Y_{6,12,13,14}$ subtypes with lower levels in cells grown in the presence of GFs (+GFs cultures; Table 1). At this time point, the new P2Y-like receptor GPR17 was found expressed only in -GFs cultures (see below; Fig. 3).

Immunocytochemical analysis of GPR17 expression showed no positive staining on GFAP+ cells displaying typical astroglial morphology (i.e., flat appearance, abundant cytoplasm) at any of the time points tested. In line with the RT-PCR data, GPR17+ cells were very rare at T0 in cultures grown in medium +GFs. Under these conditions, staining was observed only on very few, small, and rounded cells (Fig. 2A, insets) representing the 0.75±0.23% of the total cell number (n=6). Shift to DM resulted in the time-dependent appearance of highly branched GFAP-/GPR17+ cells, which morphologically looked like OPCs (red in Fig. 2A). In this set of experiments, the mean number of these cells dramatically increased from 9±2 at T0 to 358±72 cells/coverslip after 72 hours in DM (n=3; Fig. 2B). Some GPR17/GFAP double-positive cells were also observed, accounting for about the 30% of GPR17+ cells at T0 and progressively decreasing with time down to the 10% of the GPR17+ cell population after 72 hours in DM (Fig. 2B). These cells were clearly morphologically distinguishable from astrocytes (see yellow arrows in Fig. 2A). Since GFAP also represents a marker of multipotency when expressed by precursor cells (Garcia et al., 2004), we interpret these data by assuming that the population of double-labeled cells represents more undifferentiated precursors that progressively undergo differentiation towards an oligodendrocyte phenotype when grown in DM, thus loosing GFAP expression.

In cultures originally grown in medium -GFs, GPR17+ cells accounted for the 2.34±0.52% of the total cell population at T0 (n=3), a 3-time higher percentage with respect to cultures grown +GFs at this time point (see above). Also in this case, shift to DM induced an increase in the number of GPR17+ cells (see below and Fig. 3).

To further characterize the morphology of GPR17+ cells upon different culturing conditions, we performed single immunostaining studies with the anti-GPR17 antibody in cells originally grown in either +GFs or -GFs medium and then shifted to DM (Fig. 3A, C). As already shown in Fig. 2 (inset), very few GPR17+ cells with short and rare processes were detected after culturing cells for 11 days in the presence of EGF and bFGF (Fig. 3A, T0). Exposure to differentiating conditions not only promoted an increase in GPR17+ cell number (Fig. 2; see below and Fig. 6), but also deeply modified their morphology, leading to the appearance of long and branched processes that markedly stained for GPR17 (Fig. 3A, 24-72h in DM). This significant increase in GPR17 receptor staining was also detected at the mRNA level by semi-quantitative RT-PCR analysis (Fig. 3B), and quantitatively evaluated by real-time PCR (Fig. 4; see below). In cultures grown for 11 days in -GFs medium (T0), GPR17+ cells looked less rounded and more mature with respect to +GFs cultures, and already displayed very thin processes (Fig. 3C, T0; compare with Panel A, T0). Again, exposure to DM increased cell number (see below and Fig. 6 for quantification) although changes in cell morphology were less evident when compared to cultures grown in medium +GFs, likely due to the higher differentiation rate at T0. In line with this hypothesis, a smaller increase in GPR17 expression (which, at variance from cells grown +GFs, was already detectable at T0) in cultures grown -GFs shifted to DM was observed by RT-PCR (Fig. 3D) and real-time PCR analysis (Fig. 4; see below). No changes in the expression of known members of the P2Y family of G protein-coupled receptors were detected upon shifting to DM (see for example, $P2Y_1$ receptor expression in Fig. 3B, D and Fig. 4), thus suggesting a selective effect on GPR17.

ENRICA, HO SCRITTO QUESTA PARTE DA TOTALE IGNORANTE. ☺VEDI SE VA BENE E CAMBIALA COME MEGLIO CREDI. We next performed real-time PCR analysis to quantify the observed differences. GPR17 expression was found to be two-fold higher in cells grown for 11 days (T0) in medium –GFs with respect to cultures grown in the presence of GFs (Fig. 4A), thus confirming our immunocytochemical and RT-PCR data (see above; Fig. 3). Moreover, cells originally grown in medium +GFs showed a highly significant and time-dependent up-regulation of GPR17 expression when shifted to DM, reaching about 40-fold higher values after 72 hours in DM with respect to T0 (Fig. 4B, left panel) with a very slight increase in the expression of the $P2Y_1$ receptor subtype (Fig. 4C, left panel). Conversely, in cultures originally grown –GFs, GPR17 expression was only marginally affected by the exposure to DM with a 7-fold increase after 72 hours with respect to values at T0 (Fig. 4B, right panel), and again no significant changes in $P2Y_1$ receptor expression (Fig. 4C, right panel). These data fully support our morphological and RT-PCR observations (Figs. 2,3; see also below), indicating that GFs appear to restrain GPR17 expression in glial progenitors, probably by keeping cells to a less differentiated stage. Nevertheless, these cells promptly respond to differentiating conditions by significantly increasing GPR17 receptor expression and modifying their morphology. On the other hand, the more mature GPR17+ cells detected in the -GFs growing condition are less prone to react to the exposure to differentiation triggers.

Interestingly, both EGF and bFGF are necessary to keep GPR17 expression low, since GPR17 mRNA levels are comparable between cultures grown in medium -GF and cells grown for 11 days in the presence of either growth factor alone (Fig. 5A). Moreover, a full down-regulation of GPR17 receptor expression was observed when cultures grown for 11 days in -GFs medium (T0) were shifted to medium +GFs for 72 hours (Fig. 5B). Again, real-time PCR analysis fully confirmed this observation with GPR17 expression being only

0.2±0.05 after shifting cultures in medium +GFs for 72 hours with respect to values at T0 set to 1. No effect was detected on the expression of the $P2Y_1$ receptor subtype (Fig. 5B).

GPR17-expressing cells are O4+ pre-oligodendrocytes, and receptor expression decreases along with cell differentiation.

We have previously demonstrated that adult brain precursor cells and pre-oligodendrocytes express GPR17 both in vivo, and in precursor-containing primary mixed neuronal/glia cultures in vitro (Lecca et al., 2008). The morphology of the GPR17+ cells shown here clearly resembles precursor/oligodendrocyte cells, as evident from Fig. 2 and 3. Nevertheless, to unequivocally confirm this observation and to identify the differentiation stage of GPR17+ cells, we performed double immunostaining for GPR17 together with typical markers of the different oligodendrocyte maturation steps (i.e., O4, CNPase, and MBP; de Castro & Bribian, 2005). Fig. 6 shows the number of cells expressing the different cell markers in cultures grown for 11 days (T0) in medium +GFs (upper panel) or in medium –GFs (lower panel), and then shifted to DM for the indicated time periods. At T0, the oligodendrocyte cell population accounted for about the 5.65% and the 16.48% of the total cell number in cultures grown +GFs and -GFs, respectively. In both culturing conditions, the prevalent oligodendrocyte marker detected at T0 is O4 (which identifies pre-oligodendrocytes; de Castro & Bribian, 2005), representing 5.13±1.53% and 11.95±1.75% of the total cell population in cultures grown +GFs and -GFs, respectively. In the presence of sera and GFs (+GFs cultures), more mature oligodendrocyte phenotypes were very rare, with cells expressing CNPase or MBP collectively representing only the 1.8% of the total cell population. In cultures grown in the presence of HS and FBS without growth factors (-GFs cultures), 7.05% of the total cell population instead expressed CNPase and MBP, suggesting the appearance of cells with a more mature phenotype. These data were also confirmed by real-time PCR analysis of MBP expression, showing that, in cultures grown for 11 days in

medium –GFs, the levels of RNA encoding for MBP were 2.7±0.7 fold higher than the levels detected in cultures grown in medium +GFs, set to 1 (n=4). VEDERE COMMENTO DOPO PER DECIDERE SE LASCIARE As previously mentioned, the number of GPR17+ cells at T0 was extremely low in cultures grown +GFs and low but significantly higher in when culturing cells without GFs (see above and Figs. 3,4). Exposure to DM led to striking differences in the maturation/differentiation of these cells depending upon their initial culturing conditions. After 72 hours in DM, the prevalent oligodendrocyte cell population was still represented by O4+ pre-oligodendrocytes both in cultures originally grown in medium +GFs or -GFs. However, in cultures grown in medium +GFs and then kept for 72 hours in DM, a significant increase in the number of O4+ cells (corresponding to the 169% of O4+ cells at T0; $p<0.05$) was detected (Fig. 6; upper panel), while no significant changes were noticed in the expression of markers of more mature phenotypes (i.e., CNPase and MBP). On the other hand, in cells originally grown in medium -GFs, the number of O4+ cells rose to only about the 132% of cells at T0, and this was accompanied by a parallel increase in the number of CNPase (157%) and MBP (124%) expressing cells (Fig. 6, lower panel). NB. I DATI DI VARIAZIONE DI ESPRESSIONE DI MBP NON SONO STATI CONFERMATI DALLA REAL TIME, ANZI NELLE ASTRO+ PASSATE IN DM I VALORI DI MBP DIVENTANO MAGGIORI RISPETTO ALLE ASTRO-. DETTO QUESTO, TOGLIAMO ANCHE I DATI DI REAL TIME DI MBP AL T0 CHE HO AGGIUNTO SOPRA PER EVITARCI PROBLEMI CON I REFEREES (CHE PERALTRO NON CI AVEVANO CHIESTO LA REAL TIME DI MBP)? As previously shown by real-time PCR analysis of GPR17 receptor expression (Fig. 4), the different behavior between the two culturing conditions was even more remarkable for GPR17-expressing cells: in fact, a 11.6-fold increase in the number of these cells was detected in cultures grown in medium +GFs and then shifted to DM, while only a 4.5-fold increase was evident in cultures derived from cells originally grown in medium –GFs (Fig. 6).

Overall, these results suggest that: i) cells originally grown in medium +GFs are less prone to undergo differentiation beyond the O4 stage when exposed to DM, but they can significantly increase their number, and ii) GPR17 expression is unfavored when cells are more strongly committed towards a more differentiated and mature phenotype (i.e., in cultures grown in medium -GFs).

We next checked for GPR17 co-localization with the various oligodendrocyte markers. In both cultures grown in medium +GFs and –GFs shifted to DM, nearly the totality of GPR17+ cells turned out to be O4+ pre-oligodendrocytes at any of the time points tested, as clearly shown in Fig. 7 (see co-localization between GPR17 and O4 in Panels A and C, and quantitative analysis in Panels B and D, white bars). In cells grown in medium -GFs, however, a sub-population of O4+ OPCs that did not express GPR17 was also detected (Fig. 7C and black bars in D), while in cultures grown in medium +GFs, O4+ cells showed a time-dependent increase of GPR17 expression; in fact, after 72 hours, almost the 100% of O4+ cells expressed the receptor (Fig. 7A and black bars in B).

A very low percentage of GPR17-expressing cells also expressed CNPase or MBP. Fig. 8 shows the results obtained in cultures originally grown in medium +GFs and then shifted to DM. As shown by immunocytochemical analysis (Panel A), GPR17 staining colocalized with CNPase only when cells showed a more immature phenotype (i.e., at T0 and after 24 hours in DM). Mature, CNPase+ myelinating cells, as those present starting from 48 hours in DM, never co-expressed GPR17, so that at this time point CNPase+ cells only accounted for about the 5-10% of the total number of GPR17+ cells (see Panel B, white bars). When looking at the total CNPase+ cell population, a colocalization with GPR17 can be detected in about the 40-60% of cells. Most of these cells also co-expressed O4 (data not shown), thus suggesting that they belong to a less differentiated step of CNPase+ pre-oligodendrocytes maturation. It is worth noting that their absolute number is very low, being a mean of 18 cells/coverslip.

The low expression level of GPR17 in mature oligodendrocytes was even more evident in MBP+ cells, which are characterized by a flat and highly branched morphology with closely interconnected processes, typical of myelinating cells (Fig. 8C). Their expression of GPR17 is always extremely low; only about the 19% of this cell population (which corresponds to a mean absolute number of 3 cell/coverslip) expressed the receptor after 72 hours in DM. Similar results were also obtained in cultures originally grown in medium –GFs (not shown). Thus, GPR17 is down-regulated along with oligodendrocyte precursor cell differentiation.

Susceptibility of oligodendrocytes to ATP-induced cell death is influenced by GPR17 expression.

To check whether GPR17 expression could influence the behavior of progenitor cells when subjected to signals typically found in a pathological context, we exposed cultures to either 100 μM ATP or 100 μM UDPglc for 72 hours, starting from the time of the DM shift. The first condition resembles what can be observed upon brain injuries in vivo, when massive amounts of ATP are released from dying cells and act on the neighboring healthy tissue (Melani et al., 2005). UDPglc is instead one of the physiological agonist ligands for GPR17 (Ciana et al., 2006; Lecca et al., 2008; Pugliese et al., 2009); UDPglc is physiologically released extracellularly (Sesma et al., 2009), and it can be hypothesized that its extracellular concentrations can be further augmented by pathological events. At the end of the incubation period, the number of cells expressing GPR17 and the various oligodendrocyte markers was evaluated and expressed as the percentage of cells in cultures exposed to vehicle (Control). In both cultures originally grown inmedium +GFs and -GFs, the number of GPR17+ cells was dramatically reduced in the presence of ATP, with a higher effect observed in cells grown in medium +GFs (Fig. 9A). Significant differences between the two culturing conditions were instead observed when the O4+ population of cells was analyzed. In fact, after a 72-hour

exposure to ATP, a 65% reduction in the number of O4+ cells was observed in cultures grown in medium +GFs (Fig. 9A, upper panel), where virtually all O4-expressing cells also co-express GPR17 (see above and Fig. 7). The reduction of O4+ oligodendrocyte number was due to induction of cell death, as demonstrated by the appearance of condensed and fragmented nuclei in cultures grown in +GFs when exposed to ATP with respect to Control cells (Fig. 9B). No significant reduction in the number of astrocytes was observed (not shown), suggesting a selective toxic action on OPCs.

In cultures grown in medium -GFs, only a non-significant 15% reduction of O4+ cells by ATP was instead detected (Fig. 9A, lower panel). Since GPR17 co-expression with O4 is much lower in these cultures (see above and Fig. 7), we hypothesize that the different extent of receptor expression might have influenced cell susceptibility to ATP-induced cell death, and that GPR17+ pre-oligodendrocytes are more prone to death. The same trend of effect, although with lower percentages of reduction of cell number, was visible for CNPase-expressing cells (Fig. 9A), which, again, were more sensitive to cell death if co-expressing GPR17. Instead, the survival of MBP-expressing cells, which virtually never co-express GPR17 (see Fig. 8), was not influenced by exposure to ATP (Fig. 9), further confirming our hypothesis that GPR17 indeed sensitizes cells to the toxic effects of ATP. Exposure to UDPglc did not significantly affect the survival of either astrocytes (not shown) or oligodendrocyte (Fig. 9). Conversely, in accordance with our previously published data (Lecca et al., 2008), UDPglc elicited a significant increase in the number of GPR17+ cells in cells grown in medium -GFs, and a non-significant trend to increase for MBP+ cells in cultures grown in the presence of +GFs medium (Fig. 9). This confirms that GPR17 activation sustains its own expression and the transition towards a more mature oligodendrocyte phenotype (Lecca et al., 2008).

Discussion

The discovery that progenitor cells are maintained in the adult brain parenchyma and participate in the reaction to injury is greatly changing the current search for effective neuroregenerative approaches to acute and chronic diseases. In fact, in parallel to the original approach of transplantation of stem/precursor cells from an external source, the possibility to promote and foster the endogenous neurogenic/gliogenic ability of the brain has emerged as a new, safe and ethically acceptable opportunity for both acute and chronic brain diseases. However, to this purpose, it is imperative to gain a global comprehension of the signaling pathways controlling the survival, differentiation and proliferation of adult precursor cells. In fact, an in-depth understanding of the molecular basis of brain parenchyma reaction to injury would highlight possible pharmacological/biotechnological approaches to help preventing excessive tissue degeneration while enhancing local reparative mechanisms. To this purpose, in vitro studies can give interesting hints to be subsequently confirmed and expanded in vivo. Growth factors (GFs) are among the first signals that have been associated to precursor cell survival and proliferation, and are therefore largely utilized both in vivo and in vitro to promote neurogenesis and gliogenesis (Hagg, 2005). Moreover, GFs are recognized as important players in the reaction of astrocytes to injury or noxious stimuli (Buffo et al., 2010). Thus, a crucial role for GFs in the genesis of new cells can be envisaged, since parenchymal precursor cells and reactive astrocytes can cooperate to the initial healing response of the damaged brain (see Introduction).

Exposure to GFs can, in turn, regulate the expression of other key modulators of cell differentiation and survival, primarily due to GFs ability to modify the proliferative or differentiative status of precursor cells (Baldauf and Reymann, 2005). However, sometimes GFs can also act through a direct interaction with the signaling pathways activated by other receptors and factors. In this respect, extracellular purine and pyrimidine nucleotides have long been demonstrated to synergize with GFs in regulating the differentiation and

proliferation of astrocytes (Abbracchio et al., 1995; Neary and Zimmermann, 2009), and, more recently, of both embryonic and adult precursor cells (Mishra et al., 2006; Grimm et al., 2009).

On these premises, our study was aimed at evaluating the influence of GFs on primary glial cultures (containing both astrocytes and OPCs), with particular focus on the new P2Y-like receptor GPR17, which have been recognized as one of the three genes specifically expressed by adult precursor cells when compared to embryonic ones (Maisel et al., 2007). It has to be underlined that the mixed astrocytic/precursor cells cultures utilize here are particularly suitable to this purpose. These cultures can be maintained upon culturing conditions resembling the environment of reactive astrocytes and thus more closely mimic the in vivo situation where cells communicate with each other and influence their own reaction to external stimuli. For example, in cultures grown in the presence of GFs (medium +GFs) a significantly higher total number of cells was detected with respect to cells grown in medium -GFs, indicating active astrocytic proliferation as detected after brain injury in vivo (Buffo et al., 2010).

Our data clearly show that GPR17 is never expressed by cortical astrocytes irrespectively of the culturing condition in vitro, thus confirming our previous in vivo and in vitro observations (Ciana et al., 2006; Lecca et al., 2008; Ceruti et al., 2009). GPR17 expression is instead specifically confined to a population of O4+ OPCs, but can be onlydetected when GFs are not included in the culture medium, and more abundantly under differentiating conditions. This suggests that receptor expression is specifically modulated by the proliferation/differentiation state of the cells, although we cannot definitely exclude a direct role of GFs on GPR17 transcription or an indirect role played by the surrounding reactive astrocytes. Indeed, the presence of both bFGF and EGF is required to down-regulate GPR17 expression, suggesting a synergy between the two GFs in keeping cells to a less differentiated state.

RT-PCR analysis showed that our mixed astrocytes/precursor cultures express 3 out of 7 P2X receptor channels (namely, the $P2X_4$, $P2X_6$ and $P2X_7$ subtypes) and the whole family of G protein-coupled P2Y receptors at the mRNA level. Since we are dealing with a mixed cell population, based on our data we cannot distinguish between receptors expression in astrocytes or OPCs. Concerning P2X receptors, it is worth mentioning that, although clear evidence for their role in modulating astrocytic functions has been provided in vitro, no direct demonstration of their functionality is currently available in vivo where they are upregulated upon induction of reactive astrogliosis (for review, see Verkhratsky et al., 2009). Thus, results obtained in vitro could more closely resemble an "activated" state, more than a physiological situation.

Interestingly, while no significant differences between cultures grown in medium – GFs and +GFs were detected in the expression of the seven P2X or of the P2Y receptor subtypes belonging to the subgroup 1 (namely, $P2Y_{1,2,4}$), a reduced amount of the $P2Y_6$ and, more significantly, of the $P2Y_{12,13,14}$ receptors was found in the presence of GFs, i.e. in the same conditions where GPR17 is expressed at very low levels. Notably, the latter P2Y receptor subtypes are included in the subgroup 2 of the P2Y receptor family, and are very similar to GPR17 (Ciana et al., 2006). Since our work was focused on GPR17 and due to the lack of really reliable antibodies, we cannot exclude that these receptors were expressed by the astrocytic population, representing the vast majority of cells in our cultures,. Nevertheless, it may well be that some biochemical, transcriptional or structural features shared between $P2Y_{12,13,14}$ and GPR17 are at the basis of the modulation of the expression of all theses structurally related receptors exerted by GFs.

Upon shifting to differentiation medium (DM), a different behavior was observed for cells initially grown in the presence or in the absence of GFs. In cultures grown in medium +GFs, GPR17+ cells started to appear after 24 hours in DM and underwent a linear increase in number over time. All these cells were also positive for the pre-oligodendrocyte marker

O4; very few mature CNPase+ or MBP+ were seen, which never co-expressed GPR17. On the contrary, cultures grown in medium –GFs showed a much higher degree of differentiation, with a lower increase in the number of O4/GPR17 double-positive cells over time. Moreover, in these cultures, a subpopulation of O4+/GPR17- preoligodendrocytes, which has not been found in cultures grown in medium +GFs, was also detected. Again, more mature oligodendrocytes never stained positive for GPR17. The differential receptor expression on pre-oligodendrocytes also reflected in cell sensitivity to high ATP concentrations, with induction of cell death only in cells expressing GPR17. Activation of the receptor with its known agonist UDPglc instead promoted a trend to increase in the number of MBP+ cells, thus confirming our previous observation obtained in a different experimental model in vitro (Lecca et al., 2008).

These results are particularly interesting in the light of in vivo data showing that, under pathological conditions, high extracellular concentrations of adenine nucleotides, especially ATP, are reached due to increased excitotoxic neurotransmission and to the leakage of the cytoplasmic content from dying cells (Melani et al., 2005). By utilizing the GTPgammaS binding assay (that directly measures receptor activation), we have also recently demonstrated that ATP can antagonize the activation of GPR17 induced by uracil derivatives (Pugliese et al., 2009), thus counteracting the beneficial and pro-survival role possibly played by this receptor under physiological conditions (see also below). This could contribute to the death of GPR17-expressing cells at the site of injury; this hypothesis is also consistent with the present data showing that O4+ cells undergo massive cell death only when co-expressing GPR17, and are instead partially preserved by the toxic action of ATP when they do not express this receptor. A cytotoxic effect exerted on mature oligodendrocytes by high ATP concentrations through the activation of the $P2X_7$ receptor subtype has been previously demonstrated. In fact, MBP-expressing cells from the rat optic nerve exposed to 1 mM ATP undergo massive cell death, and an increased expression of $P2X_7$ on myelinating

oligodendrocytes was also observed preceding the onset of EAE, thus suggesting that this receptor subtype might represent a risk factor for the development of MS (Matute et al., 2007). Indeed, the $P2X_7$ receptor has been also demonstrated to mediate ischemic damage to oligodendrocytes in vitro (Domercq et al., 2010). Apparently, these data seem to contrast with our results, demonstrating that 100 μM ATP is only toxic to O4+ OPCs when they express GPR17, with no signs of death in the very few (about 3 cells/coverslip, see Results) more mature MBP-expressing cells. Nevertheless, it should be considered that the $P2X_7$ receptor subtype has a very low affinity (in the millimolar range) for its naturally occurring ligand ATP. In our present paper, a 10-fold lower ATP concentration have been utilized, which is high enough to antagonize GPR17 receptor function, but probably too low to activate the $P2X_7$ channel, also based on the fact that ATP is rapidly degraded extracellularly by ecto-nucleotidases. It may therefore well be that the two receptors cooperate in modulating oligodendrocyte survival and death depending upon the extracellular ATP concentrations, with GPR17 playing a predominant role at lower ATP levels and the $P2X_7$ subtype being recruited only when higher extracellular nucleotide concentrations are reached. Moreover, it should also be kept in mind that we are dealing with a mixed glial cell cultures and with have evaluated the expression of the various P2X receptor subtype on the total cell population. Thus, the levels of P2X7 expression by oligodendrocytes in our experimental setting have not been determined.

Thus, GPR17 is expressed by oligodendrocyte precursors only at specific differentiation steps, i.e. at the O4-expressing pre-oligodendrocyte state, while it undergoes a progressive down regulation along with cell maturation. Our current working hypothesis is that a basal, tonic activation of GPR17 is needed to maintain precursor cell survival and function, and also to drive their differentiation towards a more mature phenotype. This basal activation could be guaranteed by the continuous release of uracyl nucleotides as additional cargo molecules during the delivery of glycosylated proteins from the endoplasmic reticulum

to the plasma membrane (Lecca and Ceruti, 2008; Sesma et al., 2009). In line with this hypothesis, UDP and UDPglc induced the generation of outward K+ currents in cells heterogously expressing GPR17 (Pugliese et al., 2009). K+ currents represent an important protective mechanism that can preserve cell viability in the brain (Gribkoff et al., 2001). Notably, due to the high concentrations of glycosylated proteins in myelin sheets, a higher physiological release of GPR17 agonists can be envisaged at sites with ongoing active myelination (Jennemann et al., 2005). This is also in agreement with the general protective role played by uracil nucleotides and sugar-derivatives in the brain (Lecca and Ceruti, 2008), and could open new perspective in the evaluation of the role of uracil nucleotides in promoting physiological myelination with possible pharmacological outcomes for demyelinating diseases.

In our previous in vivo papers we demonstrated that a significant protection from both ischemic (Ciana et al., 2006; Lecca et al., 2008) and traumatic (Ceruti et al., 2009) injury of the CNS was obtained in vivo by the administration of an antisense oligonucleotide specifically targeting and down-regulating GPR17. This data can be reconciled with our current hypothesis of an overall physiological beneficial effect of GPR17 activation and a detrimental role for ATP as a GPR17 antagonist by considering that an upregulation of GPR17 was observed at very early stages of brain damage (Lecca et al., 2008) leading to a possible dysregulation of its functions.

A recent paper from Chen and coworkers (Chen et al., 2009) has substantially confirmed our previous and present observations that GPR17 is mainly expressed by OPCs at different levels depending upon their differentiation state (Lecca et al., 2008). According to their data (obtained in a transgenic mouse model where GPR17 was expressed under the CNPase promoter), GPR17 is a negative regulator of oligodendrocyte differentiation. In fact, the transgenic mouse shows signs of demyelination and oligodendrocyte cell death (Chen et al., 2009). Our previous and present data, instead clearly claim for a role for GPR17 in

driving and promoting oligodendrocytes maturation and differentiation, as demonstrated by the increased number of OPCs (the present paper) and of cell expressing MBP (Lecca et al., 2008) after exposure to the GPR17 agonist UDPglc. However, it may well be that GPR17 needs to be desensitized and internalized in the cell cytoplasm as a consequence of prolonged agonist exposure in order to allow terminal oligodendrocyte differentiation. It is worth noting that we performed our experiments in native systems in vitro and in vivo where GPR17 expression is significantly down regulated when oligodendrocyte precursors start expressing high CNPase levels. In the transgenic mouse model utilized by Chen and coworkers, forcing GPR17 transcription in oligodendrocytes at a maturation stage (i.e. CNPase+ cells) when they physiologically downregulate its expression (i.e., CNPase+ cells) might have created conflicting intracellular signals leading to cell suicide. Thus the conclusions drawn based on a transgenic mouse model may lead to a misinterpretation of the native function of the receptor.

In conclusion, our results demonstrate for the first time a modulation of GPR17 expression in OPCs in vitro depending upon the presence of GFs and highlight its major role in oligodendrocyte maturation and as danger signal in the presence of high extracellular ATP concentrations. GPR17 might therefore represent a potential target for the development of new therapeutic approaches to demyelinating diseases, such as multiple sclerosis.

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Figure Legends

Figure 1. In the rodent brain, neither GFAP+ astrocytes (A) nor mature MBP-expressing myelinating oligodendrocytes (B) express GPR17. The expression of GPR17 was evaluated by immunohistochemistry on cortical brain slices. A specific GPR17 staining (green in A,B) was detected on highly ramified, precursor-like cells diffused in the brain tissue. Micrographs are confocal stacks comprising 15-20 1 μm optical sections. Sporadic yellow pixels result from the overlapping of distinct optical images. No colocalization with either GFAP (red in A) or MBP (red in B) staining was observed in the single optical sections. Scale bars: 50 μM (A), 20 μM (B).

Figure 2. GFAP+ astrocytes in vitro do not express GPR17, which is expressed by oligodendrocyte-like cells. Panel A. After 11 days in medium +GFs (T0), primary astrocytic cultures were shifted to DM, fixed after 24-72 hours, and subjected to double immunostaining for the astrocytic marker GFAP (green) and GPR17 (red). Note the emission of long and thin processes by astrocytes, typical of the differentiation process in vitro, and the appearance of a significant number of GPR17+ cells from 48 hours in DM. Panel B. The numbers of GPR17+ and of GFAP/GPR17-double positive cells were counted in an identical area in each coverslip (upper panel), and the number of double-labeled cells was also expressed as the percentage of the total GPR17+ cells (lower panel). *$p<0.05$ with respect to T0 (one way ANOVA, Dunnett Post Hoc test).

Figure 3. Exposure to differentiating conditions increases GPR17 expression in cultures originally grown in medium +GFs, but not in cultures originally grown in medium -GFs. Cells were grown for 11 days in GFs-containing medium (T0; Panels A and B) or in GFs-lacking medium (T0; Panels C and D), and then shifted to differentiating medium (DM;

without sera and GFs). Coverslips were fixed at various time points (24-72 hours) and subjected to immunocytochemistry with an anti-GPR17 antibody. Nuclear staining was obtained with the fluorescent dye Hoechst33258 (Panels A and C). GPR17-expression was also evaluated by RT-PCR analysis, in comparison with $P2Y_1$ receptor expression (taken here as representative of the purinergic P2Y receptor family). β-actin expression represents an internal control (Panels B and D).

Figure 4. The upregulation of GPR17 expression in cultures grown in medium +GFs and then shifted to differentiating medium is confirmed by real-time PCR analysis. Cells were grown for 11 days (T0) in with or without GFs and then shifted to differentiating medium. Total RNA was collected at the various time points, retrotranscribed, and the expression of either GPR17 (A, and B, C left panels) or $P2Y_1$ (B,C right panels) was evaluated by real-time PCR analysis, and normalized to β-actin expression (see Method). A. A two-fold higher expression of GPR17 was detected in culture grown -GFs for 11 days with respect to cells grown +GFs, to 1. B. Upon shifting to differentiating conditions, a significant upregulation of GPR17 was detected only in cells originally grown in medium +GFs. C. No changes in $P2Y_1$ receptor expression were instead observed. Results are the mean±S.E.M. of two independent experiments run in duplicate, and normalized to the corresponding receptor expression at T0, set to 1. STATISTICA? IN REALTA' PENSO CHE ANCHE P2Y1 NELLE +GFs E FORSE ANCHE GPR17 NELLE –GFs SIANO SIGNIFICATIVI MA MOLTO MENO CHE GPR17 NELLE +GFs. ENRICA, RIUSCIAMO A FARCI UNA STATISTICA? COME POSSIAMO VEICOLARE IL MESSAGGIO AL MEGLIO?

Figure 5. A combination of EGF and bFGF is necessary to down-regulate GPR17 receptor expression in primary mouse glial cultures. Panel A. Cells were grown for 11 days (T0) in the absence (-GFs) or in the presence of EGF and bFGF either alone, or in combination (+GFs).

Only the contemporary presence of both growth factors completely inhibited GPR17 receptor expression. Panel B. Cells grown for 11 days in medium -GFs (T0) were shifted to medium +GFs for 72 hours. A full down-regulation of GPR17 receptor was observed with no effect on $P2Y_1$ receptor expression.

Data have been confirmed by real-time PCR analysis (see text for details). β-actin is shown as an internal control. A typical experiment out of 3 is shown.

Figure 6. Typical markers of oligodendrocyte development are differentially expressed in cultures grown in medium +GFs and -GFs, before and after exposure to differentiating conditions. Coverslips were fixed after 11 days in either medium +GFs or –GFs (T0) or at different time points (24-72 hours) after switching to DM. Immunostaining with antibodies against GPR17 and typical markers of the different phases of oligodendrocyte differentiation (i.e., O4, CNPase and MBP) was performed and positive cells counted in an identical area in each coverslip.

Results represent the mean±S.E.M. of cells counted in at least 4 independent experiments run in triplicate. *$p<0.05$ with respect to the corresponding marker at T0, one way ANOVA (Dunnett Post Hoc test).

Figure 7. O4+ pre-oligodendrocytes progressively express GPR17 only when originally grown in GFs-enriched medium. A, C: immunofluorescence analysis of O4 and GPR17 expression in cultures grown for 11 days in either medium +GFs(A) or in medium -GFs(C) and then switched to differentiating condtions (DM) for 48 or 72 hours. B, D: GPR17+ and O4+ cells were counted in an identical area in each coverslip, and the number of double-positive cells expressed as the percentage of the total number of GPR17+ or O4+ cells, respectively. After 72 hours in DM, almost the totality of O4+ cells co-expresses GPR17 in

cultures originally grown in medium +GFs. In cultures originally grown in medium –GFs a much lower co-localization (about 40%) was instead detected.
Results represent the mean±S.E.M. of cells counted in at least 5 independent experiments run in triplicate. *p<0.05 with respect to the corresponding T0, one way ANOVA (Dunnett Post Hoc test).

Figure 8. A very low expression of GPR17 is detected in CNPase- or MBP-expressing mature oligodendrocytes. A, B: immunofluorescence analysis of GPR17 and CNPase (A) or MBP (B) expression in cultures grown for 11 days in medium +GFs (T0) and then shifted to DM for various time periods. C, D: quantification of GPR17 colocalization with either CNPase or MBP. The number of double-positive cells is expressed as the percentage of the total number of GPR17+ or CNPase+ (C) or MBP+ (D) cells, respectively. Cells positive for CNPase, which identifies a less differentiated population with respect to MBP, partially co-expressed GPR17 (C). Only a low percentage of the very low number of MBP-expressing oligodendrocytes (D; see also Figure 3) stained positive for the receptor. Results represent the mean±s.e.m. of cells counted in at least 5 independent experiments run in triplicate. *p<0.05 with respect to T0, one way ANOVA (Dunnett Post Hoc test).

Figure 9. Exposure to ATP dramatically reduces the number of GPR17+ cells and of O4+ and CNPase+ cells only when they co-express GPR17 (i.e. in cultures originally grown in medium +GFs). A: cells were grown for 11 days in either medium +GFs or -GFs, and then shifted to DM. At the same time, cultures were exposed to either 100 μM ATP or 100 μM UDPglc; Control cultures received vehicle alone. After 72 hours in culture, cells were fixed and immunostaining performed with antibodies against GPR17 and various markers of oligodendrocyte maturation. The number of positive cells was counted in an identical area in each coverslip, and results expressed as % of corresponding Control. Values are the

mean±S.E.M. of data obtained in at least 4 independent experiments. *$p<0.05$ with respect to Control, one way ANOVA (Dunnett Post Hoc test). B: after a 72-hour exposure to ATP of cells originally grown in medium +GFs, staining with the Hoechst33258 dye revealed the appearance of condensed and fragmented apoptotic nuclei (white arrows).

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